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GROWTH KINETICS OF YEASTS

By

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(Received April 1, 1969)

Summary. Changes in growth kinetic parameters such as multiplication rate constant (k), lag phase (L), final density (D) and multiplication index (M) have been examined for *Candida beverwijkii*, *C. claussenii*, *C. krusei*, *C. utilis*, *Procandida albicans*, *Pc. tropicalis*, *Saccharomyces carlsbergensis* and *S. cerevisiae* grown aerobically in glucose, galactose, sucrose, maltose and raffinose. With the exception of maltose, in all substrates there was a close correlation between k and M values. The results have been evaluated in respect to yeast carbohydrate metabolism. The association between growth parameters in different substrates and first steps of carbohydrate metabolism (sugar transport, extra- and intracellular oligosaccharide breakdown) has been discussed.

Examination of growth characteristics may yield important data for the carbohydrate metabolism of yeasts and for the antimetabolic effect of inhibitory agents. In the knowledge of the general laws of growth kinetics [12, 13, 18], from changes in the lag phase, growth rate and maximum cell counts of bacterial and yeast populations conclusions can be drawn as to the effect of factors influencing multiplication [1, 5, 12, 34]. In the present paper an account is given of the growth kinetics in different carbohydrates of several yeast species. The results have been evaluated by comparing the characteristics of one given species in different sugars and by examining the characteristics of different species in the same substrate.

Materials and methods

Strains. Candida beverwijkii OKI CLXXV/1967; *C. claussenii* OKI 460-sect./1961; *C. krusei* OKI 238/1964; *C. utilis* OKI 287/1964; *Procandida albicans* OKI 85/1957; *Pc. tropicalis* OKI 302/1964; *Saccharomyces carlsbergensis* OKI II/1966. Strain *S. cerevisiae* OKI CXCII/1967 was identical with strain R XII used by KOTYK (Institute of Microbiology, ČSAV, Prague) and by one of the present authors (T. D.) in transport experiments [4]. The cultures were maintained on CSILLAG's molasses agar [3]. The same medium was used for preparing inocula.

Growth experiments were performed in modified Sabouraud broth (glucose, 2%; peptone, 1%; pH 5.5—5.7). When needed, galactose, sucrose, maltose or raffinose was substituted for glucose. On chromatographic checking the sugars were free from impurities except maltose which contained about 8% glucose and 1% maltotriose. As in maltose broth only known maltose-assimilating organisms were examined, there was no need to purify this sugar. All cultures were investigated for aerobic growth in glucose, sucrose and maltose. Depending on their assimilation spectrum, certain cultures were examined in galactose and raffinose. Each experiment was performed in triplicate.

Preparation of aerobic shaken cultures and measurement of growth were described previously [5]. In the present experiments, instead of 10 ml only 5 ml portions of medium were

used. Inoculation was made with suspensions adjusted to 0.05 optical density, thus the initial cell counts were approximately identical for the same species, but were different for other species (in the range between 2.5×10^6 and 18.5×10^6 per ml). Growth rate constant (k), lag phase (L) and density reached after 60 hours incubation (D) were calculated as described previously [5] except that L was determined by plotting the initial cell count characteristic of the organism. In comparing k, L, and D values obtained in different carbohydrates for the same strain the data were related to the corresponding values obtained in glucose not only for the growth constant (k_D [24]), but also for the other two parameters (L_D, D_D). Different species were compared for absolute values of growth kinetic characteristics in the same substrate with the exception of the final density (D). This, due to a difference in initial densities, could not be compared directly. Results were expressed as multiplication indices (M) showing the ratio of final and initial density. The value of M related to glucose was identical with the value of final density related to glucose ($M_D = D_D$), in other words, there was no alteration when the results obtained for the same species were compared, but the calculation of absolute M values made the comparison of data for different species more reliable.

Results

Fig. 1 presents a series of growth curves. Results expressed as k, L and D values (Tables I, II and III) were derived from such curves.

From the galactose and sucrose curves in Fig. 1 it is evident that a prolongation of the lag phase and a decrease of the growth rate constant do not necessarily lead to a lower final density, as shown by the growth in galactose and sucrose. In the case of maltose, however, the shorter lag phase and the higher growth rate were associated with a higher final density, while in raffinose an opposite effect was observed.

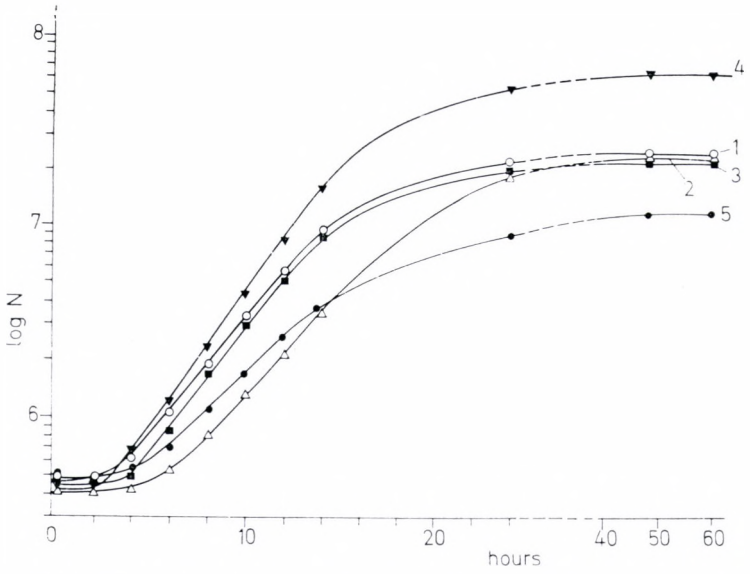

Fig. 1. Growth of *S. cerevisiae* in various substrates. Aerobic, shaken cultures. Basal medium: peptone, 1%; sugar, 2%; pH 5.6. 1 = glucose, 2 = galactose, 3 = sucrose, 4 = maltose, 5 = raffinose

Table I

Absolute and relative growth rate constants for yeasts cultured aerobically in various carbohydrates

Species	k					k_D				
	D	G	S	M	R	D	G	S	M	R
Candida beverwijkii	0.540	0.300	0.427	0.708	.	1.00	0.56	0.79	1.31	.
Candida claussenii	0.582	0.366	0.430	0.453	.	1.00	0.63	0.74	0.78	.
Candida krusei	0.330	.	.	.	.	1.00	.	.	.	.
Candida utilis	0.348	.	0.334	0.424	0.310	1.00	.	0.96	1.22	0.89
Procandida albicans	0.366	0.300	0.348	0.468	.	1.00	0.82	0.95	1.28	.
Procandida tropicalis	0.354	0.336	0.318	0.430	.	1.00	0.95	0.90	1.25	.
Saccharomyces carlsbergensis	0.145	0.096	0.148	0.140	0.130	1.00	0.66	1.02	0.96	0.90
Saccharomyces cerevisiae	0.265	0.246	0.286	0.307	0.204	1.00	0.93	1.08	1.16	0.77

k = absolute growth rate constant in hours
k_D = growth rate constant related to glucose
D = glucose, G = galactose, S = sucrose, M = maltose, R = raffinose
. = not examined

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Table II

Absolute and relative lag phase times for yeasts cultured aerobically in various carbohydrates

Species	L					L_D				
	D	G	S	M	R	D	G	S	M	R
Candida beverwijkii	8.8	9.8	7.8	6.9	.	1.00	1.12	0.89	0.78	.
Candida claussenii	8.1	9.1	7.2	8.3	.	1.00	1.12	0.89	1.02	.
Candida krusei	2.6	.	.	.	.	1.00	.	.	.	.
Candida utilis	4.1	.	5.2	4.2	5.1	1.00	.	1.28	1.02	1.24
Procandida albicans	3.8	3.7	4.9	3.7	.	1.00	0.97	1.28	0.98	.
Procandida tropicalis	4.4	5.0	4.3	3.8	.	1.00	1.13	0.98	0.87	.
Saccharomyces carlsbergensis	6.5	9.2	5.8	6.0	8.6	1.00	1.42	0.89	0.92	1.32
Saccharomyces cerevisiae	2.9	5.2	3.4	2.4	3.6	1.00	1.80	1.16	0.84	1.24

L = lag phase in hours
L_D = lag phase related to glucose
D = glucose, G = galactose, S = sucrose, M = maltose, R = raffinose
. = not examined

Table III

Absolute and relative final densities and multiplication indices for yeasts cultured aerobically in various carbohydrates

Species	D ($\times 10^6$ cells/ml)					M					D_D (= M_D)				
	D	G	S	M	R	D	G	S	M	R	D	G	S	M	R
Candida beverwijkii	88	42	78	164	.	103	50	90	193	.	100	48	88	186	.
Candida claussenii	50	39	40	74	.	91	71	73	135	.	100	78	80	148	.
Candida krusei	59	.	.	.	.	69	.	.	.	.	100	.	.	.	.
Candida utilis	93	.	84	251	70	50	.	45	136	38	100	.	90	270	75
Procandida albicans	76	57	54	175	.	89	67	63	205	.	100	75	71	230	.
Procandida tropicalis	25	22	25	58	.	100	86	100	234	.	100	86	100	234	.
Saccharomyces carlsbergensis	11	7	11	13	9	28	17	28	33	22	100	61	100	118	79
Saccharomyces cerevisiae	25	23	22	68	12	56	51	50	150	27	100	91	90	270	49

D = absolute final density
M = multiplication index
D_D, M_D = final density, multiplication index related to glucose
D = glucose, G = galactose, S = sucrose, M = maltose, R = raffinose
. = not examined

In view of the results presented in Tables I—III, a most definite association exists between k and M values (Fig. 2A, B). If the results were plotted according to different species (Fig. 2A) the correlation was not linear. The low growth rate and the consequent low final density of *S. carlsbergensis* was conspicuous. In the case of plotting according to substrates (Fig. 2B), it became

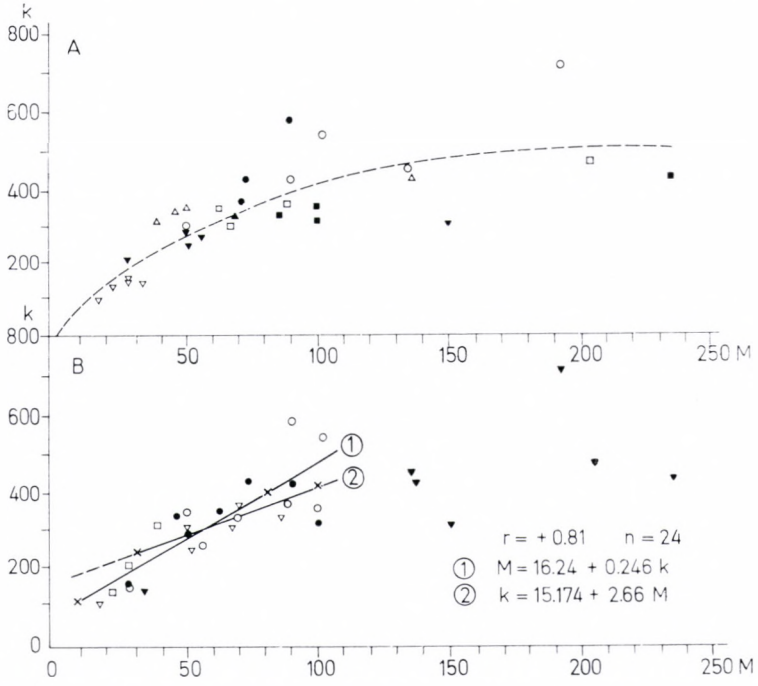

Fig. 2. Correlation between growth rate constant (k) and multiplication index (M). A: Results plotted according to species; ○ = *C. beverwijkii*, ● = *C. claussenii*, ▲ = *C. krusei*, △ = *C. utilis*, □ = *Pc. albicans*, ■ = *Pc. tropicalis*, ▽ = *S. carlsbergensis*, ▼ = *S. cerevisiae*. B: Results plotted according to substrates; ○ = glucose, ▽ = galactose, ● = sucrose, ▼ = maltose, □ = raffinose

evident that the values in maltose are responsible for the distortion of the correlation between k and M; if they were omitted, the rest showed a close correlation.

There was no correlation between k and L, and L and M, respectively. The low growth rate for *S. carlsbergensis* was in all substrates associated with a long lag phase and a low multiplication index (low final density). This organism, accordingly, showed in all respects the weakest multiplication ability. *C. beverwijkii* and *C. claussenii* were characterized by a long lag phase followed by a very rapid growth (these organisms showed the highest growth rates), although the results were not always reflected by M and D values.

Considering the growth of the same organism in different substrates it is clear that, taking into account all growth characteristics, maltose yielded

the best growth. The only exception was the k value for *C. claussenii* which was considerably lower for maltose than for glucose, despite a higher final density and a shorter lag phase in the former than in the latter sugar.

In sucrose *S. cerevisiae*, *S. carlsbergensis*, *C. utilis*, *Pc. tropicalis* and in raffinose *C. carlsbergensis* and *C. utilis* exhibited growth parameters similar to those obtained in glucose, while other strains showed more considerable differences. Growth was usually weaker in galactose than in glucose.

Discussion

The general laws of the multiplication of unicellular organisms are well known. Mathematically only the logarithmic (exponential) phase can be characterized by the multiplication rate constant (k). Since there is no general mathematical formula for the whole growth curve, it is usually characterized by the length of the lag phase (L) and the final density (D) of the culture, in addition to the k value. From the very fact that all three values are needed for characterizing the process of growth it follows that there is no generally valid correlation between these parameters. Final density may be influenced by the length of the lag phase and the value of the growth rate constant, but is determined by neither of them, since the D value depends highly also on a number of complicated processes initiated by the culture's autointoxication.

The above considerations have been confirmed by the present results. For *S. cerevisiae*, as shown in Fig. 1, L and k practically determined the final density in maltose and raffinose, but not in galactose, where despite a lower k and a higher L, the D value was the same as in sucrose. *C. beverwijkii* and *C. claussenii* were uniformly characterized by a long lag phase and a high growth rate, but the final density of the cultures varied greatly according to the substrate. For *S. carlsbergensis* the long lag phase and low growth rate were always associated with a low final density, and so with this organism the three parameters showed a definite correlation. It is, however, questionable whether this in every respect weak multiplication was a general characteristic of the species *S. carlsbergensis*. The strain examined in our aerobic growth experiments was a Schwechat-type culture well adapted to the fermentation of brewery malt mash. On the other hand, the lack of vitamins (yeast extract) in our culture medium might have influenced the growth of *S. carlsbergensis* although it seemed to exert no effect on other species.

In one respect there was a definite correlation between the growth parameters k and M. As described under "Materials and methods" index M was introduced instead of D in order to exclude the influence on final density of initial density which varied with the different species. At identical initial densities the M values were proportional to the corresponding D values. As seen in Fig. 2 the correlation between k and M was not linear; with the

increase of the multiplication index the value of the growth rate constant approached the maximum. It is a well-known property of microbial populations that neither the growth rate nor the final density (D) or, in other words, the multiplication of the initial cell count (M) can rise above a certain limit determined by culturing conditions and the characteristics of the given organism. The fact that k and M showed a linear correlation if the results for maltose were neglected, and a non-linear correlation if maltose was also considered, indicates that the various organisms approached the limit of growth more closely in maltose than in other sugars. (The high degree of growth in maltose will be discussed below.) As to the above results we refer to the experiments of VAS [37] indicating that the maximum cell count changed parallel with the growth rate constant, while there was no definite correlation between lag phase and growth rate.

In evaluating the results with respect to carbohydrate metabolism first of all the growth rate and the lag phase should be considered. The final density (*i.e.* multiplication index) depends on the autointoxication of the culture, among others, and cannot therefore be correlated directly with primary processes in carbohydrate metabolism. For a better understanding of the results, some carbohydrate metabolic characteristics of the examined yeasts are summarized in Table IV.

In the knowledge of the data presented in Table IV it might be expected that the growth kinetic parameters are identical in various substrates and in glucose when the given organism is capable of extracellular oligosaccharide breakdown. This has been confirmed for *S. carlsbergensis* and *C. utilis* in sucrose and raffinose. In these substrates *C. beverwijkii* showed a somewhat shorter lag phase and a somewhat lower growth rate as compared to glucose; this finding was attributed to a weaker invertase production. In *S. cerevisiae* the conditions are more complicated as it leads not only to an extracellular sucrose breakdown and a subsequent monosaccharide transport but also to endosaccharase activity and, at least in part, a transport of sucrose before its cleavage. The weaker growth of *S. cerevisiae* in raffinose as compared to *C. utilis* or *S. carlsbergensis* indicates a retarded and slow invertase production. *S. cerevisiae* grows better in sucrose than in raffinose; this finding confirms the presence of a pathway involving sucrose transport and intracellular cleavage parallel with invertase activity. In addition, sucrose breakdown by *S. cerevisiae* may also be promoted by maltose which acts intracellularly [30]. Melibiase in *S. carlsbergensis* together with invertase allow a perfect (3/3) utilization of the raffinose molecule. This explains why *S. carlsbergensis* utilizes raffinose relatively better than the only invertase-producing *S. cerevisiae* strain which uses 1/3 of the raffinose molecule (fructose). The late appearance of melibiase in *S. carlsbergensis* [19] may explain the prolongation of the lag phase with respect to raffinose.

Table IV

Carbohydrate assimilating and oligosaccharide splitting ability of some yeast species

Species	Carbohydrate assimilation					Oligosaccharide splitting enzyme	Reference
	D	G	S	M	R		
Candida krusei	+	−	−	−	−		
Candida utilis	+	−	+	+	1/3	invertase	6
Candida beverwijkii	+	+	+	+	1/3	invertase, endomaltase	21
Saccharomyces cerevisiae	+	+	+	+	1/3	invertase, endosaccharase, endomaltase	9, 11, 30
Saccharomyces carlsbergensis	+	+	+	+	3/3	invertase, endomaltase, melibiase	17, 19
Procandida albicans	+	+	+	+	−	endosaccharase, endomaltase	27, 28
Procandida tropicalis	+	+	+	+	−	endosaccharase, endomaltase	21
Candida claussenii	+	+	+	+	−	endosaccharase, exomaltase	21, 33

+ = assimilation, − = no assimilation, 1/3, 3/3 = degree of raffinose assimilation
D = glucose, G = galactose, S = sucrose, M = maltose, R = raffinose

It would appear that growth parameters in maltose and glucose show identical values for exomaltase-producing *C. claussenii*. The exomaltase of this organism, however, splits maltose by a transfer-type reaction [21] and thus every maltose molecule yields only one readily utilizable glucose molecule and one molecule not directly utilized maltotriose. This mechanism may explain why k and D values were lower in maltose than in glucose.

Parameters in galactose indicated a weaker growth of all species in this sugar than in glucose. This may be attributed to a probably inductive galactose metabolism of the examined yeasts. Induction may appear both in the transport and in the waldenase systems, as indicated by the fact that these organisms ferment galactose weaker and unsteadily or not at all [15, 25, 26, 35]. The inductivity of galactose transport was experimentally shown in *S. cerevisiae* [31, 32].

In the rest of the examined cultures the results should be interpreted on the basis of intracellular sucrose and maltose breakdown and the previous transport process. In this manner *Pc. tropicalis* was able to grow almost with a same intensity in sucrose and in glucose. In the case of *C. claussenii* the shorter lag phase and the lower growth rate in sucrose compared to those of glucose indicated a rapid sucrose transport but slower cleavage. As to *Pc. albicans*, the lag phase in sucrose was considerably prolonged, due to an inductive sucrose transport mechanism [23].

Endomaltase-producing yeasts *(C. beverwijkii, C. utilis, Pc. albicans, Pc. tropicalis, S. carlsbergensis, S. cerevisiae)* showed a higher growth rate (except *S. carlsbergensis*) and a shorter lag phase (except *C. utilis* and *Pc. albicans* (in maltose than in glucose. It should be noted that maltase in *S. carlsbergensis* differs from the corresponding enzyme in other species (structure-bound [20]), *C. utilis* fails to ferment maltose (its aerobic maltose utilization is therefore probably inductive) and in *Pc. albicans* glucose fermentation and maltose fermentation are constitutive processes nearly identical in reaction rate [25]. As the final density (multiplication index) was considerably higher for all species in maltose than in glucose, it may be stated that all the examined yeasts multiplied better in the former than in the latter sugar. In connection with this finding an old problem of yeast physiology, direct versus indirect fermentation, should be mentioned. The long-debated question of the cause of more rapid maltose fermentation as compared to glucose breakdown has finally been explained by an intracellular cleavage [7] and a very active transport process [22]. In some cases the presence of an active maltose transport has been experimentally confirmed [9, 10, 29]. In view of these findings the more abundant aerobic growth in maltose can be interpreted as a result of a highly active maltose transport which though the activity of endomaltases may be weak, results in an intracellular glucose concentration higher than that caused directly by the transport of glucose *per se*.

The effect of glucose-impurity in the maltose substance used remains to be discussed. Two facts should be considered. On the one hand it is known from fermentation studies that the lag phase of maltose breakdown is decreased by a small amount of glucose; on the other hand, the rate of maltose fermentation (in endomaltase-producing yeasts) is always higher than that of glucose fermentation [2, 8, 14, 16]. Consequently, the role of glucose may be explained by an improvement of maltose transport or maltase induction. This results primarily in a shortening of the lag phase and, as discussed above, in a higher growth rate due to an active maltose transport. With respect to the final density, however, the glucose impurity in maltose is of no consequence, partly because it is rapidly utilized, and partly as the D values are considerably lower in a medium containing only glucose. As a further explanation it may be supposed that certain impurities in maltose might render the cells tolerant to autointoxication and in this manner the final density increases. Another explanation is that, independently of impurities, active maltose transport may be much more intensive than glucose transport and thus it may retain metabolic processes necessary for multiplication even after a considerable decrease of the substrate concentration. For elucidating the problem further investigations are needed.

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GROWTH FACTOR REQUIREMENT OF SHIGELLA FLEXNERI AND SHIGELLA SONNEI

By

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(Received April 18, 1969)

Summary. The growth factor requirement of 271 *Sh. flexneri* and 100 *Sh. sonnei* strains has been examined on Falkow minimal medium. All *Sh. sonnei* and the majority of *Sh. flexneri* strains were prototrophic culture; only 11 auxotrophic *Sh. flexneri* strains were encountered (2.9%).

Earlier authors claimed to have shown that, unlike *Escherichia coli* which multiplies in simple defined medium, shigellae require nicotinamide or nicotinic acid [1], pantothenic acid [3] and even p-aminobenzoic acid [4]. An occasional requirement for various amino acids, nucleotide bases and thiamine has also been reported [5, 6].

Recently, LACHOWICZ *et al.* [7, 8] have refuted the general validity of observations on growth factor requirement and described many prototrophic strains and the frequent incidence of random dependence. They showed an association between antigenic structure and dependence only for *Sh. flexneri* type 6. According to SCHUH and ALDOVA [9] *Sh. flexneri* 6 was characterized by a constant thiamine requirement and the majority of the strains needed also pantothenic acid.

In earlier studies we have found that the proper composition of the minimal medium is important for avoiding errors in determining auxotrophy. This experience has made us to study random-selected *Sh. flexneri* and *Sh. sonnei* strains.

Materials and methods

Strains. A total of 271 *Sh. flexneri* and 100 *Sh. sonnei* strains collected from various parts of Hungary over a one-year period was studied. Among strains originating from one epidemiological focus only one representative culture was examined.

Culture media. Agar slant or stab cultures received were streaked onto meat infusion medium containing 2% agar. After reisolation the cultures were checked on triple-sugar-iron medium as described by NÓGRÁDY and RODLER [10].

Growth factor requirement was examined on the minimal medium of FALKOW *et al.* [11] with double glucose concentration and 2% agar content.

Serological examinations were performed with 24-hour agar cultures by slide agglutination in polyvalent and type sera. *Sh. flexneri* type determining absorbed sera were prepared as described by RAUSS [12].

Growth factor requirement was tested by two different methods for every strain. One of them consisted of streaking a small quantity of bacteria on minimal and completed minimal

media, the other of replicating [13] 5-hour agar cultures on the corresponding minimal media. The strain was regarded auxotrophic if it had failed to grow on the minimal medium after 48 hours but grew on at least one of the supplemented media.

Growth factor requirement was determined in two steps: first the auxotrophs were transferred onto plates of minimal media supplemented with 5 different growth factor pools (3 different amino acid pools, vitamin pool, and nucleotide base pool), then the culture was tested for individual components of the pool providing growth.

The minimal medium was usually completed with 20 μg substance per ml except for thiamine (4 μg/ml), threonine (40 μg/ml), leucine, serine and valine (80 μg/ml each). The concentrations were chosen on the basis of our earlier findings.

Results

Results are summarized in Table I. There was a remarkably high incidence of prototrophs while dependence was unfrequent (11 strains, 2.9%). In view of the narrow-scale type distribution in Hungary, no general conclusions could be drawn as to association between dependence and serological property. The present findings failed to confirm such association for more frequent types as *Sh. sonnei*, *Sh. flexneri* 2a, 3 and even 6.

Table I

Type distribution and growth factor requirement of Sh. flexneri and Sh. sonnei strains

Subgenus	Serotype	No. of strains	No. of prototrophic strains	No. of auxotrophic strains		
				Nia$^-$	Met$^-$	Met$^-$Trp$^-$
Sh. flexneri	1a	3	3			
	1b	9	9			
	2a	105	98	2	5	
	2b	1	—	1		
	3	116	113	1	1	1
	4a	4	4			
	4b	—	—			
	5	—	—			
	6	24	24			
	var. X	6	6			
	var. Y	3	3			
Sh. sonnei		100	100			
Total		371	360	4	6	1

Nia$^-$ = nicotinic acid requirement; Met$^-$ = methionine requirement; Met$^-$Trp$^-$ = methionine + tryptophan requirement

The few dependent strains exhibited little variety: requirement for nicotinamide or methionine was common; one culture needed methionine + tryptophan.

It should be noted that, as pointed out by LACHOWICZ *et al.* [8], subjective elements considerably influence the testing of auxotrophy. We frequently observed cultures showing poor growth on minimal medium but a good one in the presence of a given substance (mainly methionine or cysteine) or a mixture of substances. Most of these strains failed, however, to show a defined requirement for one single growth factor.

Our observations indicate that auxotrophic cultures occurred more frequently among shigellae showing multiple resistance to antibiotics than among sensitive strains. These data should be confirmed in further studies.

Discussion

In disagreement with earlier authors and in agreement with the Wroclaw team [8] we could reveal no essential growth factor requirement for *Sh. flexneri* and *Sh. sonnei*. Falkow's minimal medium [11] somewhat modified for the present study allowed a definitely better growth than Davis's minimal medium [14]. Under the rigorous criteria of readings, auxotrophy was an unfrequent character on the modified Falkow medium.

In view ot the differences in the methods our data are difficult to compare with other authors' observations. For example, the statement of SCHUH and ALDOVA [9] that *Sh. flexneri* serotype 6 is characterized by thiamine and frequently by pantothenic acid requirement cannot be compared with the present data because they considered the growth-promoting effect of these substances.

The contradictory findings in the literature are, accordingly, due mainly to differences in the methods. In our opinion the composition of the minimal medium plays an essential role in this respect. On Davis's medium which is excellent for studying *E. coli* K-12, numerous Shigella strains, which multiply well on Falkow's medium grew poorly and slowly (after 48—72 hours) or not at all.

The other source of discrepancies may be attributed to differences in readings. We frequently encountered Shigellae that grew poorly and slowly on minimal medium. These strains were similar in nature to LEDERBERG's [14] non-absolute mutants or exhibited a "narrowed synthetic capacity" — as termed for shigellae by STYPULKOWSKA-MISIUREWICZ [15]. In our opinion this character cannot be regarded as a property associated with auxotrophy.

An essential conclusion to be drawn was that, similarly to *E. coli*, Shigellae do not require organic nitrogen compounds specific of this species.

Acknowledgement. The authors are indebted to the chiefs and the staffs of the Shigella Laboratory, National Institute of Public Health, Budapest, and of the Public Health Stations in Debrecen, Szombathely, Kaposvár and Pécs for supplying the cultures.

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PROPERTIES OF THREONINE DEAMINASE IN MYCOBACTERIUM PELLEGRINO

By

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Summary. Isoleucine inhibits the activity of L-threonine deaminase isolated from *Mycobacterium pellegrino*. The degree of inhibition depends on substrate concentration. Valine exerts an activating effect and partly suspends the inhibition by isoleucine. In fresh enzyme preparations the substrate saturation curve corresponds to the Michaelis—Menten kinetics. On storage at 0°C there is an increase in activity and a decrease in isoleucine sensitivity and thermostability. The enzyme is stabilized not only by pyridoxal phosphate and isoleucine but also by valine and thiamine pyrophosphate.

We have shown that addition of valine to *Mycobacterium pellegrino* culture results in a coordinative derepression of enzymes taking part in isoleucine and valine biosynthesis [15]. The coordinative derepression involves threonine deaminase, an enzyme first observed in *Escherichia coli* by WOOD and GUNSALUS [1]. Later UMBARGER and BROWN described two different threonine deaminases in *E. coli*, a degradative threonine deaminase and a so-called biosynthetic threonine deaminase. The latter enzyme, which acts in isoleucine biosynthesis, is inhibited by isoleucine [2, 4, 5]. In these studies UMBARGER was the first to demonstrate allosteric inhibition. More recently the properties of this enzyme have been investigated in detail. On the basis of studies on *E. coli* K—12 L-threonine deaminase CHANGEUX [6, 7] devised a theoretical model for allosteric inhibition. HOLZER examined allosteric phenomena on threonine deaminase in yeasts [8]. FREUNDLICH studied the properties of the enzyme in *Salmonella typhimurium* [9]. SANWAL, who also used *S. typhimurium*, made the measurements more reliable and devised a modified model for interpreting allosteric inhibition [10]. The present studies are not merely a continuation of the above experiments but give an account of the mutual effects of coordinatively induced enzymes.

Materials and methods

Preparation of threonine deaminase. M. pellegrino was cultured as described in [15]. In the experiments 20-hour logarithmic phase cultures derepressed by valine were used. The cells were centrifuged, washed in phosphate buffer (pH 7.6, 0.4 *M*), then resuspended in buffer mixture (1 g wet cells in 6 ml). The buffer mixture consisted of: phosphate buffer (pH 7.6), 0.4 *M*; magnesium sulphate, 0.01 *M*; thiamine pyrophosphate, 0.1 *mM*. The suspension was chilled in ice bath and subjected to ultrasonic treatment in an MSE apparatus (20,000 cycles,

100 watts). The crude protein solution was separated from the cellular debris in an MSE refrigerated centrifuge at 16,000 r.p.m. The yellowish, opalescent solution was stored in ice bath until used.

The experiments were partly performed with protein solution concentrated by ammonium sulphate precipitation. Phosphate buffer (pH 7.2, 0.4 *M*) was saturated with ammonium sulphate and the pH was readjusted to 7.2 with sodium hydroxide. First 0.2 ml volume saturated ammonium sulphate was added drop by drop to the chilled protein solution and the inactive precipitate was removed by centrifugation. Then an equal volume of saturated ammonium sulphate solution was pipetted to the supernatant and the precipitate was collected by centrifugation. The precipitate contained 80—90% of the activity and was storable without loss at 0°C for a few days. Before use the precipitate was dissolved in the above described buffer mixture so that the protein concentration was 20—25 mg/ml. Finally the solution was gel filtered through Sephadex G-25 column equilibrated with buffer mixture.

Assay of enzyme activity. Threonine deaminase assay was performed in pH 7.8 tris (hydroxymethyl aminomethane) buffer in the presence of pyridoxal phosphate. The reaction mixture contained in a final volume of 0.9 ml: tris buffer, 0.1 *M*; ammonium chloride, 0.1 *M*; pyridoxal phosphate, 0.1 *mM*; L-threonine, 20 *mM*; protein, 0.2—1.0 mg. The reaction was started by the addition of the enzyme. The mixture was incubated at 28°C for 20 minutes, then the reaction was terminated with 0.1 ml 50% trichloroacetic acid. Determination of released ketoacid was carried out by the direct method of FRIEDMANN and HAUGEN [11]. To 1 ml appropriately diluted solution 3 ml dinitrophenylhydrazine reagent (0.025% dinitrophenylhydrazine in N hydrochloric acid) were added. After 20 minutes standing at room temperature hydrazone was shown by adding 1 ml 40% sodium hydroxide. The developing reddish-brown colour was read at 450 mμ. The substrate-free reaction mixture was used as blank. The unit of specific activity was expressed as μM ketoacid released by 1 ml protein in 1 hour. Protein was determined as described by LOWRY *et al.* [12]. In the experiments analytical grade commercial preparations were used.

Results

Effect of protein concentration on enzyme activity. The reaction rate was proportional to the protein concentration (Fig. 1).

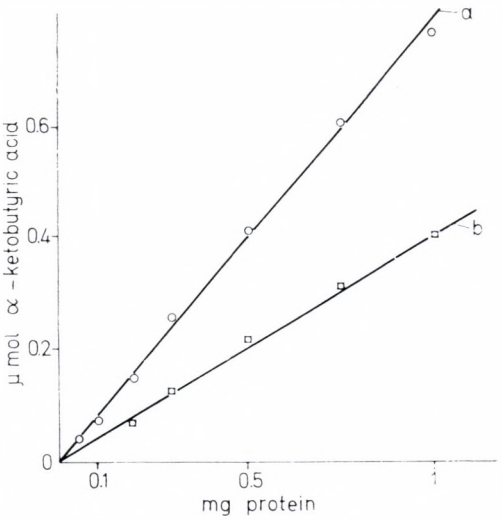

Fig. 1. Threonine deaminase activity versus protein concentration. Reaction mixture: tri buffer (pH 7.8), 0.1 *M*; ammonium chloride, 0.1 *M*; pyridoxal phosphate, 0.1 *mM*; L-threonine 20 *mM*; protein concentration as indicated. Reaction time, 20 minutes (a) and 10 minutes (b)

Effect of pyridoxal on reaction rate. A weak activating effect of pyridoxal phosphate was detectable when crude extract or ammonium sulphate-precipitated and gel-filtered protein solution was examined. Threonine deaminase was inactivated if the enzyme solution was dialysed overnight against 0.1 *M* phosphate buffer or against 0.1 *M* tris buffer. However, as an effect of pyridoxal phosphate, 70—75% of the original activity could be demonstrated (Table I).

Table I

Effect of pyridoxal phosphate on reaction rate

	Pyridoxal phosphate	
	0.1 *mM*	—
Crude protein solution	0.41	0.40
Protein solution dialysed in 0.1 *M* phosphate buffer pH 7.6	0.34	0.08
Protein solution dialysed in 0.1 *M* tris buffer pH 7.6	0.31	0.01

Reaction mixture: tris buffer, (pH 7.8), 0.1 *M*; ammonium chloride, 0.1 *M*; L-threonine, 20 *mM*; protein, 0.25 mg/ml. Reaction time, 20 minutes at 28°C. The figures indicate μmole ketoacid produced in 20 minutes.

Effect of pH on reaction rate and isoleucine sensitivity. The amount of α-ketobutyric acid released from threonine in tris buffer in the pH range 6.1—9 was determined (Fig. 2). Changing the pH from 7 to 9 resulted in a 7-fold increase of enzyme activity.

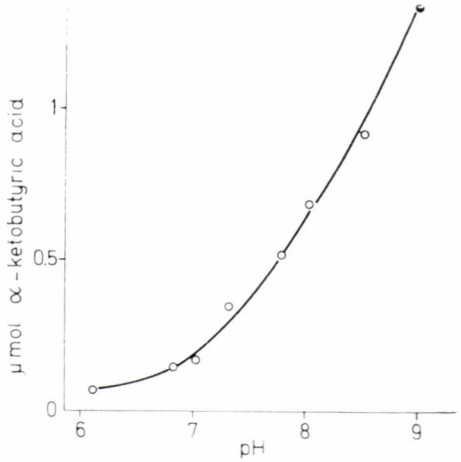

Fig. 2. Threonine deaminase activity versus pH. Reaction mixture: tris buffer, 0.1 *M*; ammonium chloride, 0.1 *M*; pyridoxal phosphate, 0.1 *mM*; L-threonine, 10 *mM*; protein, 0.2 mg/ml. Reaction time, 20 minutes at 28°C

The influence of pH on the inhibitory effect of 0.1 *mM* isoleucine is shown in Fig. 3. Between pH 6 and 8 there was no alteration, but above pH 8 isoleucine sensitivity decreased to half. The decrease occurred always in the range of pH 8—8.5 independently of the isoleucine concentration.

Effect of temperature on threonine deaminase activity and isoleucine sensitivity of the enzyme. Increasing the temperature from 20 to 38°C resulted in a 4-fold increase in reaction rate (Fig. 4). Above 38°C the activity decreased rapidly. The amount of α-ketobutyric acid produced in the presence of isoleucine increased slowly and proportionally with the increase of the temperature up to 42°C. This finding indicates that isoleucine sensitivity was highly influ-

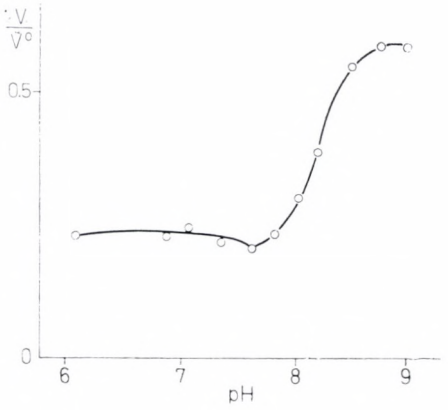

Fig. 3. Influence of pH on isoleucine sensitivity of threonine deaminase. Reaction mixture: tris buffer, 0.1 *M*; ammonium chloride, 0.1 *M*; pyridoxal phosphate, 0.1 *mM*; L-threonine, 10 *mM*; protein, 0.2 mg/ml. Inhibitory isoleucine concentration, 0.1 *mM*. Reaction time, 20 minutes at 28°C

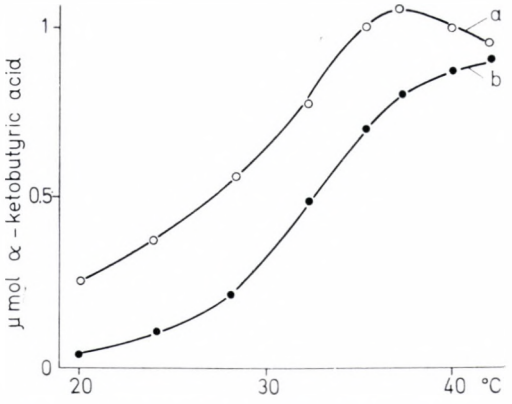

Fig. 4. Threonine deaminase activity versus incubation temperature. Reaction mixture: tris buffer (pH 7.8), 0.1 *M*; ammonium chloride, 0.1 *M*; pyridoxal phosphate, 0.1 *mM*; L-threonine, 20 *mM*; protein, 0.28 mg/ml. Reaction time, 20 minutes. a = without isoleucine, b = with 0.1 *mM* L-isoleucine

enced by the temperature (Fig. 5). At 20°C 0.1 *mM* isoleucine caused nearly 90% and at 30°C 50% inhibition but at 42°C it almost totally failed to exert any inhibitory effect. On the other hand, above 38°C the stabilizing effect of isoleucine was evident.

Effect of substrate concentration on reaction rate. When reaction rates were plotted against substrate concentration, a hyperbolic curve corresponding to the Michaelis—Menten kinetics was obtained. When plotted according to LINEVEAWER and BURK's reciprocal method (Fig. 6), the points fell along a straight line (K_M = 13.4 *mM*). In the presence of isoleucine the saturation curve approached a sigmoid shape which in the reciprocal graph appeared as a

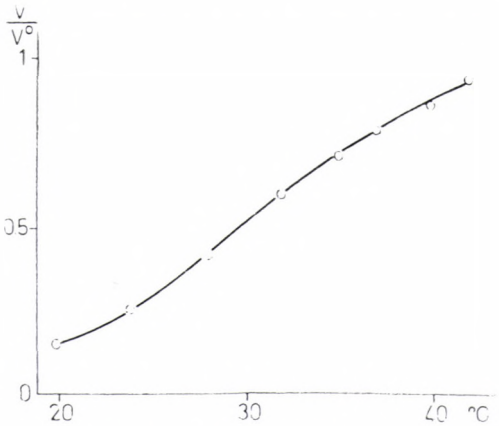

Fig. 5. Influence of incubation temperature on isoleucine sensitivity of threonine deaminase. Reaction mixture: tris buffer (pH 7.8), 0.1 *M*; ammonium chloride, 0.1 *M*; pyridoxal phosphate, 0.1 *mM*; L-threonine, 20 *mM*; protein, 0.28 mg/ml. Inhibitory isoleucine concentration 0.1 *mM*. Reaction time, 20 minutes

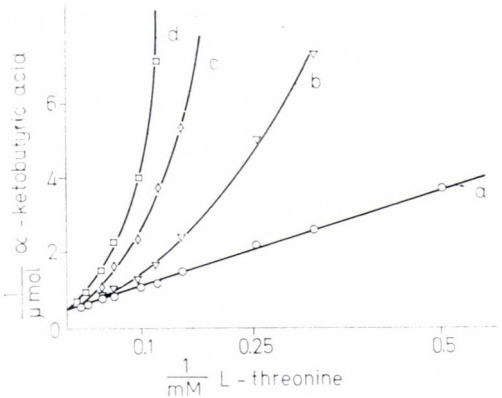

Fig. 6. Influence of substrate concentration on threonine deaminase activity in the presence of different isoleucine concentrations. Reaction mixture: tris buffer (pH 7.8), 0.1 *M*; ammonium chloride, 0.1 *M*; pyridoxal phosphate, 0.1 *mM*; protein, 0.3 mg/ml. Reaction time, 30 minutes at 28°C. a = without isoleucine, b = with 10 μM isoleucine, c = with 20 μM isoleucine, d = with 100 μM isoleucine

concave curve. At low substrate concentrations the enzyme was not inhibited by isoleucine (Fig. 7). For example, 10 *mM* isoleucine in the presence of 2 *mM* threonine caused 70%, while in the presence of 8 *mM* threonine 20% inhibition. At higher isoleucine concentrations the break in the curve indicating the weakening of the inhibitory effect showed a shift towards higher threonine concentrations. The inhibitory effect of isoleucine was very definite (Fig. 8).

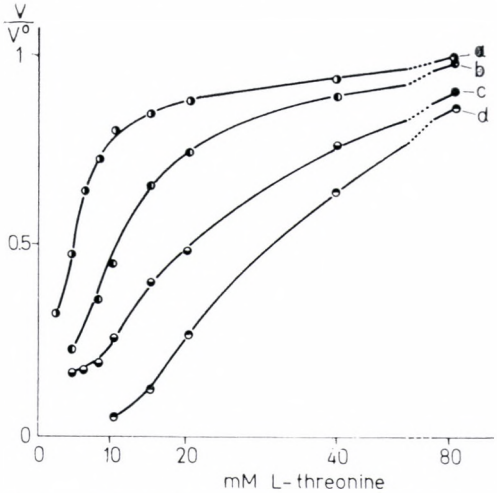

Fig. 7. Isoleucine sensitivity of threonine deaminase versus substrate concentration. Reaction mixture; tris buffer (pH 7.8), 0.1 *M*; ammonium chloride, 0.1 *M*; pyridoxal phosphate, 0.1 *mM*; protein, 0.3 mg/ml. Isoleucine concentrations: a = 10 μM; b = 30 μM; c = 100 μM; d = 300 μM

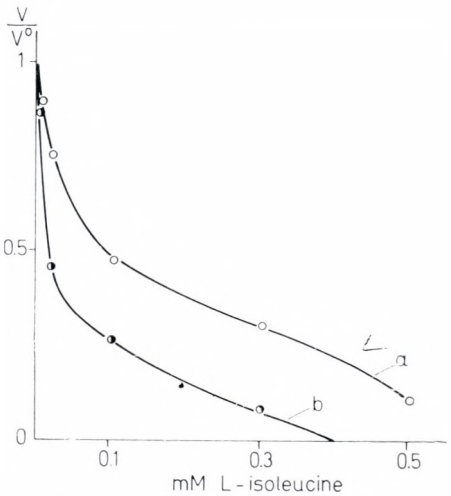

Fig. 8. Isoleucine sensitivity of threonine deaminase in the presence of 10 and 20 *mM* substrate. Reaction mixture: tris buffer (pH 7.8), 0.1 *M*; ammonium chloride, 0.1 *M*; pyridoxal phosphate, 0.1 *mM*; protein, 0.26 mg/ml. a = with 20 *mM* L-threonine, b = with 10 *mM* L-threonine

For example, at 10 *mM* threonine concentration 0.3 *mM* isoleucine caused a practically total inhibition while 0.01 *mM* isoleucine exerted not more than 10% inhibition.

The development of inhibition was the result of a rapid, reversible alteration (Fig. 9). The addition of isoleucine either at zero time or during the reaction always induced the same degree of inhibitory effect. This finding indicates that during the reaction there was no alteration in the isoleucine

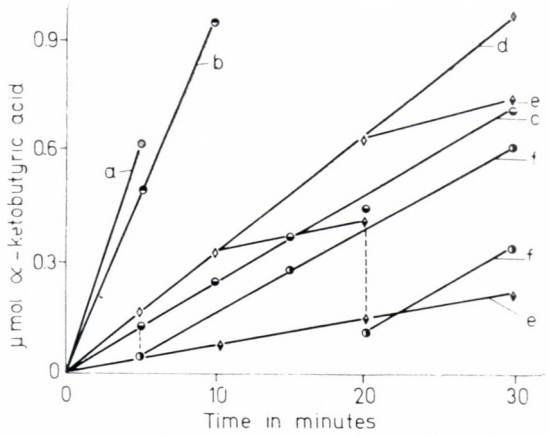

Fig. 9. Inhibition of threonine deaminase by isoleucine. Basal reaction mixture: tris buffer (pH 7.8), 0.1 *M*; ammonium chloride, 0.1 *M*; pyridoxal phosphate, 0.1 *mM*; L-threonine, 20 *mM*; protein, 1 mg/ml. The basal mixture was supplemented with 0.16 *mM* isoleucine. Dilution of isoleucine was performed by adding 3 volumes of enzyme-free reaction mixture to the above system, decreasing in this manner the protein concentration to 0.25 mg/ml and the isoleucine concentration to 0.04 *mM*. The data represent the amount of α-ketobutyric acid produced in 1 ml reaction mixture. a = basal reaction mixture, b = basal reaction mixture with 0.16 *mM* isoleucine, c = basal reaction mixture with 0.04 *mM* isoleucine, d = basal reaction mixture diluted 1 : 4, e = effect of 0.16 *mM* isoleucine added to basal reaction mixture diluted 1 : 4, f = basal reaction mixture with 0.16 *mM* isoleucine diluted 1 : 4 after 5 and 20 minutes incubation

sensitivity of the enzyme. In another series of experiments the reaction rate in an isoleucine-inhibited system was immediately increased on diluting the mixture. After dilution the inhibited state of the enzyme immediately changed so as to correspond to the new concentration of isoleucine.

Effect of valine on enzyme activity. At fairly high concentrations (1—2 *mM*) valine increased the reaction rate (Fig. 10). At higher concentrations the activating effect disappeared. At extremely high concentrations valine itself was inhibitory. Low valine concentrations decreased the inhibitory effect of isoleucine.

Other amino acids failed to influence the reaction rate. D-isoleucine and D-valine were ineffective. Norleucine and norvaline also failed to activate the enzyme at 10^{-2} *M* concentration. The inhibitory effect of D-threonine was competitive.

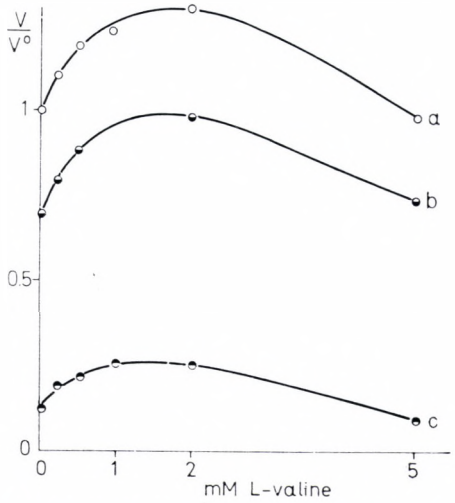

Fig. 10. Effect of valine on threonine deaminase. Reaction mixture: tris buffer (pH 7.8), 0.1 *M*; ammonium chloride, 0.1 *M*; pyridoxal phosphate, 0.1 *mM*; L-threonine, 20 *mM*; protein, 0.25 mg/ml. Reaction time, 20 minutes at 28°C. a = without isoleucine, b = with 40 μM isoleucine, c = with 0.5 *mM* isoleucine

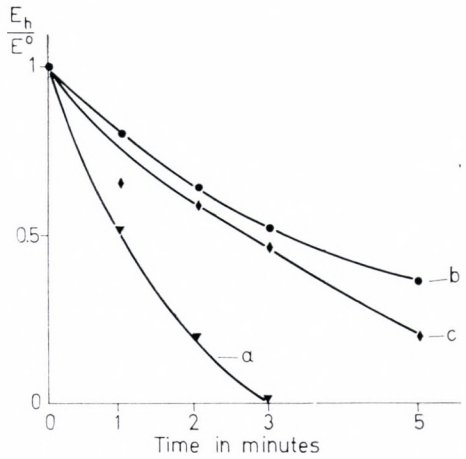

Fig. 11. Heat sensitivity of threonine deaminase. Five mg protein per ml were heated in phosphate buffer containing magnesium sulphate at 55°C. Specimens taken at one minute intervals were assayed for specific activity. Specific activity was determined also in protein solutions heated in the presence of 1 *mM* L-valine and 1 *mM* L-isoleucine. The reaction mixture contained 0.25 mg protein and 0.05 *mM* valine or isoleucine. Reaction time, 20 minutes at 28°C. a = cell-free extract heated in buffer, b = cell-free extract heated with 1 *mM* valine, c = cell-free extract heated with 1 *mM* isoleucine

Heat sensitivity of threonine deaminase. The enzyme was inactivated at 55°C in 3 minutes. Fig. 11 shows the ratio of the reaction rates for heated (E_h) and unheated (E°) enzyme versus the time of exposure. Valine and iso-

leucine protected the enzyme against inactivation approximately in the same degree. Isoleucine sensitivity of the enzyme did not change significantly during heating.

Stability of threonine deaminase at 0°C. If the gel-filtered enzyme solution without cofactors was incubated in ice bath, the specific activity increased and the isoleucine sensitivity decreased in 24 hours. In our opinion isoleucine sensitivity is characteristic of the native state of the enzyme. The decrease in isoleucine sensitivity corresponds to a change from the native state to a beginning degradation process. In the presence of thiamine pyrophosphate the inactivation of threonine deaminase was more definite, but the enzyme's isoleucine sensitivity showed no important change (Table II).

Table II

Effect of cofactors on enzyme stability

Enzyme stored with			Activity after incubation in ice bath for 24 hours	Isoleucine sensitivity (0.1 *mM*) V/V°	Activity after incubation at 37°C for 40 min.	Isoleucine sensitivity (0.1 *mM*) V/V°
TPP 0.1 *mM*	P5P 0.1 *mM*	FAD 0.1 *mM*				
—	—	—	1.01	0.72	0.64	0.85
+	—	—	1.12	0.55	0.80	0.46
—	+	—	0.90	0.77	0.75	0.52
—	—	+	0.98	0.89	0.60	0.90
+	+	—	1.08	0.50	0.82	0.46
+	+	+	0.93	0.51	0.81	0.48
Initial enzyme activity			0.86	0.48		

Phosphate buffer (pH 7.6, 0.4 *M*) containing 3.8 mg protein per ml was incubated with or without cofactor(s) in ice bath for 24 hours or at 37°C for 40 minutes, then the activity of the samples was determined. The figures indicate μ mole ketoacid produced in 1 ml reaction mixture after 20 minutes incubation at 28°C. The reaction mixtures contained 0.38 mg protein per ml.

In the presence of pyridoxal phosphate the specific activity of the solution remained unchanged, but isoleucine sensitivity decreased. Activation observed in the presence of thiamine pyrophosphate was not influenced by pyridoxal phosphate. In protein solution containing valine or isoleucine there was no change in activity during 24 hours. Kinetic experiments could not be performed with protein solutions stored without valine or isoleucine because of unstability under the applied conditions: the reaction rate changed during the reaction time.

Stability of threonine deaminase at 37°C. If the protein solution was incubated at 37°C for 40 minutes without cofactors, specific activity and isoleucine sensitivity decreased. Thiamine pyrophosphate was markedly pro-

tective: the degree of inactivation was slight and there was no important change in isoleucine sensitivity.

Discussion

The reaction rate for ammonium sulphate-precipitated and gel-filtered L-threonine deaminase showed a linear relationship to reaction time and protein concentration. Activity of the enzyme was inhibited by isoleucine. A rise in temperature up to 37°C increased the activity; at higher temperatures the reaction rate decreased steeply. With the rise of temperature isoleucine sensitivity decreased, but at the same time isoleucine exerted a protective action against the inactivating effect of heating. Thus in the presence of this agent there was no reaction rate maximum at 37°C and the amount of α-ketobutyric acid produced from threonine increased slowly and evenly with the increase of temperature.

The reaction rate was greatly influenced by the pH of the system. If the pH was increased from 7 to 8 there was a fourfold rise in activity. Slight changes in the range of pH 8 highly influenced the isoleucine sensitivity of the enzyme. In the range of pH 6—8 isoleucine sensitivity remained unaltered but at pH 8 it decreased rapidly. Activity assays were, therefore, performed always at pH 7.6. A rapid decrease in the sensitivity of the enzyme at pH 8 was reported by BURNS and ZARLENGO for *S. typhimurium* [13].

The saturation curve for the enzyme showed at pH 7.6 a hyperbolic shape corresponding to the Michaelis—Menten kinetics, in other words on reciprocal plotting a straight line was obtained. Above pH 7.6 the saturation curve became sigmoid in shape.

In *S. typhimurium* tested at high ionic strength BURNS and ZARLENGO observed a change of the sigmoid curve into a straight line, and explained the finding with an alteration in the enzyme's quaternary structure [13].

In the presence of isoleucine a rise in substrate concentration resulted in a high increase of the reaction rate even under pH 7.6; accordingly, the hyperbola indicating Michaelis—Menten kinetics in the isoleucine-free system changed into a sigmoid curve. On reciprocal plotting the latter appeared in a concave shape.

Above pH 7.6 and in the presence of isoleucine, however, the sigmoid curve obtained in the isoleucine-free system tended to become hyperbolic. The results were difficult to reproduce since the values were highly influenced by several factors such as pH, temperature, protein concentration and storing conditions. Though the inhibitory effect of isoleucine was considerable, even total inhibition could be suspended with L-threonine at high concentrations.

The structural change of the enzyme protein occurred very rapidly. The addition or dilution of the inhibitory agent caused an immediate alteration in the reaction rate.

Valine suspended or decreased the inhibitory effect of isoleucine. In *E. coli* a similar effect was described by CHANGEUX [6]. If valine was added in the absence of isoleucine the reaction rate increased; above 5 *mM* concentration, however, the activating effect changed into inhibition.

Valine, similarly to isoleucine, stabilized the fairly heat-sensitive enzyme. Heating at 55°C for 3 minutes inactivated the enzyme irreversibly, but after a similar treatment in the presence of isoleucine or valine the enzyme retained 50% of its activity.

Our experiments indicated that although pyridoxal phosphate was bound strongly to the enzyme, dialysis overnight in tris buffer inactivated the enzyme irreversibly. After the addition of pyridoxal phosphate the majority of the activity reappeared.

Adenosine monophosphate, which exerts an activating effect in *E. coli* K—12 [2] and in *Streptomyces rimosus* [14], was ineffective in *M. pellegrino*.

Isoleucine sensitivity of the enzyme on storing at 0°C was remarkably influenced by thiamine pyrophosphate, although this substance is not a coenzyme of threonine deaminase. Pyridoxal phosphate, an active group of the enzyme, exerted no such effect. It is not unlikely that threonine deaminase is loosely bound to acetohydroxyacid synthetase, the coenzyme of which is thiamine pyrophosphate. Perhaps the formation of this association plays a part in the stability of the enzyme. The correctness of this hypothesis seems to be confirmed by the results obtained with protein solution incubated at 37°C, in which thiamine pyrophosphate was found to protect the enzyme.

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VIRAL GROWTH INHIBITION BY A BIGUANIDINE DERIVATIVE IN TISSUE CULTURE

By

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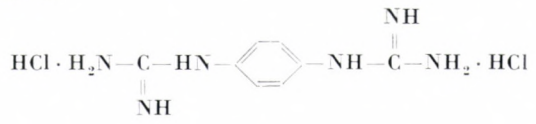
Summary. A biguanidine derivative was examined for toxic and viral growth inhibitory effects in tissue culture. The compound inhibited distinctly and fairly selectively the replication of certain myxovirus and paramyxovirus strains in roller cultures of chorioallantoic membrane fragments.

The antiviral action of biguanidine derivatives has been examined by several authors [1, 2, 3, 7, 8, 9, 10, 11]. The compounds were found to inhibit primarily the growth of myxoviruses and according to KAJI *et al.* [6], also that of adenoviruses. The biguanidine derivative N',N'-anhydrobis-(beta-hydroxyethyl) biguanidine hydrochloride [4, 5] has even been marketed as an antiviral drug under the trade name Flumidin (AB Kabi, Stockholm).

In the present paper, the growth inhibitory action of a biguanidine derivative on some strains of the myxovirus group is reported.

Materials and methods

The compound was prepared in the laboratories of the United Works of Pharmaceutical and Dietetic Products, Budapest,* by the method of BRAUN, ERIT and CROOKS [12]. Molecular weight: 265.16; melting point: > 315°C. It dissolves readily in cold water. Its designation is V-146. Structural formula:



The tissue cultures and media used, the maintenance and storage of the virus strains, plotting of the growth curves and the methods of examination and evaluation of the compound's toxic and viral growth inhibitory effects have been described in detail [13, 14]. The compound's viral growth inhibitory effect was examined by HORVÁTH's [15] roller drum infective titration method in chorioallantoic membrane fragments.

Given concentrations of V-146 and appropriate dilutions of the virus were inoculated simultaneously into the tissue cultures.

At evaluation the concentration lowering the infective titre by at least 1 logarithmic step (base 10) was considered the lowest effective concentration. The selectivity of the effect was expressed in terms of the so-called activity index:

* The compound V-146 used in the experiments was kindly supplied by the United Works of Pharmaceutical and Dietetic Products, Budapest.

$$\frac{\text{Concentration producing } ++ \text{ toxic damage}}{\text{Lowest viral growth inhibitory concentration}}$$

According to previous tests, compounds with an activity index of 100 or higher can be regarded as effective.

Results

Tables I and II show the toxic effect on primary human embryonic fibroblast cell culture and a human amniotic cell line, as plotted against time and concentration of the active substance.

Table I

Toxic effect of compound V-146 on primary human embryonic fibroblast culture

Concentration of active substance, μg/ml	Time of incubation (days)				
	3	4	5	6	7
0	0	0	0	0	0
100	0	+	++	+++	++++
50	0	0	+	++	+++
25	0	0	0	0	0

Table II

Toxic effect of compound V-146 on a human amniotic cell line

Concentration of active substance, μg/ml	Time of incubation (days)					
	2	3	4	5	6	7
0	0	0	0	0	0	0
100	0	+	+++	++++	++++	++++
25	0	0	0	0	0	+
6.25	0	0	0	0	0	0

At a concentration of 100 μg/ml the compound was distinctly toxic to both types of cell culture. At a concentration of 25 μg/ml a slight cell destruction appeared exclusively in the amniotic cell culture by the 7th day. At lower concentrations the compound was not toxic to either cell culture.

Table III shows the compound's growth inhibitory effect on various myxovirus strains. The numbers represent the differences related to the control series not containing the active substance, as expressed in logarithmic terms (base 10).

Table III

Effect of compound V-146 on growth of various myxovirus strains

Concentration of active substance, μg/ml	Influenza A-0/PR8	Influenza A-1/Paris	Influenza A-2/Sing. 1/57	Influenza B/Lee	Parainfluenza-1 (Sendai)	NDV ("H")
50	2.25*	>3.75	3.37	n.e.	3.62	>3.75
25	2.12	>3.75	2.50	n.e.	3.37	>3.50
12.5	1.87	3.25	2.12	n.e.	2.12	3.50
6.25	0.00	2.62	1.96	>3.12	1.25	3.62
3.125	0.00	2.00	1.12	2.37	0.50	2.00
2.00	n.e.	1.62	0.12	1.25	0.25	0.50
1.00	n.e.	1.12	0.00	0.37	0.00	0.00
0.5	n.e.	0.75	n.e.	n.e.	n.e.	n.e.

n.e. = not examined

* The figures indicate the difference in infective titres related to the controls, as expressed in terms of base 10 logarithmic units

The high sensitivity of the Paris and Lee strains was conspicuous, as their growth was still inhibited at concentrations as low as 1 and 2 μg/ml, respectively. The compound was distinctly active against the "H" strain of NDV and influenzavirus A-2/Sing. 1/57, and least active against the strain PR8.

In chorioallantoic membrane fragments the compound exerted a ++ toxic effect at a concentration of 8000 μg/ml and it was on the basis of this value that the activity indices (see Materials and methods) of the strains examined were calculated (Table IV). It should be noted that the activity index of adamantane was 3000 for strain A-2/Sing. 1/57.

Table IV

Activity indices of compound V-146 for various myxovirus strains

Virus strain	Activity index
Influenza A-0/PR8	800
Influenza A-1/Paris	8000
Influenza A-2/Sing. 1/57	2560
Influenza B/Lee	4000
Parainfluenza-1 (Sendai)	1280
NDV ("H")	3200

Next, it was examined how the compound would influence the infective titre of the strain PR8 when added at 50 μg/ml concentration at different points of time during the viral cycle. Results are shown in Fig. 1.

Fig. 1 shows that the compound uniformly inhibited viral growth when added at 0, 2 and 4 hours after viral infection, but failed to do so after 6 hours.

Finally, the eventual direct antiviral effect of the compound was studied by incubating the chorioallantoic fluid containing the PR8 or Sing. 1/57 strain

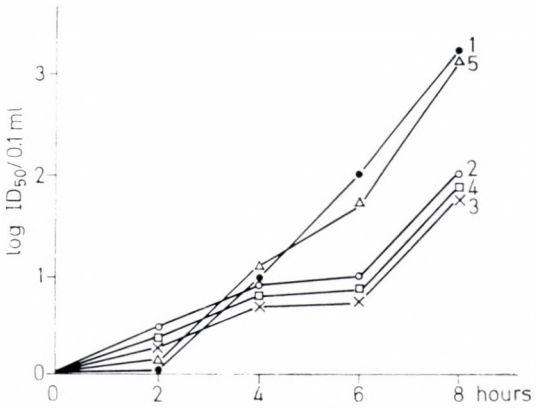


Fig. 1. Effect of compound V-146 on the replication cycle of PR8 influenzavirus. 1 = Control; 2 = active substance added simultaneously with virus inoculation; 3 = active substance added 2 hours after virus inoculation; 4 = active substance added 4 hours after virus inoculation; 5 = active substance added 6 hours after virus inoculation

in the presence of 50 μg/ml of active substance, for 3 hours at 35°C. Subsequently, infectivity titrations were done. The decrease of the infective titre (0.75 ID_{50}) was uniform in both the treated and the control cultures and no change was observed in the haemagglutination titre.

Discussion

The present results clearly indicated that the biguanidine compound V-146 has a notable growth inhibitory effect on several myxovirus strains. Of the strains tested, the growth of influenza A-1/Paris and B/Lee strains was inhibited at concentrations as low as 1 and 2 μg/ml, respectively, a favourable effect in comparison to the active concentrations of other viral growth inhibitors described in the literature.

The toxicity of compound V-146 is low. As to its mechanism of action, it had no direct virocidal effect and since it was still active when added after the 4th hour of the viral cycle, it is supposed to suppress viral maturation processes.

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SEROLOGICAL PROPERTIES OF PSEUDOMONAS AERUGINOSA

II. TYPE-SPECIFIC THERMOLABILE (FLAGELLAR) ANTIGENS

By

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Summary. In specific H antiserum depleted of O agglutinins *Ps. aeruginosa* cells grown on media promoting the development of flagella are first immobilized, then agglutinated into characteristic flaky clumps. Immunogenicity, agglutinability and agglutinin-binding capacity of the H antigens are retained after formalinization but are lost after exposure to 75°C or higher temperatures and to hydrochloric acid. Ethanol destroys agglutinability and impairs immunogenicity and agglutinin-binding capacity. It has been concluded that thermolabile antigens in *Ps. aeruginosa* are associated with the flagella.

Serological typing indicated that out of 541 *Ps. aeruginosa* isolates 288 strains were characterized by serologically uniform antigen H1, and 246 strains by complex antigen H2. The latter cultures were subdivided by partial antigens 2a, 2b, 2c, 2d, 2e and 2f. One strain was non-motile and 6 strains contained undetermined H antigens.

On the basis of various combinations of O antigens described in a previous study and of H antigens a *Ps. aeruginosa* antigenic schema containing 53 serotypes has been devised.

Investigations performed in the last decade have satisfactorily elucidated the nature of somatic thermostable antigens in *Pseudomonas aeruginosa* [5, 6, 9, 12—15]. It was supposed for long that this microorganism contained also thermolabile antigens [1—4, 10, 11], but no evidence was presented that these receptors were associated with flagella or corresponded to the "envelope" or "capsular" antigens usual in the family *Enterobacteriaceae*. The investigations of KLEINMAIER, SCHREINER and GRAEFF [7], KLEINMAIER, SCHREIL and QUINCKE [8] and VERDER and EVANS [14] suggested that thermolabile antigens in *Ps. aeruginosa* may be assigned to flagella.

The purpose of the present work was to continue studies on the serology of *Ps. aeruginosa* [9] and to elucidate the properties of the thermolabile factors. Another aim was to supplement the *Ps. aeruginosa* O antigenic schema with H antigens in order to make it suitable for the determination of serotypes. As, in agreement with the above-mentioned authors, the present data also indicate that thermolabile antigens in *Ps. aeruginosa* are flagellar factors, the term H antigen is used throughout this paper.

Materials and methods

Ps. aeruginosa strains. Twenty-three type strains of the antigenic schema described in [9] and 518 strains isolated from a wide variety of sources and selected so as to represent the average incidence of O antigens were used.

Preparation of immune sera. Agar slant cultures were transferred to U tubes containing semisolid agar (agar, 2 g; nutrient broth, 1000 ml). When the culture had appeared in the other arm of the U tube after a few days incubation at 37°C, it was inoculated onto soft agar medium somewhat thicker than the usual plates: Bacto-agar (Difco), 5 g; Bacto peptone (Difco), 10 g; NaCl, 2 g; K_2HPO_4, 2 g; KH_2PO_4, 1 g; 121°C, 30 minutes.

After incubation at 37°C overnight, about 10 ml 0.42% NaCl solution was pipetted onto each swarm plate. The dishes were left to stand at room temperature for about 1 hour, then the suspensions were transferred to Erlenmeyer flasks and left to stand for 3—4 hours at room temperature. Finally the bacteria were killed with 0.2% formalin.

The density of suspensions for immunization was adjusted so as to correspond approximately to the density of a standard suspension giving 44% transmittance at 1 cm light path in the Beckman DU spectrophotometer set at 0.03 mm slit and 530 mμ wavelength. Rabbits were injected with graded intravenous doses of 0.5, 1.0, 2.0 and 4.0 ml twice weekly. The sera were preserved with 0.5% phenol and stored at 4°C. Heated, alcoholized, saturated NaCl-treated and HCl-treated suspensions used in studying the properties of H antigens were prepared as described in [9].

Tube agglutination. The bacterial suspensions were prepared as antigens used for immunization except that they were preserved with 0.5% formalin and adjusted to 65% density. Tube agglutination was performed with 0.5 ml serum dilution and 0.5 ml bacterial suspension. Readings were made after incubating the tubes at 37°C for 18—20 hours.

Absorption of agglutinins. First O agglutinins were removed by absorbing the serum with the homologous culture heated at 100°C for 1 hour (1 ml serum + 39 ml saline absorbed with bacteria harvested from 5 Roux flasks).

From pure H sera obtained in this manner agglutinins for partial H antigens were removed as follows. After passage in U tube, 0.1 ml culture was transferred into broth. After incubation at 37°C for 4—6 hours a 1 ml aliquot of the culture was pipetted into each Roux flask, the nutrient agar medium in which had been overlayered with 5 ml sterile broth. The Roux flasks were incubated at 37°C overnight, then the culture was harvested. The suspension was left to stand at room temperature for 3—4 hours, then centrifuged. Finally, the bacteria were resuspended in H sera depleted previously of O agglutinins. For absorption of 1 ml undiluted serum, bacteria harvested from 20—40 Roux flasks were usually sufficient. For O or H agglutinin absorption the serum—bacteria mixture was incubated at 37°C for 2 hours, then at 4°C overnight. The absorbed sera were preserved with 0.5% phenol.

Immobilization test. Before studying the specific inhibitory effect of antibodies on motility, phenol-free OH sera were absorbed with the heated suspension of the homologous strain. Then 0.05 ml 18—20-hour broth culture of the organism was pipetted into 0.5 ml serum diluted 1 : 40—1 : 80 with saline. The tubes were incubated at 37°C for 1 hour then motility was examined under the darkfield microscope with low-power objective.

Results

1. *Serological methods for studying type-specific antigens in Ps. aeruginosa.* Although in fresh broth culture most *Ps. aeruginosa* strains showed definite motility, broth was not the best medium for the preparation of H antigens. After passaging in semisolid agar the motility of the cultures improved so that they swarmed on suitable soft agar plates. It should be noted that in U tubes *Ps. aeruginosa* differed in behaviour from motile *Enterobacteriaceae* strains. The diffuse growth travelling away from the site of inoculation was entirely absent in *Ps. aeruginosa* cultures. The successful passage of this organism through the semisolid agar column was indicated by the starting of growth on the surface of the medium in the other arm of the U tube. The passage of less motile strains was improved if prior to inoculation small air bubbles were forced into the semisolid medium by means of a sterile pipette. The time of travelling through the U tubes was variable: the first passage in a semisolid

agar column 8—10 cm in length took 2—10 days; the time needed for subsequent passages was usually shorter.

The best antigen for H agglutination was obtained when soft agar medium thicker than the usual plate was used. From *Ps. aeruginosa* it was more difficult to prepare suitable H antigens than from *Enterobacteriaceae*. The swarming of *Ps. aeruginosa* was highly influenced by the quality of agar, peptone and other ingredients of the medium. The medium found the most suitable contained only Bacto peptone and Bacto agar in addition to minerals. If the suspension harvested from this medium was left to stand at room temperature for a few hours before formalinization, the agglutinability of the antigen definitely improved as compared to bacteria killed immediately after washing off (disentangling of flagella?).

Despite of formalinization, dense suspensions of many strains showed a partial clearing during storage, as if the bacteria had been undergoing lysis. In dense suspensions many strains had lost their homogeneity and the bacteria formed flaky aggregates. In order to prevent these difficulties the suspensions were prepared with 0.42% sodium chloride and their density was adjusted so that instead of 0.5 ml serum dilution + 0.05 ml antigen employed in O agglutination, 0.5 ml serum dilution and 0.5 ml antigen were pipetted into the tubes. Cultures failing to give homogeneous suspensions even in this manner were homogenized by mild shaking for about 30 minutes before formalinization. *Ps. aeruginosa* suspensions prepared as described above gave in specific sera flaky, loose clumps easy to shake up. The type of agglutination was similar to that shown by *Enterobacteriaceae* H antigens.

Differentiation of H antigens and thermolabile somatic antigens (the existence of which in *Ps. aeruginosa* had been supposed by some authors) was attempted by culturing the strains on media disadvantageous for the development of flagella: blood agar containing sodium desoxycholate and agar supplemented with anionic detergent. The development of flagella was definitely decreased but not entirely suppressed on these media. The cultures were weakly and much less typically agglutinated in pure H sera than bacteria passed through U tubes and subcultured in broth or on swarm agar plates. As compared to bacteria with well-developed flagella, organisms harvested from inhibitory media produced H antibodies lower in titre. When applied in sufficient amounts, cultures with less developed flagella completely absorbed H antibodies.

Slide agglutination was unsuitable for the reliable determination of H antigens, although the soft agar culture of many strains reacted readily in pure H sera.

2. *Specific immobilization.* Under suitable conditions the motility of *Ps. aeruginosa* is inhibited by specific H serum. The experiments were performed with broth cultures in which the majority of cells showed rapidly advancing

movements characteristic of polar flagellate bacteria. To the serum dilutions relatively small numbers of bacteria were added since microscopic agglutination taking place in dense suspensions disturbed the reading of the immobilization reaction. The best results were obtained in systems incubated for 1 hour. In specific serum the active movement of the cells ceased or was reduced to a few cells, while in the control sera (antisera not reacting in the tube agglutination test) the majority of cells showed brisk motility. When the mixture of serum and bacteria was incubated overnight, considerable multiplication took place and many actively motile rods were detected not only in the control but also in the specific H serum.

Immobilization and agglutination tests yielded identical results for all *Ps. aeruginosa* type strains. Strains characterized by H antigen 1 showed complete cross-immobilization in the corresponding H sera. Sera prepared from strains containing complex H antigen 2 failed to inhibit the movement of H1 cultures. Among H2 strains those bearing identical H antigens inhibited the motility of one another. At lower serum dilutions a definite cross-immobilization was demonstrated between H2 strains containing different partial H antigens. H1 sera did not inhibit H2 cultures.

The inhibitory effect of specific serum on motility was more or less definitely demonstrated in semisolid agar: in U tubes supplemented with specific H serum the culture appeared in the other arm 3—8 days later than in U tubes containing the control serum.

3. *Properties of Ps. aeruginosa H antigens. Agglutinability.* Experiments shown in Table I were performed with motile cells of strain 170 002 harvested from swarm plates. After heating or exposure to chemical agents the bacteria were used as antigens for immunization and for agglutination. Prior to the agglutination test the O agglutinins were absorbed from the sera with heated cells of the homologous strain. From Table I it is evident that in H agglutination there was no difference between living and formalinized cells. Bacteria treated with saturated sodium chloride or heated at 60°C for 1 hour showed weak, indefinite agglutination in the first tubes. Agglutinability of the culture was lost after heating at 75°C or higher and after ethanol and hydrochloric acid treatment.

Immunogenicity. Table I shows that living and formalinized bacteria produced H agglutinins in high titres. Immunogenicity was also unaffected by heating at 60°C for 1 hour and by treatment with saturated sodium chloride. Alcoholized bacteria induced H agglutinins only in traces. H immunogenicity was lost after heating at 75°C or higher as well as after hydrochloric acid treatment.

Agglutinin-binding capacity is shown in Table II. It is evident that H agglutinins were completely removed not only by living and formalinized bacteria, but also by cultures subjected to heating at 60°C and to saturated

Table I

Effect of heat and chemical agents on the agglutinability and immunogenicity of Ps. aeruginosa H antigens

(Reciprocal agglutination titres in tubes incubated at 37°C for 20 hours. Prior to testing O agglutinins were absorbed with the homologous heated culture.)

Antigens	Antigens used for immunization						
	170 002 living	170 002 1 hr. 60°C	170 002 1 hr. 75°C	170 002 0.5% formalin	170 002 saturated NaCl	170 002 50% ethanol	170 002 *N* HCl
170 002 living	20 480	10 240	0	10 240	10 240	160	0
170 002 1 hr. 60°C	×	×	0	×	×	0	0
170 002 1 hr. 75°C	0	0	0	0	0	0	0
170 002 0.5% formalin	10 240	5 120	0	5 120	2 560	80	0
170 002 saturated NaCl	×	×	0	×	×	0	0
170 002 50% ethanol	0	0	0	0	0	0	0
170 002 *N* HCl	0	0	0	0	0	0	0
170 002 2 1/2 hr. 100°C + 1 hr. 130°C*	0	0	0	0	0	0	0

* Control for O agglutinin absorption
× = Weak agglutination in the first tubes

Table II

Effect of heat and chemical agents on the agglutinin-binding capacity of Ps. aeruginosa H antigens

(Reciprocal agglutination titres in tubes incubated at 37°C for 20 hours. Prior to absorption with antigens treated in different manners the O agglutinins were removed with the homologous heated culture.)

Formalinized antigens	Serum 170 002 prepared with formalinized antigen and absorbed by							
	–	170 002 living	170 002 1 hr. 60°C	170 002 1 hr. 75°C	170 002 0.5% formalin	170 002 saturated NaCl	170 002 50% ethanol	170 002 *N* HCl
170 002	5120	0	0	5120	0	0	×	5120
170 008	5120	0	0	5120	0	0	×	5120

× = Weak agglutination in the first tubes
Strains 170 002 and 170 008 contained identical H, but different O antigens

sodium chloride. Absorption by alcoholized antigen decreased the H agglutinin titre; this partial absorption could, however, be attained only with great amount of alcoholized bacteria. The H agglutinin content of the sera was not influenced by absorption with bacteria heated at 75°C or higher. Acid treatment also resulted in a total loss of H agglutinin-binding capacity.

Table III presents the properties of *Ps. aeruginosa* H antigens compiled on the basis of the above experiments. Essentially the same results were obtained in preliminary experiments with other strains [9]. The only difference

Table III

Properties of H antigens of Ps. aeruginosa

Antigen	Agglutinability	Immunogenicity	Agglutinin-binding capacity
Living	++++	++++	++++
1 hr. 60°C	+	++++	++++
1 hr. 75°C or higher temperature	–	–	–
0.5% formalin	++++	++++	++++
Saturated NaCl	+	++++	++++
50% ethanol	–	+	++
N HCl	–	–	–

was that the method used in the present work allowed an optimal development of flagella and, consequently, the titres were higher and the readings were more reliable.

Phase variation. An attempt was made to induce H phase serologically different from the original flagellar antigen. As the results were negative with a number of strains tested in broth and U tubes containing homologous H serum, it has been concluded that *Ps. aeruginosa* is probably characterized by monophasic H antigens.

4. *Diagnostic antigenic schema.* Cross-agglutination experiments indicated that in respect of H antigens, *Ps. aeruginosa* isolates can be divided into two groups. One of these, the characteristic H antigen of which has been termed H1, is practically uniform in flagellar antigenic structure. Strains belonging to the other group (H2) are related by one or more common partial H antigens. H1 and H2 strains differ sharply in antigenic structure: cross reactions between them are insignificant and unfrequent.

In H1 strains cross-absorption revealed only one unimportant partial

Table IV

Cross-absorption experiments with H1 antigens

(Reciprocal agglutination titres with formalinized antigens in tubes incubated at 37°C for 20 hours. Prior to H agglutinin absorption the O agglutinins were removed with the homologous heated culture.)

Strain	H serum 170 001 absorbed by		H serum 170 003 absorbed by	
	–	170 003	–	170 001
170 001	20 480	160	20 480	0
170 003	20 480	0	20 480	0
Agglutinins	1a, 1b	1b	1a	–

Result of antigenic analysis: 170 001 = 1a, 1b
170 003 = 1a

Table V

Cross-absorption experiments with H2 antigens

(Reciprocal agglutination titres with formalinized antigens in tubes incubated at 37°C for 20 hours. Prior to H agglutinin absorption the O agglutinins were removed with the homologous heated culture.)

Strain	H serum 170 002 absorbed by					H serum 170 016 absorbed by				
	–	170 016	170 012	170 018	170 021	–	170 002	170 012	170 018	170 021
170 002	5120	2560	5120	2560	2560	640	0	0	1280	0
170 016	160	0	0	0	0	1280	640	320	2560	0
170 012	80	0	0	160	160	1280	0	0	5120	0
170 018	1280	160	2560	0	2560	160	0	0	0	0
170 021	0	0	0	0	0	640	320	80	640	0
Agglutinins	(2a), 2b	2b,...	2b,...	2b,...	2b,...	2a, 2c	2c	2c	2c,...	—

Strain	H serum 170 012 adsorbed by					H serum 170 018 adsorbed by				
	–	170 002	170 016	170 018	170 021	–	170 002	170 016	170 012	170 021
170 002	160	0	0	0	0	320	0	0	160	0
170 016	640	0	0	0	0	0	0	0	0	0
170 012	5120	1280	1280	1280	1280	80	160	0	0	0
170 018	160	80	160	0	160	5120	5120	2560	5120	5120
170 021	160	0	0	0	0	640	640	640	80	0
Agglutinins	2a, 2d	2d	2d	2d	2d	(2a), (2d), 2e, 2f	(2d), 2e, 2f	2e, 2f	2e, (2f)	2e

Strain	H serum 170 021 absorbed by				
	–	170 002	170 016	170 012	170 018
170 002	640	0	0	640	0
170 016	1280	1280	0	640	2560
170 012	320	0	0	0	0
170 018	2560	1280	320	1280	0
170 021	2560	1280	640	1280	1280
Agglutinins	2a, 2c, 2f	2c, 2f	2f	2c, 2f,...	2c

Results of antigenic analysis:
170 002 = (2a), 2b
170 016 = 2a, 2c
170 012 = 2a, 2d
170 018 = (2a), (2d), 2e, 2f
170 021 = (2a), 2c, 2f

antigen present in a small number of strains (Table IV). This insignificant factor (H1b) was not included in the antigenic schema. In H2 strains, in contrast, certain well-defined, diagnostically important partial antigens were demonstrated (Table V).

Data shown in Table V summarize the result of repeated absorption experiments performed in sera prepared in at least two different rabbits. From Table V it is seen that strains containing complex antigen H2 are characterized not only by bilateral but also by unilateral antigenic relationships. Sera prepared with strains 170 016, 170 012, 170 021 and others agglutinated all strains containing complex antigen H2; the factor responsible for this reaction was termed antigen H2a. At 1 : 80 dilution sera for several strains (*e.g.* 170 002 and 170 018) failed to agglutinate certain other H2 strains. This finding indicated that antigen H2a was weakly developed in such strains or gave a unilateral reaction. From Table V it is also evident that in H2 strains other unilaterally reacting antigens may also be present. As the purpose of the present work was to establish a practical diagnostic schema, only well-defined antigens giving bilateral reactions were used for typing. These antigens were designated by symbols 2b, 2c, 2d, 2e and 2f.

Only 1 out of the 541 strains examined was non-motile. This strain, the type culture for O antigen subgroup 4a, 4c, had probably lost its motility in the course of laboratory maintenance, as in a serum prepared with its freshly isolated culture years ago, H2 agglutinins were demonstrated in the present study.

Table VI presents the division into serotypes of *Ps. aeruginosa* O antigen groups and subgroups [9]. It should be noted that several strains designated with the same antigenic formula were not always identical in antigenic structure; such strains failed to give complete cross-absorption, only decreased the H agglutinins of one another considerably. In view of the practical aim of serological typing these minor differences were not included in the antigenic schema.

The last column of Table VI presents the distribution of the 541 strains according to serotypes. Although the strains had been isolated from a wide variety of sources, in most serogroups there was but one predominant type. Strains bearing two or three different H antigens occurred in larger numbers only in serogroups 2, 5a, 5b, 5d, 6, 10a and 11. Many of the unfrequent serotypes, represented by a few isolates or by one single strain, had been isolated from water, sewage and other materials, though the majority of cultures originated from clinical specimens.

The number of cultures belonging to serotypes represented by 4 strains or less was 45, out of which 22 had been isolated from clinical materials, 17 from water or sewage, and 6 from miscellaneous faecal specimens.

Table VI

Ps. aeruginosa diagnostic antigenic schema
Number of strains: 541

Group	O antigen	H antigen*	Type strain	Number of strains
	1	1	170 001 (Ps 304)	62
1	1	2a, 2b	171 059	1
	1	2a, 2c	171 031	1
	2	1	171 076	11
2	2	2a, 2b	170 002 (Ps 21)	10
	2	2a, 2c	171 074	1
	3a, 3b	1	170 003 (Ps 11)	25
	3c	1	170 004 (Ps 340)	25
	3a, 3d	1	170 005 (Ps 317)	35
3	3a, 3d	2a, 2b, 2f	171 175	1
	3a, 3d, 3e	1	170 006 (Ps 469)	12
	3a, 3d, 3e	2a, 2c	171 188	1
	3d, 3f	1	170 007 (Ps 48)	24
	3d, 3f	2a, 2b, 2f	171 192	1
	4a, 4b	2a, 2b	170 008 (Ps 161)	17
	4a, 4b	2a, 2d, 2e, 2f	171 289	1
	4a, 4c	—	170 009 (Ps 217)	1
	4a, 4c	1	171 230	1
4	4a, 4c	2a, 2b, 2f	171 252	16
	4a, 4c	2a, 2c	171 269	3
	4a, 4c	2a, 2c, 2f	171 248	1
	4a, 4d	2a, 2b, 2f	170 010 (Ps 323)	24
	4a, 4d	2a, 2c, 2f	171 272	2
	5a, 5b, 5c	1	170 011 (U 645)	6
	5a, 5b, 5c	2a, 2d	171 304	1
	5a, 5b, 5d	1	171 312	5
5	5a, 5b, 5d	2a, 2d	170 012 (Ps 319)	8
	5a, 5d	1	170 013 (Ps 194)	29
	5a, 5d	2a, 2c	171 348	2
	5a, 5d	2a, 2d	171 338	1
	6	1	171 350	12
6	6	2a, 2b	170 014 (U 72/59)	18
	6	2a, 2c	171 365	11

Table VI continued

Group	O antigen	H antigen*	Type strain	Number of strains
7	7a, 7b	1	171 401	4
	7a, 7b	2a, 2c	170 015 (U 118/59)	42
	7a, 7b	2a, 2d	171 420	4
	7a, 7c	1	171 447	1
	7a, 7c	2a, 2b	171 445	1
	7a, 7c	2a, 2c	170 016 (B 415)	3
8	8	1	170 017 (U 900/60)	3
9	9	2a, 2d	171 453	2
	9	2a, (2d), 2e, 2f	170 018 (Ps 910)	1
10	10a	1	170 019 (Ps 196)	1
	10a	2a, 2b, 2f	171 464	13
	10a	2a, 2c	171 455	10
	10a, 10b	1	171 478	4
	10a, 10b	2a, 2c	170 020 (Ps 275)	1
11	11	1	171 483	15
	11	2a, 2c	171 492	5
	11	2a, 2c, 2f	170 021 (Ps 898)	11
12	12	1	170 022 (L 83)	5
	12	2a, 2c	171 518	1
13	13	1	170 023 (V 142a)	1
Total				497

* Antigen H2a is in many strains weakly developed

Strains not included in the schema:

2: ND	1	10a, 10b: 2a,...	1
3a,... : 1	6	11: ND	1
3a,3d: ND	2	ND: 2a, 2b	3
4a,...: 1	1	ND: 2a, 2c	4
4a,...: 2a,...	3	ND: 2a, 2c, 2f	1
4a,...: 2a, 2b	15	ND: ND	2
4a,...: 2a, 2c	3		
4a,...: 2a, 2c, 2f	1	Total	44

In order to study a wide variety of strains, the isolates were chosen so as to represent as many hospital units as possible. Accordingly, only limited conclusions could be drawn as to the stability of the serotypes. It may be stated that, as a rule, the same serotype or serotypes occurred in patients treated in the same ward and there was no alteration on repeated examinations. The serotypes were stable *in vitro*: on subculturing or freeze-drying they showed no change in antigenic structure.

5. *Routine determination of H antigens.* The single tube technique is suitable for routine examination. The bacterial suspension is prepared as described under "Materials and methods" and added at 0.5 ml aliquots to tubes containing 0.5 ml adequately diluted (not cross-reacting) "H1" and "polyvalent H2" sera. The tubes are incubated at 37°C overnight, then read. Strains showing typical H agglutination in serum "H1" are regarded as bearing antigen H1. If characteristic H agglutination occurs in serum "polyvalent H2", then the suspension is tested in a similar manner in absorbed sera H2b, 2c, 2d, 2e and 2f and the result is read on the next day. For determining antigen H2a, the sera must be prepared from two different strains containing well-developed H2a antigen. Preparation of H sera for routine typing is presented in Table VII.

Table VII

Determination of the H antigens of Ps. aeruginosa

H antigens	Tube agglutination in
1	Serum 170 001 absorbed by 170 001 1 hr. 100°C culture
2 complex	Polyvalent serum pool "H2" (mixture of H sera 170 002, 170 016, 170 012, 170 018 and 170 021, each absorbed by heated homologous culture)
2a	Serum 170 016 absorbed by 170 016 1 hr. 100°C culture and serum 170 012 absorbed by 170 012 1 hr. 100°C culture
2b	Serum 170 002 absorbed by 170 002 1 hr. 100°C + 170 012 living + 170 018 living culture
2c	Serum 170 016 absorbed by 170 016 1 hr. 100°C + 170 002 living culture
2d	Serum 170 012 absorbed by 170 012 1 hr. 100°C + 170 002 living culture
2e	Serum 170 018 absorbed by 170 018 1 hr. 100°C + 170 021 living culture
2f	Serum 170 021 absorbed by 170 021 1 hr. 100°C + 170 016 living culture

Discussion

The first investigators of the serological properties of *Ps. aeruginosa* were unable to distinguish clearly between O and H antigens. BRUTSAERT [1] in 1924 attempted to show O and H antigens in this species. He prepared O antigens by boiling the cultures for 2 hours — a procedure certainly destroying H antigens. His choice for "O + H" antigens — exposure of the suspension to 56°C — was, in view of our present knowledge, less fortunate, since this antigen is suitable neither for O nor for H agglutination. GABY [3] used formalinized and alcoholized suspensions for the demonstration of H and O agglutinins, respectively. As shown by subsequent authors, in sera containing O agglutinins the determination of H antigens is difficult or impossible even with formalinized suspensions, and alcoholized bacteria are unsuitable for the determination of O antigens.

MAYR-HARTING [11] was the first to conclude that the classical methods were inadequate for studying the serology of *Ps. aeruginosa*, and described that the organisms retained their immunogenicity but lost their agglutinability after heating and alcohol treatment. Unlike other authors, who had assumed that the agglutination of living *Ps. aeruginosa* was due entirely to flagellar antibodies, she regarded the reaction correctly as O agglutination.

CHRISTIE [2] in 1948 divided 138 *Ps. aeruginosa* strains into 13 serological groups. By means of CHRISTIE's method FOX and LOWBURY [4] and LOWBURY and FOX [10] prepared sera with formalinized bacteria and tested the agglutination with living and alcoholized suspensions. They regarded the high titre reaction of the former as H and the low titre reaction of the latter as O agglutination. As mentioned above, in unabsorbed OH serum living bacteria react with somatic antibodies so strongly that H agglutination remains totally suppressed.

HABS [5] in 1957 by the use of heated antigens and sera prepared against them, established the first antigenic schema suitable for the precise determination of *Ps. aeruginosa* O antigens. On the basis of HABS's schema KLEINMAIER, SCHREINER and GRAEFF [7] have shown that the agglutination of thermolabile antigens can be distinguished from that of thermostable antigens only in sera which have been deprived of antibodies reacting with heat stable factors. They showed a marked difference between granular-type agglutination due to thermostable antigens and fluffy-type agglutination due to thermolabile antigens. In their excellent study they, however, failed to answer the question whether thermolabile antigens were associated with flagella. The precautious approach of the problem was justified, since in view of the many kinds of thermolabile somatic antigens in the family *Enterobacteriaceae* several authors had suggested the presence of such receptors in *Ps. aeruginosa*. That KLEINMAIER *et al.* assumed these factors to be H antigens is indicated by the fact that — for the first time in the history of *Ps. aeruginosa* serology — they laid stress upon preparing their antigens from actively motile cultures and worked on a medium allowing optimal development of flagella. In 1959, the morphological studies of KLEINMAIER, SCHREIL and QUINCKE [8] made it very probable that the thermolabile factors were flagellar antigens.

In 1961 VERDER and EVANS [14] devised an antigenic schema of *Ps. aeruginosa* O and H antigens. They described the thermolabile antigens definitely as H factors, though they remarked that somatic thermolabile antigens might also be present. The technique used by VERDER and EVANS conforms to modern principles of bacterial serology. They determined O antigens with heated suspensions in sera containing pure O agglutinins. For H antigen determination they used formalinized motile cultures and sera prepared with motile bacteria and absorbed with heated suspension in order to remove O agglutinins. Only the amount of antigen applied by them in cross-absorption

experiments for the demonstration of H partial antigens seems to be too small. In the present studies, for the complete absorption of H partial agglutinins considerably higher (about tenfold) amounts of bacteria were needed.

The present study has furnished a further proof of the thermolabile antigens in *Ps. aeruginosa* being associated with flagella: characteristic H agglutination was obtained only with bacteria bearing well-developed flagella and only in sera prepared with such cultures; bacteria grown on media unfavourable for the development of flagella agglutinated much less readily than cells cultured on the medium devised for H antigen production; there was a close agreement between agglutination and immobilization test in specific sera.

The statement of KLEINMAIER *et al.* and of VERDER and EVANS that, because of the strong reactivity of somatic and the weak reactivity of flagellar antigens, the latter should be determined in sera free from O agglutinins, has also been confirmed. H antigens may be determined in unabsorbed OH sera prepared with strains different in O antigen from the culture under investigation. As the removal of O agglutinins presents no difficulties (in fact, if the suspension is re-heated, it can be used for repeated absorptions) it is more advisable and more simple to use pure H sera.

The determination of *Ps. aeruginosa* H antigens is not an easy task even with suitable pure H sera, as special methods are needed for the preparation of homogeneous suspensions giving typical agglutination. An important aim of this study was, therefore, to elaborate the proper technique of the preparation of antigens. Macroscopic H agglutination of *Ps. aeruginosa* cells is a slow procedure and thus slide agglutination has not been found suitable for the purpose.

Although the H antigens of *Ps. aeruginosa* and of *Enterobacteriaceae* may be similar in physicochemical properties, it is clear that they differ in certain other respects, especially as regards the conditions suitable for their determination. Thus it is no wonder why several authors' contribution was needed to elucidate the nature of *Ps. aeruginosa* H antigens.

It is interesting that after treatment with saturated sodium chloride *Ps. aeruginosa* H antigens lose their agglutinability but retain their immunogenicity and agglutinin-binding capacity. A similar effect was observed after heating at 60°C. The phenomenon is probably due to slime production assumed to be associated with the swelling of a non-antigenic envelope covering *Ps. aeruginosa* cells [9].

The method elaborated in this study for the practical determination of H antigens, though not so simple as slide agglutination for grouping according to O antigens, is suitable for a further differentiation of *Ps. aeruginosa* isolates. The H antigenic schema presented has been devised for practical purposes and contains, therefore, only well-defined, stable antigens.

As shown in Table VI, 497 out of 541 strains could be serotyped according

to the schema. The remaining 44 strains were not included in the schema because of irregular or not determined O antigens (34 strains), H antigens (5 strains) or O and H antigens (5 strains).

As to the practical usefulness of differentiation of *Ps. aeruginosa* serogroups and subgroups into serotypes by H antigens, the following may be said. The serotypes have proved stable *in vitro* and, as far as it may be concluded from data for isolates collected from a wide variety of sources, they do not change their antigenic structure *in vivo*. Thus, determination of H antigens in addition to O grouping seems to be suitable for epidemiological examinations. On the other hand, the value of differentiation by H antigens is limited to a certain degree, as the overwhelming majority of strains belonging to frequent serogroups and subgroups (O1, O3a, 3b, O3d, 3f, O4a, 4c, O4a, 4d, O5a, 5d, O7a, 7b) contained in each group one kind of H antigen. In groups O6 and O11, which were also frequent, strains characterized by different H antigens were distributed more or less evenly, so that a complete serological typing of such cultures may be of more practical value.

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ISOLATION AND CHARACTERIZATION OF SOME NEWLY ISOLATED B. CEREUS PHAGES

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Summary. The isolation and properties of phages newly isolated from two groups of *B. cereus* differing in cell wall structure are described. The newly isolated phages produced clear, target-like and turbid types of plaques. The turbid (wild) type contained clear and target-like plaque mutants in 5—10%. Examination of the host range revealed that part of the isolates were type specific and the rest group specific. Some of the phages were of polyvalent nature, being specific for both *B. anthracis* and *B. cereus*. Antigenically there was a sharp difference between the phages of the two *B. cereus* groups. The newly isolated *B. cereus* phages were examined also for burst size, latency period, and sensitivity to heat, pH and UV irradiation.

Isolation of several *B. cereus* phages was reported previously [4]. It seemed worthwhile to examine whether these isolates were suited for the genetic study of *B. cereus* and whether they might be used in transfection experiments. Therefore, further *B. cereus* phages have been isolated and studied.

The phages of *B. cereus* have a considerable influence on *B. anthracis* strains. This phenomenon is ascribed to the phylogenetic relationship of the two bacterial species [5, 7]. Phages highly specific for *B. anthracis* were isolated from a lysogenic strain of *B. cereus* [11, 12]. Many phages acting on both *B. cereus* and *B. anthracis* were isolated from soil samples [3, 6, 14, 15]. Strains resistant to streptomycin were found particularly suitable for the isolation of *B. anthracis* phages [6] and the method has been adapted for isolation of *B. cereus* phages [4]. Phages isolated from soil samples on *B. cereus* are markedly active also against *B. anthracis* [15]. As to the designation of these phages, there are certain nomenclatural differences depending on their isolation on *B. cereus* [3, 14, 15] or *B. anthracis* [9]. However, their nomenclatural differentiation is not justified before their systematic positions have been cleared. They very likely represent host range mutants of one and the same phage according to the organism on which the isolation had been made. It has therefore been suggested to term such phages "A phage" [9]. Attempts have already been made for the systematic classification of such phages on the basis of biological properties [9]. Some biologically dissimilar phages have distinct receptors in the cell wall of *B. anthracis* [10]. The phages designated A, B, C and D, isolated by Norris [13] on *B. entomocidus*, act exclusively on the *B. cereus* strains not producing lecithinase.

Thus the phages described in the literature and partly available for study were on the one hand polyvalent and on the other hand specific for not satisfactorily characterized strains. The present experiments were undertaken with the purpose to isolate *B. cereus* phages with fairly limited specificity for the bacterial strains used in the previous experiments [5, 7] and characterized by their biochemical and antigenic properties.

Materials and methods

Media. "PY" medium, containing 1% of peptone, 0.25% of yeast extract (YE, DIFCO) and 0.5% of NaCl. The medium was solidified with 1.5% of agar. For use as a diluent, the PY medium was diluted 1 : 5 with saline.

Bacterial strains. The aerobic sporogens *B. anthracis*, *B. cereus* and *B. subtilis* were of different origin. In the experiments, spore suspensions of strains from our collection [5] were used. The biochemical activity of the strains was examined by the lecithinase [16, 17], phosphatase [2] and haemolysis tests and the strains were tested for penicillin sensitivity as described earlier [5].

Isolation of the phages from soil samples was carried out with the streptomycin method [6] adapted by us for the isolation of *B. cereus* phages [4].

Sensitivity to UV irradiation of the phages examined was tested with a Hanau UV-lamp, using LATARJET's apparatus and method [8] for measuring the radiated energy. The phages were exposed in PY diluent.

Thermosensitivity was examined at 56°C and assessed by determining the survivor phage count.

Sensitivity to pH was estimated in acetate buffer solution. To aliquots of 0.5 ml acetate buffer solution adjusted to different pHs, the phage suspension was added in equal volumes and the mixtures were incubated at 37°C. In samples taken at different time intervals the viable phage count was determined by titration.

Host-range examinations were made by placing 10^{-4}, 10^{-6} and 10^{-8} dilutions of phages with 10^9 titre on bacterial cultures embedded in a soft layer of PY agar.

Phage adsorption experiments were carried out in PY medium containing 0.002 *M* $CaCl_2$. From the samples taken at different time intervals, the bacteria were removed by centrifugation and the non-adsorbed phage content was determined by titration. Adsorption rate was calculated according to ADAMS [1].

One-step growth curves were plotted on the basis of ADAMS' [1] standard experimental method.

Immune sera for serological examination were prepared in rabbits and the K-values were measured according to ADAMS [1].

Results and discussion

Isolation of phages was attempted from soil samples taken in different areas and designated with serial numbers from 1 to 5. The suspension prepared from the samples in PY medium was divided into two parts and enriched selectively as described earlier [4]. To one part, *B. cereus*-569 was added, whose cell wall extract does not precipitate the anthrax antiserum [7]. To the other part, *B. cereus*-114 cells were added whose cell wall polysaccharide does precipitate the anthrax antiserum. The enriched suspensions were aerated and incubated at 37°C for 4—6 hours. Subsequently, bacterial cells were removed by centrifugation and the supernatant was examined for the presence of phages. The strains *B. cereus*-569 and *B. cereus*-114 were used as indicator bacteria.

On the basis of plaque morphology, the phages isolated from the soil samples were of three different types, *viz.* clear, target-like and turbid, which were designated *a* (clear), *b* (target-like) and *c* (turbid) with the *B. cereus*-569 host and α, β and γ, respectively, with the *B. cereus*-114 host. Thus the following designations of the isolated phages were used: CPa, CPb, CPc and CPα, CPβ, CPγ, respectively. The Roman numerals attached as indices indicate the numbers of the soil samples.

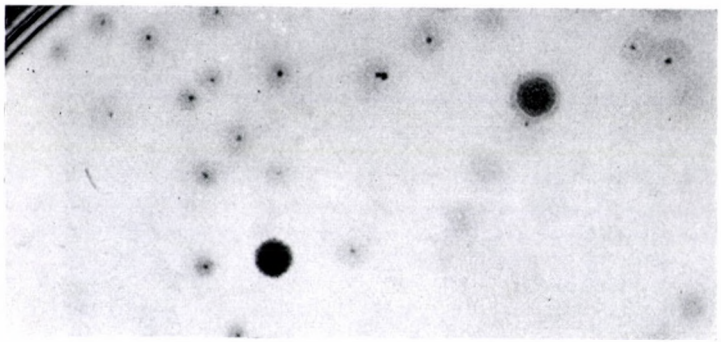

Fig. 1. Phage CPγ_1: Clear, target-like and turbid plaques

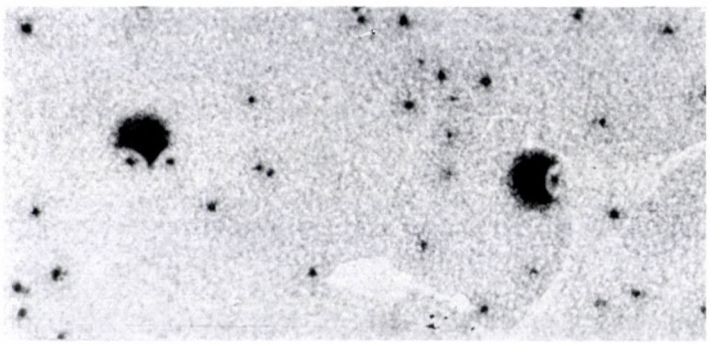

Fig. 2. Phage CPγ_1: Lysis inhibition and turbid plaques

The optimal medium for the propagation of the phages was the PY medium, which was supplemented with 10^{-3} *M* and 10^{-4} *M* $MnSO_4$ for *B. cereus*-569 and *B. cereus*-114 phages, respectively.

The phages CPa, CPb, CPα and CPβ were isolated in pure form and their plaque diameters were 4—5 mm. The plaques of the CPc and CPγ phages were 1—2 mm in diameter, and clear and target-like types were always present in 5—10%. The CPγ phages showed a conspicuous lysis inhibition. This phenomenon and the basic plaque types are shown in Figures 1, 2 and 3.

For host range studies, 10 pathogenic strains each of *B. anthracis*, *B. subtilis* and *B. megatherium*, as well as 25 *B. cereus* strains producing leci-

thinase, phosphatase and haemolysin and resistant to penicillin, were used. For control, experiments were set up with McCLOY's Wα phage. Results are shown in Table I.

As shown in Table I, (1) the phages propagating on *B. cereus*-569 (CPa, CPb, CPc) had no effect on the *B. anthracis* strains, while (2) some of the phages propagating on *B. cereus*-114 (CPα, CPβ) had a lytic effect also on *B. anthracis*, owing to common polysaccharide components in the cell wall [7]. (3) The differentiated host range specificity of the phages was noteworthy,

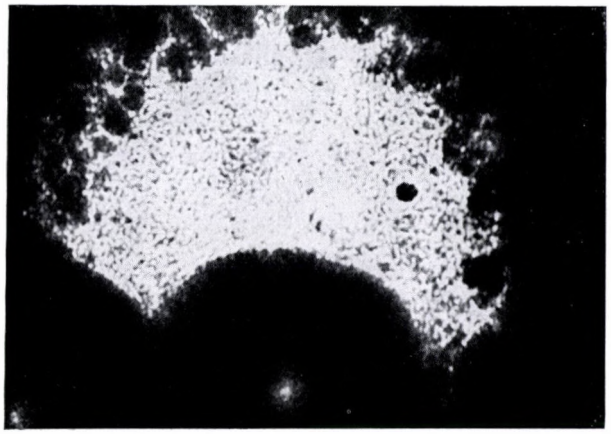

Fig. 3. Lysis inhibition of CPγ_1 by CPγ_1 turbid plaque

as e.g. the phages CPa, CPb were strictly type specific, with minute differences in the specificity range. (4) Phage CPα_1, but particularly CPβ_2 were of polyvalent nature: they had a lytic effect not only on anthrax bacilli but also on most examined strains of the *B. cereus*-569 group. (5) According to the host range studies, a third group of the *B. cereus* strains was not sensitive to any of the phages tested. (6) The negative results obtained with *B. subtilis* and *B. megatherium* strains have not been included in Table I.

The serological results were complementary to the findings made in the host-range studies. Table II shows the K-values obtained with CPα_1, CPβ_2, CPa_1 and CPb_3 antisera.

Data in Table II show that the respective phages of the two *B. cereus* groups different in cell wall structure were clearly distinguishable serologically. The polyvalent phage CPα_1 was serologically unrelated to the other phages examined, including those of its own group. Antibodies to phage CPβ_2 neutralized also phage CPα_1. The other common property of these two phages was their lytic effect on *B-anthracis*. The antigenic patterns of the various phages of the *B. cereus*-569 group differed only slightly. Only with phages CPa_2 and

Table I

Host range of B. cereus phages
(Number of tested and sensitive strains)

Phage	No. of bacterial strains			
	10 *B. anthracis*	10 *B. cereus* I (114)	10 *B. cereus* II (569)	5 *B. cereus* III
Wα	10	0	0	0
$CP\alpha_1$	10	10	7	0
$CP\alpha_2$	0	6	0	0
$CP\beta_1$	0	7	0	0
$CP\beta_2$	8	7	0	0
$CP\gamma_1$	0	6	0	0
$CP\gamma_2$	0	6	0	0
CPa_1-a_5	0	0	10	0
CPb_3	0	0	10	0
CPb_5	0	0	10	0
CPc_3	0	0	10	0
CPc_4	0	0	10	0
CPc_5	0	0	10	0

Table II

Neutralization rate constants ($K\ min^{-1}$) determined by exposure of phages to antisera
(Samples taken at 5, 10 and 15 minutes)

Phage	Antisera			
	$CP\alpha_1$	$CP\beta_2$	CPa_1	CPb_3
$CP\alpha_1$	600	225	0	0
$CP\alpha_2$	0	0	0	0
$CP\beta_1$	0	0	0	0
$CP\beta_2$	0	585	0	0
$CP\gamma_1$	0	0	0	0
$CP\gamma_2$	0	0	0	0
CPa_1	0	0	250	550
CPa_2	0	0	75	200
CPa_3-a_5	0	0	250	550
CPb_3	0	0	250	550
CPb_5	0	0	80	100
CPc_3	0	0	250	550
CPc_4	0	0	200	500
CPc_5	0	0	125	400

CPb_5 was the neutralization rate constant indicating that the phages of similar plaque morphology, but isolated from different soil samples were not identical. Accordingly, further examinations were carried out exclusively with those phages which differed both morphologically and serologically.

One-step growth curves were plotted on the basis of growth experiments in PY medium at 37°C.

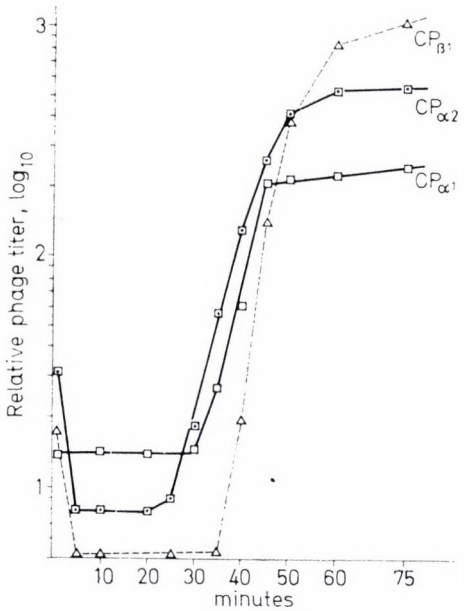

Fig. 4. One-step growth curves of phages $CP\alpha_1$, $CP\alpha_2$ and $CP\beta_1$ on *B. cereus* 114

With some phages, the slope of the one-step growth curve was, after the latency period, steep and ran practically parallel, reaching the plateau at different heights (burst size). The data read from the curves are summarized in Table III, which shows the latency periods and burst sizes.

The latency period varied from 30 to 45 minutes, and average burst size from 10 to 70. Adsorption of the phages to homologous host cells was 99% and to heterologous cells 0%.

Testing of the phages for acid sensitivity was carried out first at pH 4.6, which simultaneously represented the optimum pH for producing mutants by nitrous acid treatment. Phage sensitivity varied in pH 4.6, 0.25 *M* acetate buffer, as shown in Fig. 6.

Fig. 6 shows the pH sensitivity of the phages of the *B. cereus*-114 group. The polyvalent phage $CP\alpha_1$ appeared to be the most sensitive; the number of surviving phages fell by two logarithmic orders in 2 minutes, while with the other phages such a decrease did not occur before the 5th to 10th minute.

In view of these findings, pH sensitivity was tested also in higher ranges. In addition to other markers, these data may be helpful in characterizing the properties of the different phages. In this case, too, the examinations were carried out in 0.25 *M* acetate buffer, at pH 5, 5.6 and 6.2. Results are summarized in Figures 7—12.

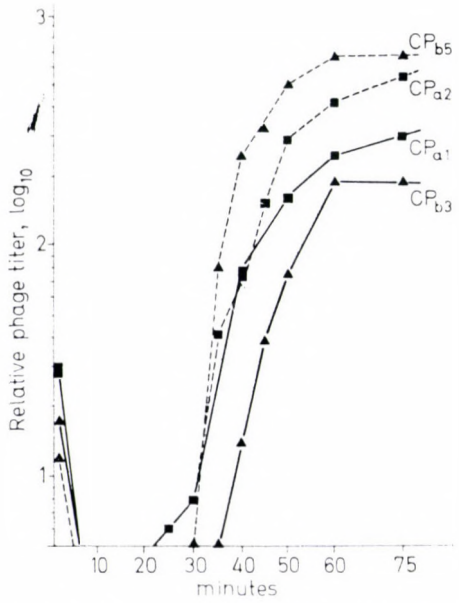

Fig. 5. One-step growth curves of phages $CP\alpha_1$, CPa_2, CPb_3 and CPb_5 on *B. cereus* 569

Table III

One-step growth characteristics of the phages in the hosts B. cereus 569 and B. cereus 114

Host	Phage	Latent period, minutes	Average burst size
B. cereus 114	$CP\alpha_1$	33—35	20
	$CP\alpha_2$	33—35	20
	$CP\beta_1$	40—42	70
	$CP\beta_2$	45—47	70
B. cereus 569	CPa_1	38—40	10
	CPa_2	30—33	20
	CPb_3	40—45	10
	CPb_5	33—35	50

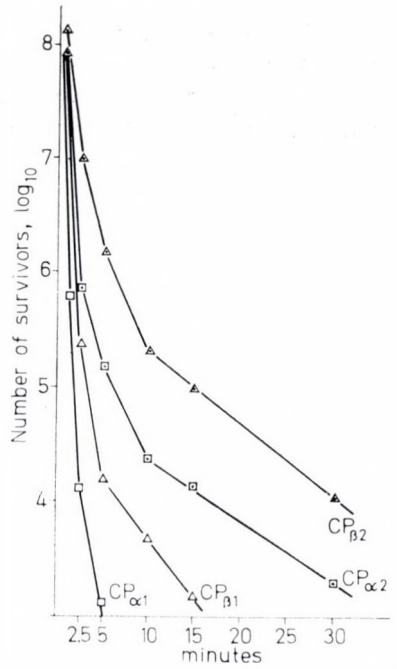

Fig. 6. **Sensitivity to acid at pH 4.6 of phages CPα_1, CPα_2, CPβ_1 and CPβ_2**

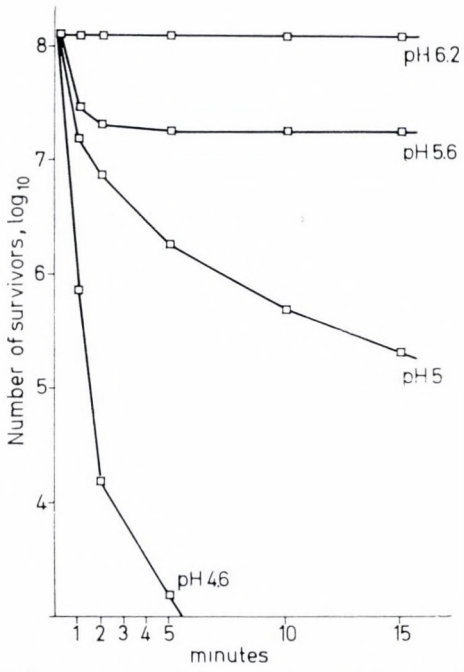

Fig. 7. **Inactivation of phage CPα_1 at different pHs**

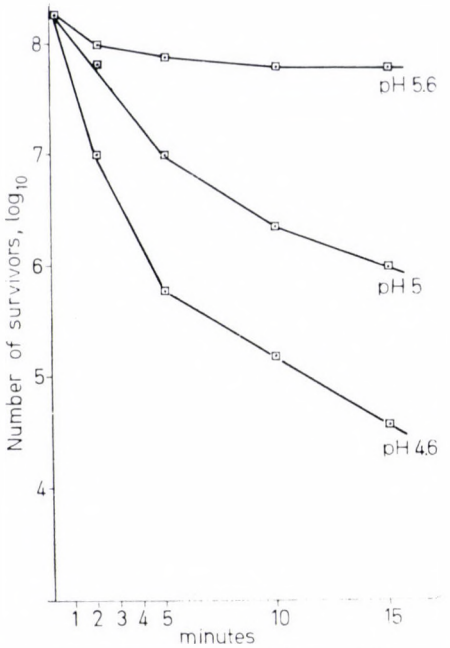

Fig. 8. **Inactivation of phage CPα_2 at different pHs**

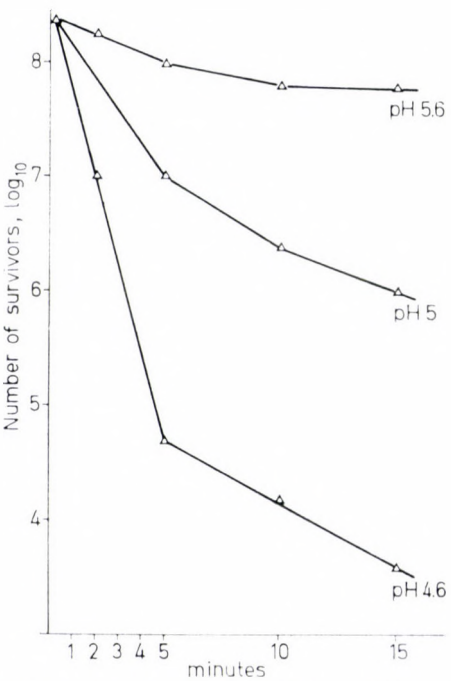

Fig. 9. **Inactivation of phage CPβ_1 at different pHs**

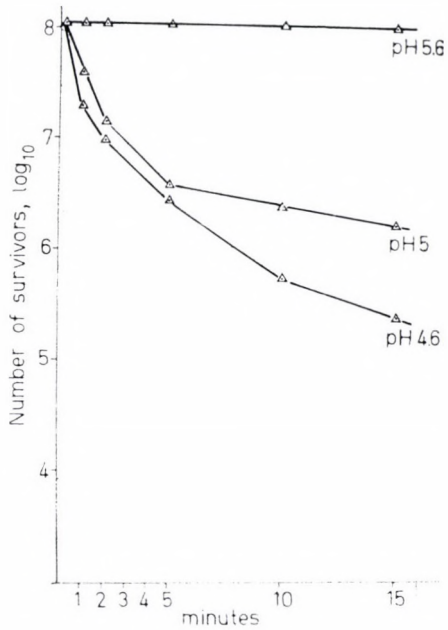

Fig. 10. Inactivation of phage CPβ_2 at different pHs

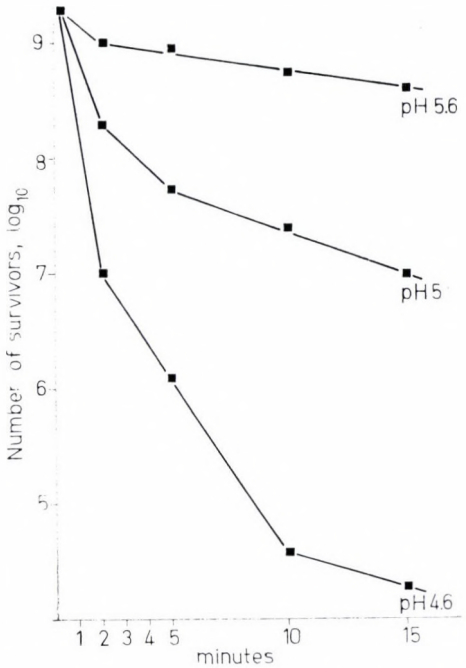

Fig. 11. Inactivation of phage CPa$_1$ at different pHs

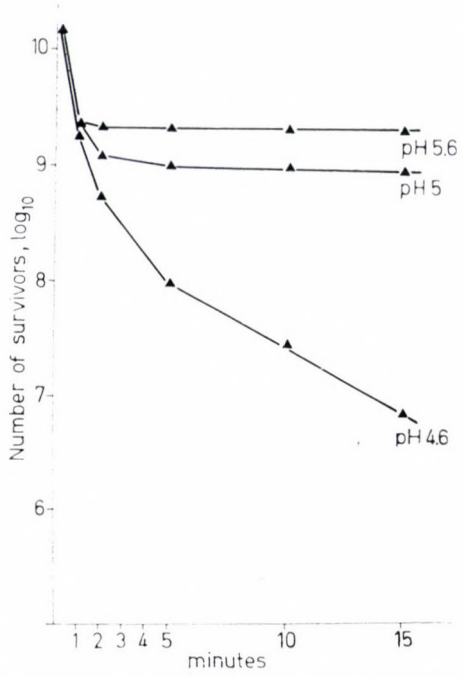

Fig. 12. Inactivation of phages CPb_3 and CPb_5 at different pHs

As shown by the inactivation curves, the pH sensitivity of the different phages presented a characteristic and fairly varied picture. The high pH sensitivity of the phages $CP\alpha_1$, $CP\beta_1$ and CPa_1 was conspicuous in comparison to that of the rest of *B. cereus* phages. These characteristics will be helpful in later experiments on mutant production, hybride analysis, etc.

The sensitivity of the phages to UV irradiation is shown in Fig. 13.

The curves presented in Fig. 13 show the data of inactivation kinetics. Except for phage $CP\alpha_1$, all phages were exposed at 17 cm distance to 6.76 $erg/mm^{-2}/sec^{-1}$ radiation energy. Phage $CP\alpha_1$ was extremely sensitive to UV irradiation, when it was exposed at 30 cm distance to 5.3 $erg/mm^{-2}/sec^{-1}$. The UV sensitivity of the *B. cereus*-569 phages may be characterized by two curves. The phages producing clear and target-like plaques (representant CPa_1) could mostly be classified into a group in which the number of survivors decreased by two logarithmic exponents in 40 seconds following exposure at 270 erg/mm^{-2}. Phage CPa_5, which was considerably more resistant, was an exception. The UV-sensitivity of the phages CPc_1 and CPc_5 was identical with that of CPa_3. A similar inactivation of CPc_3 required 135 erg/mm^{-2}.

Of the phages of the *B. cereus*-114 group, the polyvalent phage $CP\alpha_1$ was most sensitive to UV irradiation. The number of the surviving phage particles decreased by two exponents after exposure at 100 erg/mm^{-2}. The

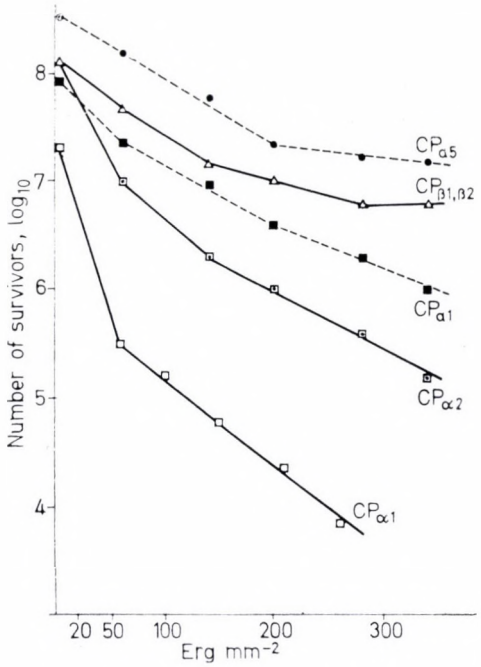

Fig. 13. **UV-inactivation**

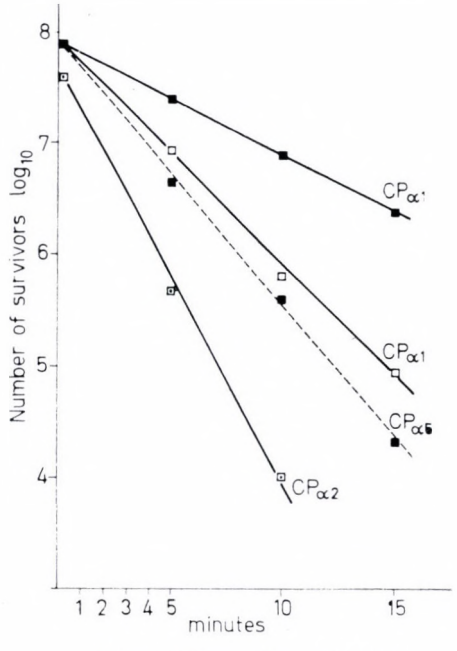

Fig. 14. **Thermal inactivation**

relative UV-resistance of the target-like plaque types $CP\beta_1$ and $CP\beta_2$ was conspicuous.

Some curves representing heat inactivation experiments are shown in Fig. 14.

Inactivation at 56°C usually decreased the number of surviving phage particles by two exponents in 5—10 minutes. The phages examined can be regarded markedly thermosensitive. The heat inactivation curves of the phages not shown in Fig. 14 coincided with one of existing curves and are thus representants of the phages grouped by identical thermosensitivity. Thus, the heat inactivation curve of phage $CP\alpha_2$ coincided with those of phages $CP\beta_1$ and $CP\beta_2$, not shown in Fig. 14.

The dissimilar appearance of the UV and heat inactivation curves was ascribed to their different sites of action, *viz.* phage DNA and protein coat. This accounts also for the absence of a parallelism between UV sensitivity and heat resistance of the phages.

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THE EFFECT OF BORDETELLA PERTUSSIS VACCINE AND ADRENAL HORMONES ON 5-HYDROXYTRYPTAMINE LEVEL IN RAT TISSUES

By

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Summary. The effect of *Bordetella pertussis* vaccine (BPV), adrenalectomy and cortisone treatment on 5-hydroxytryptamine (5-HT) level in rat tissues has been examined. Administration of BPV resulted in an increase of the 5-HT level in the pyloric stomach and duodenum and in an increase of 5-hydroxyindoleacetic acid content in the urine. After adrenalectomy the level of 5-HT increased in all tissues except in the blood and the lung. Cortisone treatment decreased the 5-HT content in all tissues but the pyloric stomach and the duodenum. In the pyloric stomach cortisone increased the amount of 5-HT. It is assumed that BPV influences the tissue 5-HT level indirectly, through the adrenals.

Bordetella pertussis vaccine (BPV) treatment or adrenalectomy are known to increase the sensitivity of rats to histamine, 5-hydroxytryptamine (5-HT) and anaphylactic shock [1]. In earlier experiments we have shown that BPV causes a change in the histamine metabolism of rat tissues. It has been assumed that the change was associated with the histamine sensitivity-increasing effect of BPV [2]. Adrenalectomy induced similar but more definite alterations in tissue histamine metabolism [3]. BPV exerts its effect mainly through the adrenals causing probably a reversible cortical insufficiency [2].

The present experiments were performed in view of the assumption that BPV influences tissue 5-HT metabolism and that this effect, similarly to that of histamine, may be analogous to changes occurring after adrenalectomy. In order to confirm that hypothesis we examined the effect of BPV, adrenalectomy and cortisone on the 5-HT level in rat tissues. Changes in 5-hydroxyindoleacetic acid (5-HIAA) content of the urine after BPV treatment were also determined.

Materials and methods

Wistar rats of both sexes weighing 150—200 g were used. The animals were fed on a standard diet and were given water *ad libitum*.

BPV treatment consisted of one intraperitoneal injection of 3×10^{10} cells. Groups of animals were sacrificed 24 hours, 4 days and 12 days after injection.

Bilateral adrenalectomy was performed under hexobarbital anaesthesia. Two groups of rats were sham adrenalectomized and sacrificed 24 hours and 12 days after operation.

Cortisone acetate (Adreson, N. V. Organon) was given intramuscularly to one group of animals in single doses of 20 mg/kg, to the other group in 10 mg/kg doses at two-day intervals. The rats were sacrificed 24 hours after the last injection.

Tissue 5-HT level was determined by the fluorimetric method of BOGDANSKI *et al.* [4]. Amine levels were expressed as μg 5-HT base/g or ml tissue. All data represent average values for at least 9 specimens. Significance was tested by STUDENT's two-sample t test.

For examining *5-HIAA excretion*, as there was no sex difference in this respect, 4 male and 4 female rats were used. The animals were placed in metabolic cages one week before the experiment in order to accustom them to the special circumstances. Urinary 5-HIAA content was determined by the method of DREUX and DELEUNEUX [5]. The control values corresponded to 3 days mean excretion before BPV injection. The 5-HIAA content of 24 hour urine samples was determined 1, 4, 8 and 12 days after BPV administration. Significance was tested by STUDENT's one-sample t test.

Results

Twenty-four hours after BPV injection, the 5-HT level was considerably lower in the blood and significantly higher in the pyloric stomach and duodenum than the respective control values (Fig. 1). After 4 days the blood level became normal; in the pyloric stomach and duodenum it remained high between the 4th and 12th day. The peak in these organs was observed on the 4th day. There was no alteration in liver, lung and skin 5-HT levels.

The amount of 5-HIAA excreted in the urine increased significantly after BPV injection (Table I). After the peak on the 4th day the values decreased and by the 12th day there was no significant difference from the control.

The level of 5-HT decreased significantly in the blood and increased significantly in the liver, skin and especially in the pyloric stomach and duodenum as soon as 24 hours after adrenalectomy (Fig. 2). After sham operation only the blood level changed, approaching the values found in adrenalectomized

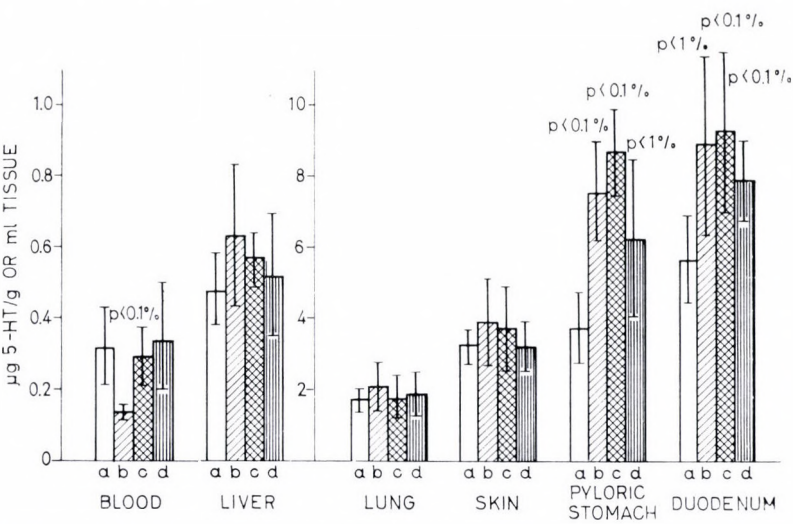

Fig. 1. Effect of *Bordetella pertussis* vaccine on rat tissue 5-HT level. a = control, b = 24 hours after BPV injection, c = 4 days after BPV injection, d = 12 days after BPV injection

rats. Tissue levels determined 12 days after adrenalectomy were similar to the 24-hour values, but there was a definite increase in the liver. The 5-HT levels in animals sacrificed 12 days after sham adrenalectomy were omitted from Fig. 2 as they were identical with the control values.

Table I

Effect of Bordetella pertussis vaccine on 5-HIAA excretion in rats

Designation and sex of animals	5-HIAA excreted in urine, μg/24 hours				
	Control	1 day	4 days	8 days	12 days
	after BPV injection				
1 ♂	24.1	20.0	37.5	22.7	21.1
2 ♀	21.6	43.3	34.0	29.4	17.2
3 ♀	18.7	32.1	47.8	26.6	29.5
4 ♀	15.1	23.0	26.6	26.4	23.8
5 ♂	21.2	32.7	36.8	22.1	23.8
6 ♂	15.0	18.1	30.6	31.4	17.8
7 ♂	13.2	25.3	29.3	20.2	26.2
8 ♀	14.5	19.5	28.2	28.3	26.1
Mean ± SD	17.9 ± 3.8	26.7 ± 8.7	35.8 ± 7.2	25.9 ± 3.9	23.2 ± 4.24
Significance		$p < 2\%$	$p < 0.1\%$	$p < 1\%$	

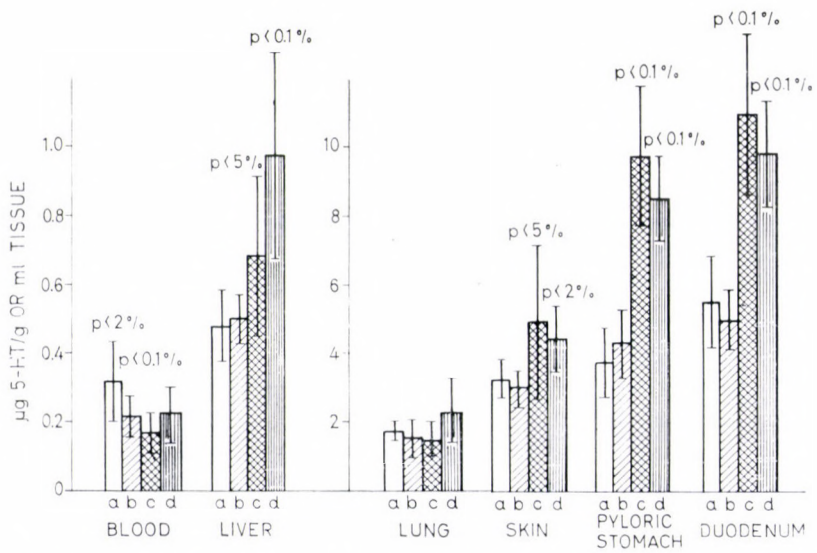

Fig. 2. Effect of adrenalectomy on rat tissue 5-HT level. a = control, b = 1 day after sham operation, c = 1 day after adrenalectomy, d = 12 days after adrenalectomy

One single dose of cortisone caused some increase in the 5-HT level only in the pyloric stomach (Fig. 3). Prolonged cortisone treatment, however, decreased the 5-HT level in all organs save the duodenum and pyloric stomach. In the latter organ a definite rise was observed while in the duodenum there was no change as compared to normal values.

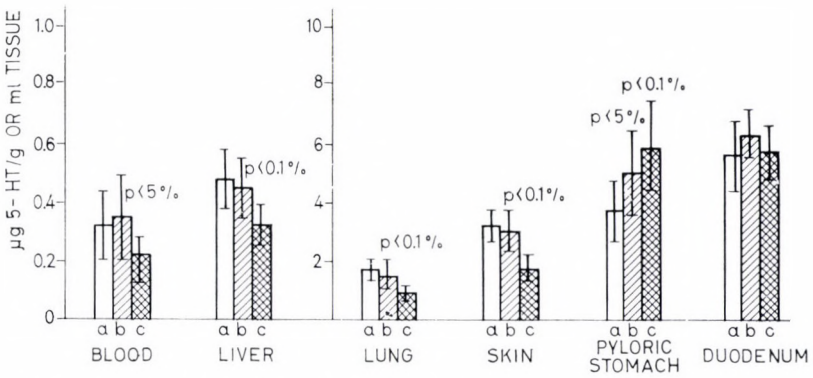

Fig. 3. Effect of cortisone treatment on rat tissue 5-HT level. a = control, b = single cortisone injection (20 mg/kg), c = prolonged cortisone treatment (10 mg/kg at 2-day intervals for 12 days)

Discussion

BPV injection caused a change in the 5-HT level in the blood, pyloric stomach and duodenum. The decrease in blood level 24 hours after treatment reflected probably not an alteration of 5-HT metabolism, but the release of the agent from thrombocytes under the effect of certain components of the vaccine. In contrast, the definite and prolonged increase of the 5-HT level in the pyloric stomach and duodenum was presumably due to alterations in tissue amine metabolism. This concept was confirmed by our results as to the excretion of 5-HIAA, the most important final product of 5-HT. It is known that the 5-HIAA content of urine originates mainly from the gastrointestinal tract [6]. Accordingly, the simultaneously observed high 5-HIAA excretion and high 5-HT levels in the gastrointestinal tract may be explained by an increased 5-HT synthesis and by an increased "turnover" *in vivo*. It should be mentioned that in rats treated with pertussis vaccine, SANYAL and WEST [7] observed no change in the 5-HT level of the spleen, lung and subcutaneous connective tissue but they demonstrated decreased values in the jejunum. As we examined different tissues, our results cannot be compared with those of SANYAL and WEST, but concerning the lung the data showed a fairly good agreement. Our conclusions, however, are different from those of SANYAL and WEST.

There are contradictions in the literature as to the 5-HT-regulating role of the adrenals. According to RESNICK *et al.* [8] various hormonal effects failed to influence the 5-HT content of the tissues. In contrast, TELFORD and WEST [9] are of the opinion that the adrenal gland, or more exactly the glucocorticoids, play an essential part in the regulation of tissue histamine and 5-HT levels. Our results support the latter conception. After adrenalectomy 5-HT levels increased in all organs except the blood and lung. An especially high increase was observed in the pyloric stomach and duodenum. In agreement with other authors [10] we demonstrated decreased amine levels in the blood after adrenalectomy. The similar but less definite alteration in sham operated animals makes the specificity of this effect doubtful. Cortisone treatment decreased considerably the 5-HT level in all rat tissues save the pyloric stomach and the duodenum; *i.e.* its effect was the opposite of that of adrenalectomy. The incompleteness of this effect and data in the literature [11] indicate that, in addition to the primary role of glucocorticoids, other factors are also involved in tissue amine level regulation.

Cortisone treatment increases 5-HT content as well as histamine content [3] in the pyloric stomach. The cause of this effect is not known. For its elucidation, systematic investigations are needed into 5-HT anabolism and catabolism in the gastrointestinal tract.

The changes occurring after BPV treatment were similar to those found one day after adrenalectomy. In both cases a definite increase in the 5-HT level in the pyloric stomach and duodenum was noted. It is known that after adrenalectomy as well as BPV injection the urinary 5-HIAA content increases [13]. BPV-treated and adrenalectomized rats and mice react similarly to different stresses [1]. Although morphological proofs are lacking, it seems that BPV causes adrenal insufficiency [2 14,]. Thus it may be supposed that BPV, similarly to histamine metabolism, exerts its effect on tissue 5-HT levels indirectly, through the adrenals.

Our results failed to prove, but did not disprove the existence of a direct association between the BPV-induced higher sensitivity to 5-HT and the changes in tissue 5-HT levels. In view of the fact that 4 days after BPV injection, at the peak of sensitivity, there was no change in the blood 5-HT level, a direct association seems improbable. After BPV treatment the increase in gastrointestinal 5-HT levels may enhance the anaphylactic hypersensitivity.

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IMMUNOFLUORESCENT STUDIES ON THE REACTIVITY OF "EARLY" AND "LATE" HERPES-IMMUNE RABBIT SERA WITH VIRUS-INDUCED ANTIGENIC FORMATIONS OF HEp-2 CELLS*

By

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Summary. The reactivity of sera of herpes-infected rabbits was studied with antigenic formations of herpes simplex virus-infected (HSV) HEp-2 cells during the course of immunization. Serum samples taken one week after the onset of immunization stained diffuse cytoplasmic, perinuclear, and some intranuclear finely granular elements in HSV infected HEp-2 cells. Immune sera taken 2 weeks later revealed only large inclusion-like bodies and some large granules in the nuclei of the same preparations, in addition to a diffuse cytoplasmic and perinuclear fluorescence. At the same time a significant increase in the titre of virus neutralizing antibodies was noticed.

The "early" and "late" immune sera reacted with perinuclear antigenic formations in cells treated with cytosine arabinoside.

The "early" antibodies corresponded to IgM immunoglobulins.

Lippelt and Söltz-Szöts [1] described that antibodies against the soluble herpes-specific antigenic components develop earlier than those reactive with the sedimentable ones. Tokumaru [2] established that the increase of the titre of IgM immunoglobulin is characteristic of the early immune response in herpes simplex virus (HSV) infections, which 14 days later is followed by the predominance of IgA and IgG antibodies.

It had been shown by Roizman *et al.* [3, 4] and confirmed by others [5, 6] that HSV induces different antigenic formations in infected cells. Though each of the immunofluorescent elements consists of more than one antigen, some data are available on differences in their nature [3, 4].

Based on these findings it has been supposed that the deviation in time of appearance of antibodies against different herpes-specific antigens manifested itself also in connection with immunofluorescent formations containing different antigenic components as for their early or late, structural or non-structural, sedimentable or non-sedimentable nature. This paper reports on studies on the reactivity of "early" (taken 1 week after the first immunizing dose) and "late" (taken 2—3 weeks or later after the onset of immunization)

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rabbit immune sera against the antigenic formations of HSV infected HEp-2 cells, treated (CA^+) or untreated (CA^-) with cytosine arabinoside.

Materials and methods

Tissue cultures. Preparation of human embryonic fibroblast, coverslip cultures of HEp-2 cell line, and maintenance of HEp-2 cells were as described previously [5, 7, 8].

Virus. Syncytium-forming HSV strain 532 in passages 543—550 maintained on HEp-2 cell cultures. The virus strain was isolated from the conjunctival exudate of a patient with keratitis. Before the experiments the strain was passaged in primary human embryonic cell cultures.

Syncytium-forming HSV-strain "M" in passage 1 on human embryonic fibroblast cells isolated from a labial vesicle fluid.

Virus assay. Infectivity titrations were performed on human embryonic fibroblast cells. Titres were calculated according to REED and MUENCH and expressed in TCD_{50}.

HEp-2 cells cultivated on coverslips for indirect immunofluorescent technique were inoculated with HSV at a multiplicity of 10 TCD_{50} per cell as described previously [5, 7]. The medium added to certain tubes contained 10 μg per ml of cytosine arabinoside (CA^+ cells) while others were grown without cytosine arabinoside (CA^- cells).

Immune sera. Antisera to HSV were prepared in young adult rabbits which were separated into 4 groups.

Group 1. These rabbits were inoculated intravenously with an infective suspension of virus strain 532 derived from human embryonic fibroblast cells. Rabbits were injected with 2 ml of virus suspension, 9 times at weekly intervals. Blood samples were taken before each injection. Rabbits Nos 1, 2 and 3 belonged to this group.

Group 2. Rabbits belonging to this group were inoculated once intracutaneously with 1 ml of the suspension of HSV strain "M" propagated on human embryonic fibroblast cells. The site of inoculation was infiltrated with 0.2 ml (30 I.U.) of hyaluronidase prior to infection. Four days after infection skin lesions measuring 30×30 mm in diameter developed. Blood samples were taken before infection and later at weekly intervals. Rabbits Nos 4, 5 and 6 belonged to this group.

Group 3. Rabbits Nos 7, 8 and 9 of this group were immunized by corneal scarification with vesicle fluid of a labial herpetic lesion. Keratitis developed in each of the animals within 7 days after scarification. The causative agent proved to be Type 1 HSV at serological identification and pock-production on chick embryo chorioallantoic membrane. Blood samples were taken before immunization and later at weekly intervals.

Group 4. Rabbits of this group were inoculated intravenously 9 times at weekly intervals with 2 ml of the suspension of HSV strain 532, cultivated on human embryonic fibroblast cells. Before inoculation the virus suspension was inactivated at 56°C for 1 hour. Blood samples were taken from the animals before every inoculation. Rabbits Nos 10, and 11 belonged to this group.

Preparation of virus suspension for immunization. The human embryonic fibroblast cell cultures used for the propagation of virus were inoculated with HSV at a multiplicity of 1 TCD_{50} per cell. Cytopathogenic effect involving the whole cell sheet developed after incubation at 37°C for 2—3 days. The maintenance medium used for inoculated cell monolayers was Parker's medium No. 199 without serum (0.25% $NaHCO_3$). The cell cultures showing complete cytopathogenic effect were frozen at —78°C, thawed once, centrifuged at 2500 r.p.m. for 10 minutes, and the supernatant titrated for virus content. The mean titre was $10^{7.5}$ per 1 ml.

Removal of non-specific staining components. Immune rabbit sera and fluorescein isothiocyanate conjugated goat anti-rabbit globulin (supplied by SEVAC, Prague) were adsorbed with HEp-2 cells and mouse liver powder, as described previously [5, 7].

Fixation and staining of cell monolayers for indirect immunofluorescent technique were done as described previously [5, 7].

Neutralization test. Equal parts of virus suspension (HSV-532) containing 200 TCD_{50} and undiluted or tenfold dilutions of unheated immune sera were added, mixed and incubated overnight at 4°C. On the following day, 0.1 ml of the virus—serum mixtures were inoculated on HEp-2 cell sheets. The inoculated cell cultures were incubated with Parker's medium No. 199 containing 2% calf serum, and read for cytopathogenic changes daily. Simultaneous titration of the virus suspension was carried out by adding to the virus suspension instead of serum an equal amount of diluent. The suspension was incubated overnight at 4°C, then tested for infectivity.

Treatment of immune sera with 2-mercaptoethanol. Inactivated serum was diluted 1 : 4 in 0.13 *M* 2-mercaptoethanol, incubated at 37°C for 30 minutes, and dialyzed against several changes of Hanks' balanced salt solution containing 0.5% lactalbumin hydrolysate in the refrigerator until odourless [9].

Results

Immunologic reactivity of serum samples during immunization. For the determination of the course of development of antibodies which stain immunofluorescent elements of different formations in HSV infected HEp-2 cells,

Figs 1—3. HEp-2 cells infected with herpes simplex virus, fixed 6 hours after infection and stained with serum samples of rabbit No. 1 which was immunized with infective virus

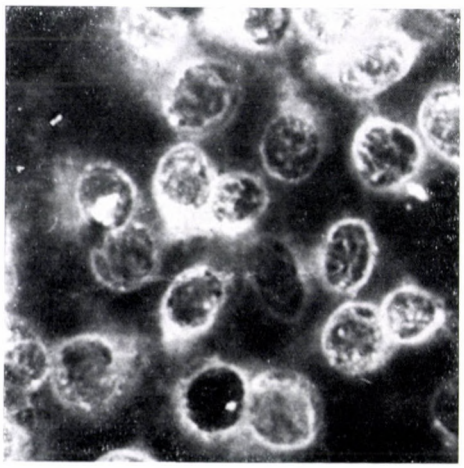

Fig. 1. Indirect stain of CA⁻ HEp-2 cells with immune serum taken on 7th day after the first immunizing dose

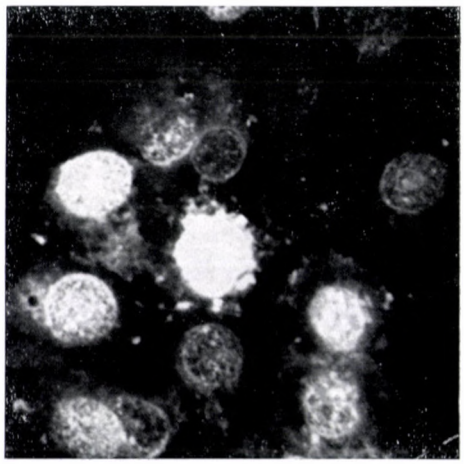

Fig. 2. Indirect stain of CA⁺ HEp-2 cells with immune serum taken on 7th day after the first immunizing dose

the rabbits of Group 1 were injected with infective suspension of HSV strain 532 intravenously, the members of Group 2 were immunized intracutaneously on a single occasion with freshly isolated infective virus strain "M", and the rabbits of Group 3 were immunized by corneal scarification with the vesicle fluid of herpetic eruptions, as described under "Methods". Blood samples taken at different intervals after infection were tested for reactivity with HEp-2 cells fixed 6 hours after HSV infection. In some preparations the production of antigens coded by the progeny viral DNA was inhibited with cytosine arabinoside.

On the 7th day after the onset of immunization with live HSV, the sera reacted with the cytoplasmic, perinuclear and finely granular intranuclear antigenic elements of HEp-2 cells, treated or untreated with cytosine arabinoside (Figs 1 and 2, Table I).

The reactivity of immune sera with the intranuclear inclusion-like large bodies and large granules of CA^- cells developed 1—2 weeks later. It was not influenced by the route of immunization or by the number of injections (Fig. 3, Table I).

Table I

Development of antibodies against HSV specific antigenic formations in rabbits immunized with infective or heat inactivated virus

Antigenic formation	Day of appearance of antibodies against the indicated antigenic formations			
	Group			
	1	2	3	4
In CA^- cells				
1. Diffuse cytoplasmic	7	7	7	7
2. Perinuclear	7	7	7	7
3. Intranuclear diffuse granules	7	7	7	7
4. Intranuclear inclusion-like bodies	21	21	14—21	21
5. Intranuclear large granules	21	21	14—21	21
In CA^+ cells				
1. Perinuclear	7	7	7	7—14
2. Intranuclear diffuse granules	7	7	7	No reaction

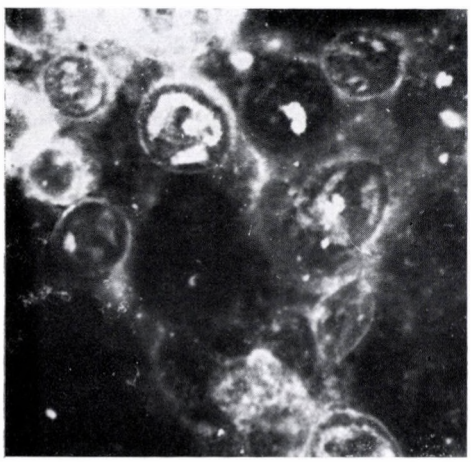

Fig. 3. Indirect stain of CA^- HEp-2 cells with immune serum taken on 21st day after the first immunizing dose

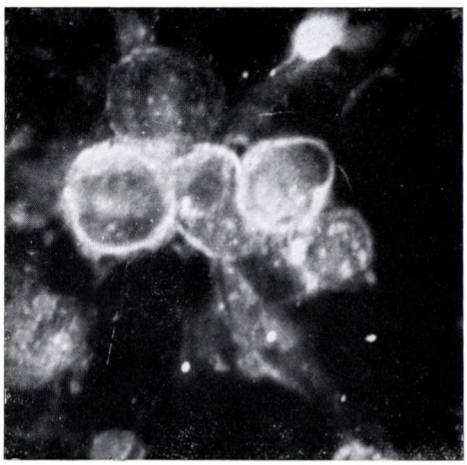

Fig. 4. CA^+ HSV infected HEp-2 cells fixed 6 hours after infection, stained with immune serum of rabbit No. 11, taken on the 21st day after the first immunizing dose. The rabbit was injected with heat inactivated virus. Magnifications about ×750

Reactivity of immune sera derived from rabbits injected with heat-inactivated virus. Rabbits of Group 4 were immunized 9 times at weekly intervals with heat-inactivated suspensions of HSV propagated in human embryonic fibroblasts. HSV infected HEp-2 coverslip cultures were stained with serum samples taken before every immunization. There was no difference in the course of the antibody response in these animals as compared to those of groups Nos 1, 2 and 3 with the exception that no antibodies had developed against the diffusely distributed intranuclear finely granular antigens of CA^+ cells (Fig. 4, Table I), although one of these sera also reacted with diffusely distributed granules of the nuclei of CA^- cells.

There was also some difference in the appearance of the intranuclear inclusion-like formations depending on the immune serum used for staining. The sera of rabbits immunized with live virus stained the inclusion-like bodies more intensely and diffusely than did the sera of animals immunized with heat-killed virus. The formations stained with the latter showed a less dense, weakly staining spongious structure.

2-Mercaptoethanol sensitivity of "early" antibodies. After treatment with 2-mercaptoethanol the "early" immune sera lost their reactivity with the cytoplasmic, perinuclear and intranuclear antigenic formation of CA^- and CA^+ cells. Control immune serum not treated with 2-mercaptoethanol but kept at 37°C for 30 minutes at 1 : 4 dilution in Hanks' balanced salt solution retained its reactivity with the antigenic formations mentioned above.

Table II

Development of virus-neutralizing antibody titres in serum of rabbits of Group 3 immunized with HSV by corneal scarification

Rabbit No.	Day after infection		
	7	14	21
7	0	1 : 30	1 : 1000
8	1 : 3	1 : 50	1 : 500
9	1 : 10	1 : 50	1 : 3000

Individual differences in reactivity of immune sera. Immune serum No. 2 derived from a rabbit immunized with live virus did not react with the large granules localized in the nucleus along the nuclear membrane of CA^- cells, while other members of the same group were reacting with it. Immune serum No. 10 of a rabbit immunized with heat-killed virus stained neither the diffusely distributed, nor the large granules in the nuclei of CA^- cells, while serum No. 11, belonging to the same group, failed to react with the intranuclear

inclusion-like bodies of CA^- cells. Serum No. 11, like No. 10, did not stain the diffusely distributed intranuclear granules of CA^+ cells but, unlike serum No. 10, it did react with the small granules diffusely distributed in the nuclei of CA^- cells. The serum of rabbits Nos 4 and 5, immunized with live virus, did not react with the inclusion-like intranuclear formations of CA^- cells, and serum No. 6 of the same group did not stain the large intranuclear granules localized along the nuclear membrane of CA^- cells. None of the pre-immune rabbit sera reacted with any of the HSV induced antigens. All the immune sera retained their immunologic reactivity throughout, but none of the immune sera negative at 21 days after the first immunizing dose against the mentioned antigenic formation had later become positive.

Discussion

Though the indirect immunofluorescent technique proved a valuable method for studying the multiplication of HSV, it has limitations for the determination of antibody response against the numerous HSV induced proteins, as (i) one immunofluorescent formation consists of more than one antigenic component, (ii) the herpes-specific proteins are synthetized in the cytoplasm, and subsequently some of them are transferred into the nucleus. Thus the same antigen may have different locations inside the cell. (iii) In the nucleus where the nucleocapsids are assembled the macromolecular aggregates may have an immunologic specificity different from that of their components [9].

Although the antigenic constituents of the different HSV induced immunofluorescent elements in HEp-2 and other cells are not precisely known, there are some data about their nature. We know the sequence of their development [3, 4, 5, 6, 7]. ROIZMAN *et al.* [4] showed that the intranuclear and cytoplasmic granules are macromolecules, synthetized late after the infection. Their properties fit herpes virions as well. The diffuse nuclear and cytoplasmic antigens appear early and consist of structural and nonstructural elements. Immunofluorescent elements containing only "early" structural and nonstructural proteins are produced in infected cells treated with cytosine arabinoside [5].

There was some regularity in the development of antibodies reactive with different antigenic formations of HSV infected HEp-2 cells. On the basis of present knowledge the following conclusions may be drawn from our findings.

(1) The "early" immune sera reacted with those antigenic formations of CA^- and CA^+ cells, which are the earliest fluorescent products in infection (cytoplasmic, perinuclear and finely granular intranuclear formations). The "early" antibodies proved to be IgM immunoglobulins, in agreement with TOKUMARU's finding [2].

(2) "Late" immune sera stained many intranuclear antigens of different forms and arrangements in CA^- cells, in the same preparations in which only intranuclear small granules could be detected with the "early" serum samples. The intranuclear inclusion-like formations appeared 4 hours or later after infection. Since in the nuclei no new virus-specific protein is synthetized, they might be macromolecular aggregates of virus specific proteins transferred from the cytoplasm into the nuclei, and they might have an immunologic specificity different from their components [9].

This hypothesis is supported by the observation that the reactivity of immune sera with the intranuclear large granules coincides with the increase of virus neutralizing antibody titres (Table II).

(3) Immune sera of rabbits immunized with heat-inactivated virus did not react with the intranuclear small granules of CA^+ cells, though one of them stained such formations in CA^- cells. According to SABIN [10] the non-virion herpes specific early antigens are heat labile. The heat inactivation of antigens used for immunization had a double purpose: (a) to denature "early" antigens in the virus suspension to be injected and, (b) to prevent the injection of live virus which could induce production of non-virion early proteins. Based on the non-reactivity of immune sera of Group 4 with the diffuse granular elements of CA^+ nuclei, these formations can be interpreted as the intranuclear location of heat labile, ether labile, soluble early antigens detected earlier by the complement fixation technique [8].

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THE EFFECT OF TEMPERATURE ON THE DEVELOPMENT OF IMMUNOFLUORESCENT ELEMENTS IN HERPES SIMPLEX VIRUS INFECTED BS-C-1 CELLS

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Summary. The incubation of HSV inoculated BS-C-1 cells at 40°C did not influence the development of "early" antigenic formations but delayed the appearance of large, intranuclear inclusion-like bodies which are late products in infection. Both "early" and "late" virus-specific complement-fixing antigens were produced, the former in larger quantities. The titre of intracellular infective virus was markedly reduced.

Incubation at room temperature resulted in a latent virus infection of cells, with the accumulation of "early" antigenic formations, completely blocking the synthesis of "late" antigenic elements and infective virus.

The temperature of incubation has a significant effect on the herpes simplex virus (HSV) production of cells: the largest number of virions is synthetized at 34, 35 and 37°C. Cell cultures incubated at room temperature show little or no evidence of virus production, while incubation at 39.5—40°C retards the synthesis of viral DNA and proteins, causing an abnormal infective cycle and a considerable decrease in the number of newly synthetized virions [1, 2, 3].

As shown by Roizman *et al.* [4] and confirmed by others [5, 6, 7], some of the antigenic formations induced by HSV in infected cells are characteristic of the early phase of infection, while others are typical products of the later events. The studies to be reported have dealt with the effect of suboptimal and supraoptimal temperatures on the development and compartmentalization of antigenic elements in infected cells, with the purpose of establishing which of the phases of viral multiplication are influenced by unfavourable temperatures.

Materials and methods

Tissue cultures. The procedures for the maintenance of BS-C-1 and HEp-2 cells, and the preparation of coverslip cultures of BS-C-1 cell line, were described previously [6, 7].

Virus. Syncytium-forming HSV strain 532 in passages 540—542 on HEp-2 cells [6] with an infective titre of $10^{7.5}$ TCD_{50} per 0.1 ml when titrated on BS-C-1 cells.

Immune sera. Anti-HSV-infected cell extract immune guinea pig serum F—T and human convalescent serum No. 42 at 1 : 2 dilution, as described previously [6]. Both reacted with the HSV-induced cytoplasmic, diffusely distributed small intranuclear granules and nuclear

inclusion-like masses of HSV-infected cells, and with the diffusely distributed perinuclear, and intranuclear antigenic formations of HSV infected cells treated with cytosine arabinoside.

HSV immune rabbit sera R4 and R6. The same sera were used in our previous experiments [8]. These sera were identical in anti-viral complement fixing antibody titre [1 : 16(32)]. R6 did not react with the early extract of cytosine arabinoside treated HSV infected cells while R4 reacted with this preparation at a lower titre than with the viral ones [1 : 8(16)].

BS-C-1 cells cultivated on coverslips for indirect immunofluorescence technique were inoculated with HSV at a multiplicity of 10 TCD_{50}/cell, as described previously [6]. The medium added to certain tubes contained 10 μg per ml of cytosine arabinoside (CA^+ cells), while others were grown without the compound (CA^- cells). The coverslip cultures were incubated at 20—22°C, 30°C, 37°C, 40°C and 45°C. Coverslips were removed and fixed 2, 3, 4, 6, 8 and 10 hours after inoculation, and in the case of incubation at room temperature, 2, 4 and 7 days after infection.

Fixation and staining of cell monolayers for indirect immunofluorescence technique was the same as described previously [6].

Preparation of CF antigens and technique of CF tests carried out was the same as described previously [8].

Results

The effect of supraoptimal temperatures on the development of HSV specific immunofluorescent elements in BS-C-1 cells. The observations were made on coverslip cultures fixed 2, 3, 4, 6, 8 and 10 hours after exposure to 10 TCD_{50} virus per cell, and then stained with immune serum F—T and with labelled antibody preparations. Some coverslip cultures were incubated in the presence of 10 μg/ml of cytosine arabinoside.

Two hours after inoculation no virus-specific antigenic formations occurred in the cells.

Three hours after infection, in CA^- cells incubated at 40°C the diffuse cytoplasmic fluorescence was weaker than in those incubated at 37°C. Diffusely distributed intranuclear granules were present in cells incubated either at 37°C or at 40°C (Table I).

Four hours after infection, in the cells incubated at 40°C the cytoplasmic fluorescence was still weaker than in those kept at 37°C. In the nuclei, diffusely distributed small granules were only present (Fig. 1). In this period, in the cells incubated at 37°C, inclusion-like intranuclear large bodies developed; they were separated by a narrow unstained zone from the nuclear membrane (Fig. 2).

Six hours after inoculation, in cells incubated at 40°C most of the nuclei were filled with diffusely distributed granules but in the centre development of dense small bodies were noticed. In cells incubated at 37°C no change was seen in the appearance of elements as compared to preparations fixed 4 hours after infection.

Eight hours after infection, in the nuclei of cells incubated at 40°C inclusion-like fluorescent bodies appeared; they were similar to those observed at 37°C 4 hours earlier. The only difference was that at 40°C these bodies were connected to the nuclear membrane with less dense fluorescing material. The nuclear membrane also showed a well-defined fluorescence not seen in prep-

Table I

Effect of 40°C on the development of HSV induced immunofluorescent elements in BS-C-1 cells

Antigenic formation	Location of antigen	Time of appearance of antigen at 40°C (hours after inoculation)	Difference in appearance of antigen as compared to those formed at 37°C
In untreated cells			
1. Cytoplasmic diffuse	fills cytoplasm	3	less intense lacy network
2. Nuclear small granules	dispersed throughout nucleus	3	no difference
3. Nuclear inclusion-like mass	fills nucleus	8	appearance 4 hours. later; It is surrounded by a less dense fluorescing material
4. Nuclear membrane	along nuclear membrane	8	developed only at 40°C
In cells treated with cytosine arabinoside			
1. Perinuclear	nucleocytoplasmic junction	3	no difference
2. Intranuclear small granules	dispersed throughout nucleus	4	no difference

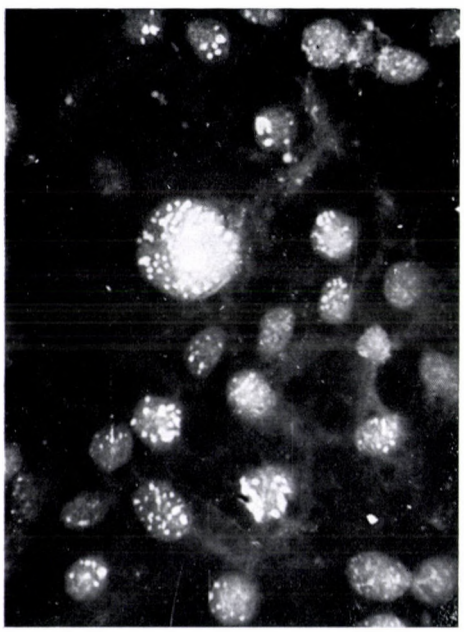

Fig. 1. CA⁻ cells 4 hours after inoculation, incubated at 40°C

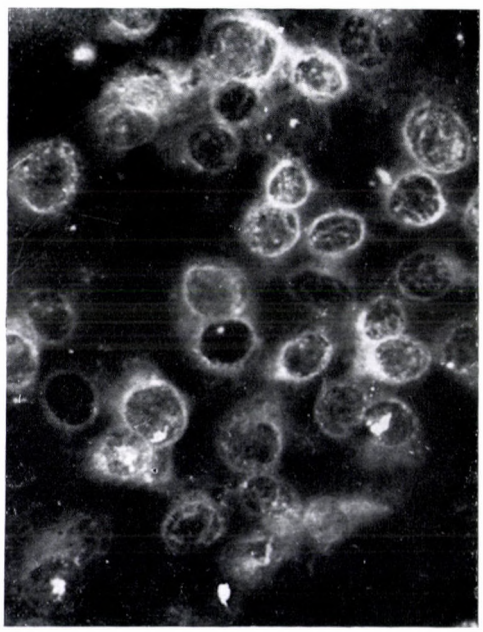

Fig. 2. CA⁻ cells 4 hours after inoculation, incubated at 37°C

BS-C-1 cells fixed at different intervals after inoculation with HSV. All the preparations were stained with immune serum F—T. (Magnification about ×500)

arations incubated at 37°C (Fig. 3, Table I). The cytoplasmic fluorescence was still weaker than at 37°C.

The formation of antigenic elements in cells treated with cytosine arabinoside was the same at 37°C and at 40°C. Three hours after infection cytoplasmic and perinuclear fluorescence was dominant, and was followed one hour later by the appearance of diffusely distributed granules in the nuclei (Figs 4, 5, 6, 7; Table I).

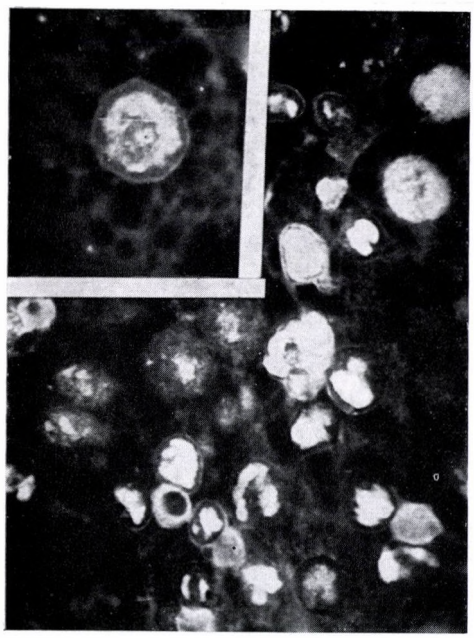

Fig. 3. CA$^-$ cells 8 hours after inoculation, incubated at 40°C

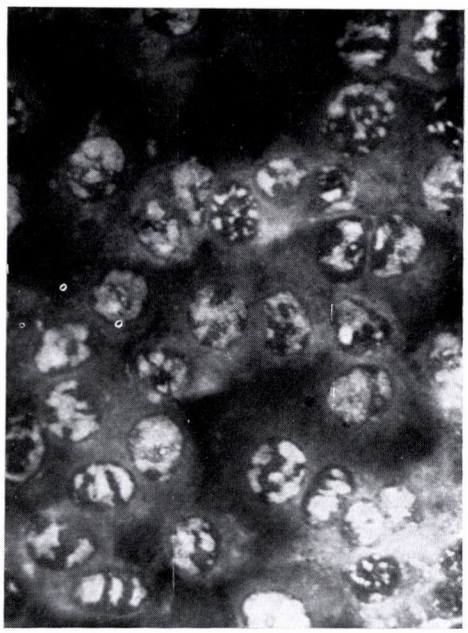

Fig. 4. CA$^+$ cells 3 hours after inoculation, incubated at 37°C

At 45°C rapid degeneration of cells was observed without any sign of virus multiplication.

Effect of suboptimal temperatures on the development of HSV specific immunofluorescent elements in BS-C-1 cells. In preparations incubated at 30°C no difference in the appearance of antigenic elements was observed as compared to those incubated at 37°C, but for a 3-hour delay in their development.

In cells kept at 20—22°C no cytopathogenic changes developed. The preparations stained with anti-HSV immune sera showed densely distributed small granules inside the nuclei and a weak diffuse cytoplasmic fluorescence (Fig. 8). The picture reminded of the immunofluorescence of HSV infected

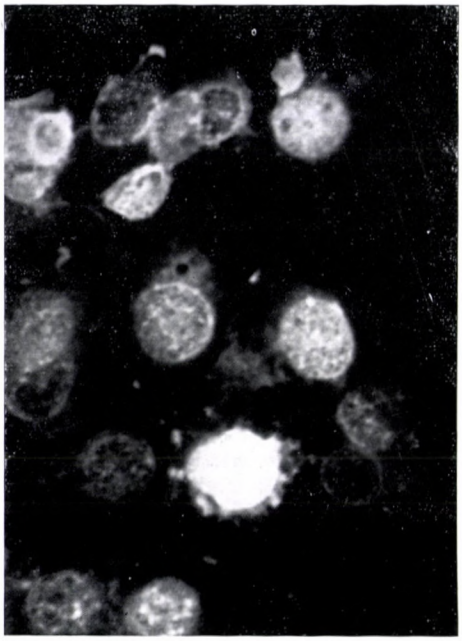

Fig. 5. CA^+ cells 4 hours after inoculation, incubated at 37°C

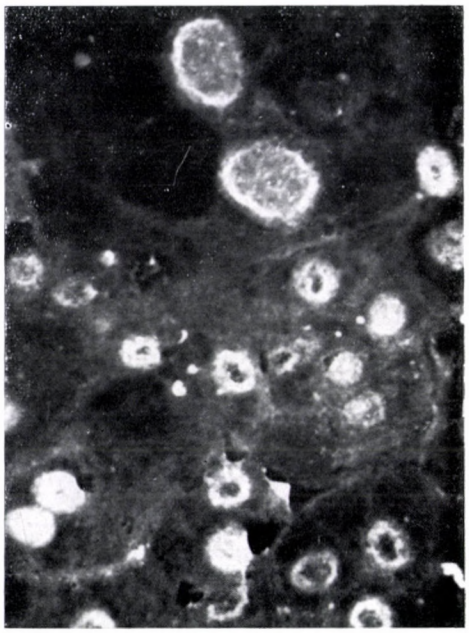

Fig. 6. CA^+ cells 3 hours after inoculation, incubated at 40°C

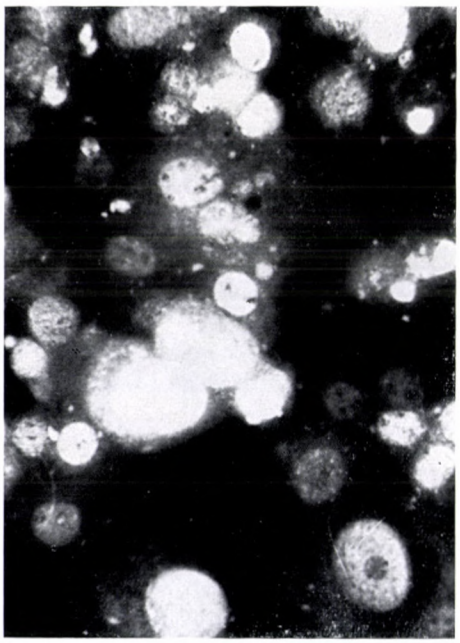

Fig. 7. CA^+ cells 4 hours after inoculation, incubated at 40°C

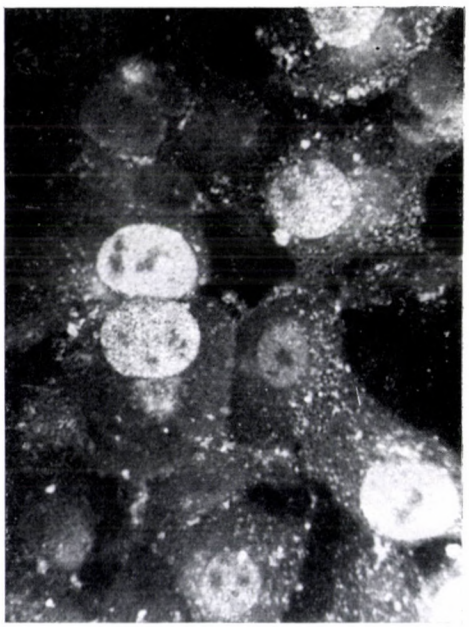

Fig. 8. CA^- cells 48 hours after inoculation, incubated at 20°C

cells which had been treated with cytosine arabinoside and stained four hours after infection.

Titration of complement fixing antigens and infectivity of HSV-infected BS-C-1 cells incubated at 22°, 37° and 40°C. The "early" and "late" antigen content in the extract of HSV-infected cells was determined with the use of R4 and R6 immune sera [8], identical in anti-viral titre. R6 failed to react with the early extract of HSV-infected cells, while R4 reacted with this component at a lower titre than with the viral ones.

Table II

Titre of intracellular herpes simplex virus-induced complement-fixing antigens synthetized in BS-C-1 cells at different temperatures

Time of preparation of antigen (hours after infection)	22°C			37°C			40°C		
	Complement-fixing antigenic units titrated with serum		Infectivity log TCD_{56} per 0.1 ml	Complement-fixing antigenic units titrated with serum		Infectivity log TCD_{56} per 0.1 ml	Complement-fixing antigenic units titrated with serum		Infectivity log TCD_{50} per 0.1 ml
	R4	R6		R4	R6		R4	R6	
6	N.T.*	N.T.	N.T.	8	4	4.5	4	und**	4.74
12	N.T.	N.T.	N.T.	32	16	6.74	8	und	3.24
18	4(8)	0	2.74	32	16	7.24	4	und	3.24
48	2(4)	0	1.5	N.T.	N.T.	N.T.	N.T.	N.T.	N T.
96	2	0	0.74	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.

* not tested
** undiluted

Most complement-fixing antigens were synthetized at 37°C. At 40°C the production of "early" antigens decreased less than that of the "late" structural components. In cultures incubated at room temperature no detectable amount of "late" viral antigens was synthetized, but the titre of "early" antigens reacting with serum R4 was equal to the amount of "early" antigens produced in CA^+ cells at 37°C detected in our previous experiments [8].

The greatest amount of intracellular infective virus was produced at 37°C, while the smallest quantity was detected in the extract of cells incubated at room temperature.

Discussion

The disadvantageous effect of elevated temperatures on the multiplication of HSV has been studied by several investigators [1, 2, 3]. At 39.5—40°C practically no extracellular infective virus was detected, but the amount of intracellular infective virions was also reduced to one-third as compared to that synthetized at 34—35°C.

YOSHINO *et al.* [3] established that the suppression of HSV plaque formation at 40°C was not equivalent with the complete block of virus production. The mechanism of partial inhibition differs from that of bovine herpesviruses, where the synthesis of viral DNA is blocked at high temperatures [11].

Our observations can be summarized as follows: (*i*) The supraoptimal temperature (40°C) failed to inhibit the synthesis of "early" antigenic formations in CA^+ cells, which are coded by the parental viral DNA, but it retarded the development of immunofluorescent elements which are formed in CA^- cells late (4 hours) after infection.

These intranuclear large inclusion-like bodies can be macromolecular aggregates of virus-specific proteins in the period when nucleocapsides are assembled in the nuclei having an immunological specificity different from those of their components [9]. (*ii*) The appearance of perinuclear fluorescence late after infection at 40°C might be due to an accumulation of nucleocapsides along the nuclear membrane which were inhibited by the elevated temperature in their transport into the cytoplasm. (*iii*) Complement fixation tests and infectivity titrations made with extracts of cells incubated at 40°C have confirmed the data of HOGGAN and ROIZMAN [2], WHEELER [1], YOSHINO *et al.* [3], that only a small amount of intracellular infective viruses is produced at 40°C; they further showed that the formation of "late" antigenic components was damaged. (*iv*) Incubation at room temperature (20—22°C) did not inhibit the formation of "early" antigenic elements of CA^+ cells. The diffusely distributed granules in the nuclei of CA^- cells incubated at 20°C were like the formations of CA^+ cells kept at 37°C. The development of "late" antigenic formations (inclusion-like bodies) was completely blocked at room temperature. This finding was in accordance with the low degree of infectivity of cell extracts prepared 18, 48 and 96 hours after infection (Table II), which was probably due to absorbed but not penetrated virus particles. This was confirmed also by the fact that with immune serum R6 no complement fixing antigens were detected, while serum R4 revealed 4 (8) antigenic units in the preparations. This finding is in correlation with that of KITAHARA and MELNICK [10], who found that more "early" "tumour-like" antigens were synthetized in the cells if they had been incubated at 30°C after infection with SV-40.

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COMPARATIVE STUDY ON SENSITIVITY TO TOXOPLASMA GONDII OF HUMAN PRIMARY AMNIOTIC CELL CULTURE AND OF MICE

By

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(Received July 23, 1969)

Summary. Primary human amniotic cell cultures and albino mice were tested for sensitivity to the RH strain of *Toxoplasma gondii*. Sensitivity of the cultured cells proved equivalent or superior to that of mice by both qualitative and quantitative evaluation. The findings have confirmed the observation that the virulent viable Toxoplasma count decreased considerably after 24 hours of storage.

A number of reports have dealt with the testing of a variety of tissue cultures for the cultivation of *T. gondii* [1—15], and several authors have studied in animal experiments the factors influencing the demonstration of the protozoon from test materials [16, 17, 18, 19]. However, information is scanty on comparison of sensitivity to *T. gondii* of cell cultures and experimental animals [20, 21]. In view of the favourable results obtained in this laboratory with culturing in primary human amniotic cell culture, it seemed worthwhile to compare the latter's susceptibility with that of the mouse, the animal commonly used in studies of *T. gondii* [15—22]. In addition, experiments were carried out to clarify, how storage in the refrigerator for 24 hours would influence the virulent viable parasite count in a phosphate buffer solution.

Materials and methods

Toxoplasma strain. The international reference strain RH of *T. gondii* was used throughout. Peritoneal exudate from mice was obtained by a technique described previously, with the modification that rinsing of the abdominal cavity was made with pH 7.5 phosphate buffer containing saline [15]. To remove intracellularly located parasites, the exudate was passed through a G3 glass filter without applying negative pressure. The parasite count in the filtrate was assessed by haemocytometry and the different dilutions were prepared with regard to protozoal counts.

Tissue culture. Primary human amniotic cell cultures were prepared by the conventional trypsinization technique. Preparation of glass plate tube cultures and the composition of the nutrient medium were the same as described previously [15].

Experimental animals. Male Swiss albino mice weighing 15—20 g were used throughout.

The experiments were performed in two steps. The first series was set up 6 hours after sacrifice. After cell counting in the filtered exudate, serial dilutions with low toxoplasma counts were prepared with regard to the high virulence of the RH strain. Four dilutions were

prepared in nutrient fluid. Subsequently, in all the three experiments, each dilution was inoculated into 6 tube cultures and 10 mice. The nutrient medium was discarded and replaced by 1 ml of the appropriate dilution, while the mice received the material in 1 ml intraperitoneal doses. The animals were observed for 1 month during which number and time of losses were recorded.

After inoculation, the tube cultures were incubated at 37°C. The glass plates were removed on the 4th, 6th and 8th days and stained with Giemsa's stain. In the stained preparations, the number of infected amniotic cells was assessed by microscopic examination. On the 10th day, the tissue culture was exposed to trypsin for 4 hours as described previously [22] and the cell count in 1 ml was estimated in a haemocytometer.

In the second experimental series the parasites were suspended in pH 7.5 phosphate buffer containing saline (PBS pH 7.5) and stored prior to the experiment for 24 hours at +4°C. Storage was made in highly diluted suspension (*e.g.* 50,000 cells/ml). After 24 hours, aliquots of this suspension were adjusted by dilution in nutrient medium to the cell counts used in the first experiment, and each dilution step was inoculated into 6 tube cultures and 10 mice as described above. Results were evaluated in the same way as above, with the modification that the plates were removed from the tubes on the 6th, 8th and 10th days of incubation and trypsinization was carried out on the 12th day.

Results

In the animal experiments, exudates with an identical protozoa count yielded different results depending on their storage for 6 or 24 hours. The majority of the mice inoculated with 6-hour exudate died between the 7th and 9th days and a small number during the rest of the 1-month observation period. Losses amounted to 90% or higher. With the 6-hour exudate, the different parasite counts had no notable influence on the number and time of deaths.

In contrast, from the mice inoculated with the exudate stored at +4°C for 24 hours, only 30% died between the 10th and 12th days. Results are summarized in Table I.

Table I

Losses among mice infected with different doses of Toxoplasma from exudates stored for 6 and 24 hours

(Figures represent averages of several experiments)

Inoculated toxoplasma count per ml	Time of storage (hours)	Number of animals died on day										Percentage of losses
		7	8	9	10	11	12	13	14	15	15—30	
4600	6	10	13				1	1	2			90
	24				6	1	6					43.3
2300	6	8	14	1		1		2	1	1		93.3
	24			1	4	1		1				23.3
1150	6	8	10	2	4	3	1				1	96.6
	24			2	3	1			1			23.3
575	6	8	12	5				1			1	90
	24				1			1				6.6

In the tissue cultures inoculated with exudates of identical parasite count and stored for 6 or 24 hours, the following phenomena were observed. In the cells inoculated with the 6-hour exudate, toxoplasmal growth was apparent in 10—13% by the 4th day and in 90% by the 8th day. In contrast, in the tubes inoculated with the 24-hour exudate, only 1—2% of the cells showed toxoplasmal growth by the 6th day and this proportion did not notably increase by the 10th day.

The quantitative values for parasite growth in the tissue cultures inoculated with exudates stored for 6 and 24 hours did also differ. As shown in Table II, while the growth rate was of the order of several hundreds in the tubes inoculated with the 6-hour exudate, it was only 20—30-fold in those inoculated with the 24-hour exudate, despite the fact that trypsin treatment had been carried out 2 days later in the latter series.

Table II

Toxoplasma counts in trypsin-treated amniotic cell cultures infected with peritoneal exudate stored for 6 and 24 hours

(Figures represent average values)

Inoculated toxoplasma count/ml	Toxoplasma count/ml after storage for 6 hours	Toxoplasma count/ml after storage for 24 hours
4600	3.750,000	160,000
2300	3.700,000	40,000
1150	3.077,000	31,000
575	1.085,000	11,000

Discussion

The growth of *T. gondii* in tissue cultures, which have been applied for its maintenance in the last 15 years, was seldom compared to the growth of the parasite in experimental animals traditionally used for that purpose. BUTTITTA [16] studied a strain from the Paris Pasteur Institute in mice, guinea-pigs and chick embryos. While 100% of the mice became infected on the first inoculation, of the guinea-pigs only a few died and in chick embryos Toxoplasma was demonstrable only in the second passage. ABBAS [21] compared the susceptibility of mice to that of the chick embryo, and HEp-2 and HeLa cell cultures. The mice were found 16 times more sensitive than the chick embryo and 316 times more sensitive than HEp-2 and HeLa cell cultures [21].

In the present experiments, the susceptibility of the primary human amniotic cell culture was compared to the susceptibility of the mouse, the

experimental animal commonly used in studies of Toxoplasma. Applying for inoculation a peritoneal exudate stored for 6 hours, the sensitivity of the amniotic culture was identical or sometimes slightly superior to that of mice. While none of the mice died before the 7th day after inoculation, in the tissue cultures toxoplasmal growth was always apparent by the 4th day. By the 8th—10th day all amniotic cells adhering to the tube wall had become infected and the maximum attainable parasite count of the order of several millions was demonstrated by haemocytometry. A much less difference in sensitivity was manifest with the 24-hour exudate. Among the mice inoculated with the latter material, no losses occurred before the 10th day, while in the tube cultures, though only in a few, protozoal growth was demonstrable on the 6th day.

Using HeLa cells, LYCKE [24] found a direct relation between the quantity of inoculated Toxoplasma and their growth rate. In the present experiments, with the 6-hour exudate we failed to observe this correlation. The growth rates did not notably vary with the concentrations applied, either in mice or in the cell culture. This may be explained by our earlier finding that the LD_{50} of the strain used was only about 100 [22].

The stability of Toxoplasma in various fluids has extensively been studied. According to JACOBS [25], physiological saline is unsuitable for the maintenance of Toxoplasma even for a short time. This has been confirmed by SCHULZE [26], who found that of the fluids tested saline was the least suitable for storing Toxoplasma and that sodium hydrocarbonate buffer seemed more advantageous. SHIMIZU [27], too, has noted that Toxoplasma suspended in protein-free fluid is liable to rapid deterioration.

The present experiments have confirmed the above findings. The virulent viable Toxoplasma count decreased considerably on storage in phosphate buffered saline at +4°C for 24 hours. This circumstance should be considered in the maintenance of, and all kinds of experiments with, Toxoplasma.

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INDUCTION OF MEMBRANE-BOUND PENICILLINASE SYNTHESIS IN B. CEREUS 569

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Summary. The induction of membrane-bound penicillinase and exopenicillinase was examined by using penicillin and high concentrations of phosphate as inducers. Both penicillin and phosphate induced the synthesis of membrane-bound penicillinase, but the proportion of bound enzyme to total enzyme production was considerably lower in the presence of high electrolyte concentrations. The possible origin of the membrane-bound enzyme is discussed.

According to POLLOCK [1] 6—8% of the total penicillinase production of induced *B. cereus* 569 cells is membrane bound and may be distinguished from exopenicillinase secreted into the medium. The main differences between membrane bound enzyme and exoenzyme are the greater solubility of the bound enzyme in ammonium sulphate solutions, its sensitivity to iodine treatment and its inability to react with exopenicillinase antiserum. POLLOCK could exclude the possibility that the bound penicillinase was a precursor of exopenicillinase, but the possibility of their independent origin as well as that of exopenicillinase being a precursor of the bound enzyme has remained.

Since the bound enzyme is of a considerable biological significance and may have some function in the regulation of penicillinase synthesis, we have studied its biosynthesis and properties. This paper deals with the induction of the bound enzyme.

Materials and methods

The culture medium used and the conditions for the growth of *B. cereus* as well as those of its aspecific induction by high electrolyte concentrations have been described earlier [2, 3].

The activity of bound enzyme was determined by POLLOCK's [1] slightly modified method in the following way. The samples of cell cultures were treated with 50 μg/ml chloramphenicol to prevent further growth, and centrifuged, and the harvested cells were washed four times with a solution containing 1.0 *M* sodium chloride, 0.2 *M* sodium citrate and 50 μg/ml chloramphenicol; the pH was adjusted to 8.0. After washing the cells were suspended in 3 ml of 0.5% gelatin containing 50 μg/ml chloramphenicol and penicillinase activity was determined iodometrically as described earlier [4].

Results

Induction with penicillin. A logarithmically growing culture of *B. cereus* 569 was induced with 6 μg/ml of sodium G-penicillin. Samples were taken in

intervals as indicated in Fig. 1A; exopenicillinase and bound penicillinase and the optical density of the cells were determined.

The kinetics of the synthesis of the two enzymes showed some remarkable differences. Exopenicillinase production followed an exponential curve while the production kinetics of the found enzyme was evidently linear. In the case of exoenzyme a certain "lag phase" was observed by POLLOCK [5]. On the basis of our experiment it is obvious that the lag phase represents only the first part of an exponential curve and not an inhibited or delayed synthesis

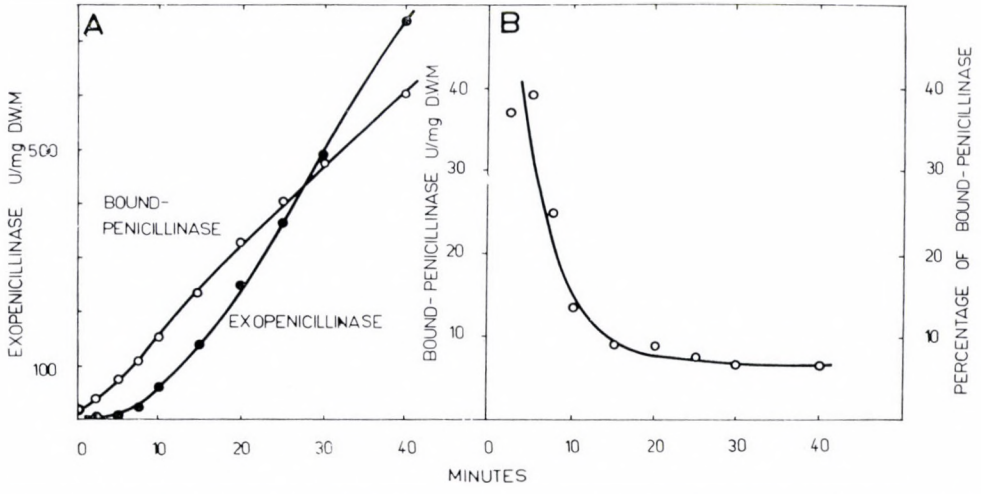

Fig. 1. Induction of exopenicillinase and bound penicillinase with penicillin. A logarithmically growing culture of *B. cereus* 569 was induced by 6 μg/ml penicillin. A. ●—●—●— exopenicillinase, ○—○—○— bound penicillinase; B. ○—○—○— amount of bound penicillinase in percentage of the total amount of enzyme production

The production of bound penicillinase showed no lag phase.

Another interesting feature of the synthesis was revealed by expressing the amount of bound enzyme produced at a given time as a percentage of the total enzyme production. As it can be seen in Fig. 1B, immediately after the induction the proportion of bound enzyme was 20 to 50% in different experiments while after the lag phase its amount was only 8—10% of the total production, as found also by POLLOCK [1].

Induction by electrolytes. As it has been shown earlier, penicillinase is inducible by high electrolyte concentrations [3]. In the following experiments the influence of 0.5 *M* phosphate on bound enzyme synthesis was examined.

Three logarithmically growing cultures were induced by 6 μg penicillin, 0.5 *M* phosphate and by penicillin and phosphate together, respectively. The production of bound enzyme in percentage of the total amount of penicillinase can be seen in Fig. 2.

Unlike penicillin induced cells, the production of bound enzyme began slowly in the case of phosphate induction, and little enzyme was obtained in the first phase of synthesis.

The effect of penicillin and phosphate together used as inducer was the same as that of phosphate alone.

In some unpublished experiments the bound enzyme content of the penicillin induced cells showed no difference whether they had been washed

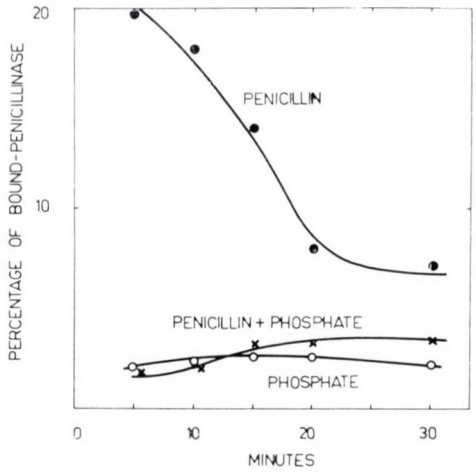

Fig. 2. Induction of bound penicillinase with high electrolyte concentrations. Three logarithmically growing cultures of *B. cereus* 569 were induced by 6 μg/ml penicillin, 0.5 *M* phosphate and by both penicillin and phosphate, respectively. ●—●—●— induced by penicillin; ○—○—○— induced by phosphate; ×—×—×— induced by penicillin and phosphate

with 1 *M* sodium chloride + 0.2 *M* sodium citrate or with 0.5 *M* phosphate. Thus the lower bound enzyme content of the cells growing in the presence of 0.5 *M* phosphate was not due to the eluating effect of the phosphate.

Discussion

Both penicillinases formed by *B. cereus* are induced beside penicillin, the natural inducer, with high concentrations of electrolytes. The kinetics of cell bound penicillinase synthesis is remarkably different from that of exopenicillinase. Exopenicillinase formation follows an exponential curve in the early phase of synthesis, then it becomes linear, while bound enzyme formation is linear immediately after the induction. Following induction by penicillin the proportion of bound enzyme is considerable, but it is low and very slow if

induction takes place in phosphate at high concentrations, an inducer by itself. It is known from the experiments of CITRI *et al.* [6] that the adsorption of exopenicillinase can be prevented by high electrolyte concentrations.

These two effects of electrolytes, the inhibition of exopenicillinase adsorption and the prevention of great amounts of bound enzyme immediately after induction, suggest an explanation for the regulation of the two enzymes.

The possibility of bound penicillinase being a precursor of exopenicillinase has been excluded by POLLOCK, thus we suggest that exopenicillinase is a precursor of the bound enzyme, which latter is formed through an adsorption process, as supported by the experiments presented in this paper. Further experiments are, however, necessary to prove this assumption.

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THE EFFECT OF LYSOZYME AND METICILLIN ON THE GROWTH OF METICILLIN RESISTANT AND SENSITIVE STAPHYLOCOCCUS AUREUS STRAINS

By

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Summary. In the presence of lysozyme the meticillin resistance of naturally meticillin resistant staphylococci increased slightly. Under the same condition, strains sensitive to the antibiotic or adapted to meticillin resistance showed an increased sensitivity.

In the presence of meticillin + lysozyme the lag phase for naturally resistant staphylococcal cultures decreased to about one-half, while that for sensitive and adaptively resistant strains increased 4—7-fold of the value observed with meticillin alone. When sublethal concentrations of meticillin + lysozyme were added in the logarithmic phase, the number of surviving bacteria decreased by 1 exponent in naturally resistant cultures and by 5—6 exponents in sensitive and adaptively resistant strains.

Meticillin resistant *Staphylococcus aureus* strains distributed in nature show a phenotypic resistance [2, 5, 16] associated with penicillinase production and a so-called intrinsic or inherent factor [2, 3, 5, 8, 16]. Penicillinase-producing *Staph. aureus* strains inactivate semisynthetic penicillins, antibiotics known as resistant to penicillinase activity [3, 8, 9, 14, 15, 18, 19]. As inactivation of these drugs begins and increases gradually only in the stationary phase, it may be regarded as a secondary phenomenon not being a direct cause of resistance.

Little is known about the intrinsic factor. Osmotic [1, 2], acriflavine [2, 5], UV irradiation, acridine [10] and ultrastructural examinations of meticillin resistant staphylococci as well as the isolation and study of meticillin resistant mutants which fail to produce penicillinase [6, 8, 16, 17] have contributed to the knowledge of, but did not suffice to explain the reason for, meticillin resistance.

In this respect new perspectives have been opened by our studies on the dynamics of growth of naturally and adaptively meticillin resistant staphylococci [13—15].

From these experiments it was concluded that naturally meticillin resistant staphylococci differ from sensitive cultures in cell wall synthesis and it is probable that the former organisms are capable of synthesizing their whole cell wall in the presence of the antibiotic. In order to prove this hypothesis, we have examined the behaviour of naturally resistant, adaptively resistant and sensitive *Staph. aureus* strains in the presence of meticillin and lysozyme.

Materials and methods

Strains. Staph. aureus 5814/R was isolated in our laboratory [13] on a medium containing 500 μg meticillin/ml; the strain is a naturally meticillin resistant, penicillinase-producing organism inhibited by 1600 μg/ml of meticillin during 48 hours incubation at 37°C. *Staph. aureus* 100/110 was kindly supplied by Prof. G. T. STEWARD and Dr. R. J. HOLT; it is a naturally meticillin resistant strain [19] inhibited by 1000 μg/ml of the antibiotic. *Staph. aureus* 18R was kindly supplied by Dr. MARY BARBER; it had been trained to meticillin resistance (96 μg/ml) by serial passages on meticillin medium. *Staph. aureus* 80/81 was isolated in our institute; the strain is sensitive to 4 μg/ml of meticillin and produces penicillinase. *Staph. aureus* 100 is sensitive to 1 μg/ml and fails to produce penicillinase.

Medium. One per cent glucose-casitone (Difco) broth, pH 7.2 [21].

Lysozyme. Crystalline product from egg white (Reanal, Budapest).

Meticillin, produced by Chinoin, Budapest.

Determination of meticillin sensitivity in the presence of lysozyme. Meticillin was serially diluted at 1 ml volumes in casitone broth containing 0, 20, 200 and 500 μg lysozyme per ml. Meticillin concentrations were 1600—1500—1400— etc. μg/ml for strains 5814/R and 100/110 96—48—24— etc. and 128—64—32—16— etc. (two parallel rows), for strains 18R and 100. Each tube was seeded with 0.1 ml of a 24 hour broth culture diluted 1 : 100. Incubation lasted for 48 hours at 37°C. Readings were made with naked eye after 24 and 48 hours.

Growth in the presence of meticillin and meticillin + lysozyme. The cultures were grown at 37°C in the "Bonet Maury et Jouan Biophotometre" in broth without additives and with lysozyme, meticillin, or both. Each cuvette containing 10 ml medium was seeded with 10^7 cells.

Effect of meticillin, and meticillin + lysozyme on logarithmic phase cultures. Ten ml broth were seeded with 10^7 cells and incubated in the biophotometer. When the logarithmic phase was reached, lysozyme or meticillin or their combination was added to cuvettes at concentrations indicated in the Figures and Tables. Then the cuvettes were incubated further and after 22—24 hours the number of colony-forming units was determined as described previously [13].

Results

Changes in meticillin sensitivity in the presence of lysozyme. Table I shows the effect of various concentrations of lysozyme on the meticillin sensitivity of the examined staphylococci.

Table I

Effect of lysozyme at various concentrations on meticillin sensitivity of Staph. aureus strains

Lysozyme μg/ml	Incubation, hours	Minimal inhibitory concentrations for meticillin* μg/ml			
		5814/R	80/81	18R	100
0	24	1200	2	48	1
	48	1600	4	96	1
20	24	1600	1	32	1
	48	2000	1	64	1
200	24	1600	1	24	1
	48	2400	1	48	1
500	24	2000	0.5	24	0.5
	48	3200	1	32	0.75

* Average values for 6 experiments

Lysozyme did not influence growth. In the presence of lysozyme naturally resistant strain 5814/R was more resistant to meticillin than in lysozyme-free medium. Increasing of the amount of lysozyme increased slightly the resistance to meticillin. Similar results were obtained with strain 100/110 [15].

The meticillin sensitive, penicillinase positive strain 80/81 exhibited an increased meticillin sensitivity even at low lysozyme concentrations. At 500 μg lysozyme/ml the minimal inhibitory concentration of meticillin decreased to 1/4 of the level shown in lysozyme-free medium.

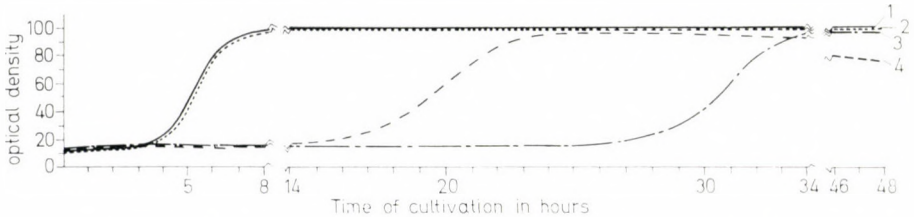

Fig. 1. Growth curves for naturally resistant *Staph. aureus* strain 5814/R in 1% glucose-casitone broth. 1 = control; 2 = lysozyme 500 μg/ml; 3 = meticillin 800 μg/ml; 4 = meticillin 800 μg/ml + lysozyme 500 μg/ml

The meticillin resistance of strain 18R (adapted to meticillin resistance, penicillinase negative) decreased in the presence of lysozyme. At higher lysozyme concentrations the decrease became more marked: with 500 μg/ml of lysozyme the minimal inhibitory concentration of meticillin was 1/2—1/3 of the value found in the control medium.

Meticillin sensitive, penicillinase negative *Staph. aureus* strain 100 was used as a control for strain 18R. Strain 100 showed increased sensitivity to meticillin only at a concentration of 50 μg/ml of lysozyme.

The results indicate that naturally meticillin resistant strains differ from adaptively resistant and sensitive ones; in the presence of lysozyme the former show a slightly increased meticillin resistance while the latter become more sensitive to the antibiotic.

Dynamics of growth in the presence of meticillin and meticillin + lysozyme. From the above experiments it is evident that the effect of lysozyme which is the opposite in naturally resistant and in adaptively resistant or sensitive cultures, is well-defined at 500 μg/ml concentration. Accordingly, in further experiments lysozyme was used at 500 μg/ml.

Fig. 1 shows growth curves for the naturally resistant strain 5814/R, in the absence and in the presence of lysozyme, meticillin and of meticillin and lysozyme.

Growth was similar in the control medium and in the presence of lysozyme (curves 1 and 2). In the absence of meticillin the optical density increased rapidly from the 3rd hour onward and reached the maximum after 5 hours

(curve 1). If the medium contained meticillin, the optical density began to increase only after 24 hours and reached the maximum after another 11 hours of incubation (curve 3). In meticillin + lysozyme medium optical density began to increase after 14 hours and reached the peak after further 10 hours, to remain there for 7—8 hours, then slowly to decrease (curve 4). This finding indicates that naturally resistant bacteria begin to multiply earlier in meticillin + lysozyme than in meticillin medium. Similar observations were made for strain 100/110 [15].

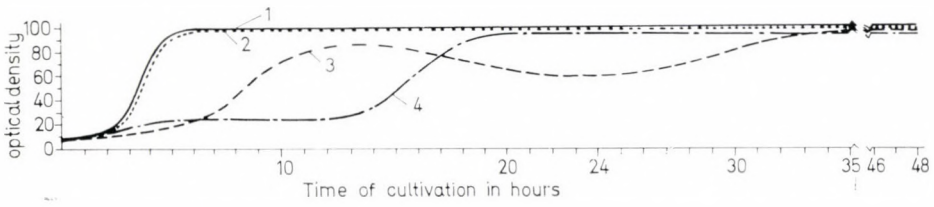

Fig. 2. Growth curves for penicillin sensitive, adaptively meticillin resistant *Staph. aureus* strain 18R in 1% glucose-casitone broth. 1 = control; 2 = lysozyme 500 μg/ml; 3 = meticillin 16 μg/ml; 4 = meticillin 16 μg + lysozyme 500 μg/ml

The growth dynamics of adaptively resistant and sensitive staphylococcal strains were similar in meticillin and in meticillin + lysozyme media. The results for strain 18R are presented in Fig. 2.

It is evident that lysozyme *per se* did not influence the growth of the adaptively resistant strain (curve 2); the course of multiplication was the same as in the control medium (curve 1). In the presence of 16 μg/ml of meticillin (curve 3), growth began nearly after the same period as in the control medium but optical density, especially until the 6th—7th hour, increased very slowly. Later it increased more rapidly, but never reached the peak in the control and began to decrease after 13—14 hours. The decrease lasted until the 23rd—24th hour of incubation. In meticillin + lysozyme (curve 4), after a lag of 3 hours the optical density slightly increased and remained at that level until the 12th hour. After incubation for another 9 hours the peak was reached, then for a 9 hour period the density remained unaltered; after the 30th hour of incubation a slight lysis was observed.

With 32 μg/ml of meticillin the growth curve for strain 18R was similar in course to that with 16 μg/ml, only the lag phase lasted longer and lysis was more marked [13]. In broth containing 32 μg meticillin + 500 μg lysozyme/ml the culture failed to multiply within 24 hours.

Lysozyme in the presence of meticillin retarded the beginning of growth of adaptively resistant and sensitive strains; when multiplication had started the cells were practically resistant to the lytic action of meticillin and lysozyme. This was shown by the fact that the optimum density in meticillin + lysozyme

remained at a low level for long, because the multiplying cells synthetized the cell wall structure inadequately and thus the cells were lysed rapidly. The process lasted until cells sensitive to the given meticillin concentration had disappeared from the culture.

The main difference between naturally meticillin resistant staphylococci and adaptively meticillin resistant or sensitive staphylococci is as follows. Naturally resistant strains in meticillin + lysozyme show a lag phase almost two times shorter than the lag phase in meticillin. In contrast, the lag phase of adaptively resistant and sensitive strains is 4—7 times longer than in meticillin medium. That is, lysozyme in the presence of meticillin promotes the multiplication of naturally resistant strains but retards the growth of adaptively resistant or sensitive cultures.

Effect of lysozyme, meticillin and meticillin + lysozyme on logarithmic phase cultures. In these experiments we examined the effect of meticillin and meticillin + lysozyme on logarithmic phase cultures of strains of different meticillin sensitivity. In addition,it was studied whether a damage or destruction of cells had occurred.

The strains were cultured in the biophotometer in broth containing neither meticillin nor lysozyme. In the logarithmic phase lysozyme, meticillin and meticillin + lysozyme were added to different cuvettes and the alteration in the course of growth was recorded.

The growth of naturally meticillin resistant strain 5814/R was not influenced by 500 μg/ml lysozyme, 800 μg/ml of meticillin or 800 μg meticillin + + 500 μg lysozyme/ml. The effect of 1200 μg/ml of meticillin added in the logarithmic phase is shown in Fig. 3.

It is seen that the optical density for strain 5814/R was not influenced by 500 μg lysozyme or by 1200 μg meticillin/ml (curves 1 and 2). Meticillin + lysozyme also failed to alter the optical density for 8 hours, then a slight degree of lysis occurred (curve 3).

Logarithmic phase cultures of adaptively meticillin resistant strain 18R and of meticillin sensitive strains reacted similarly to lysozyme, meticillin and meticillin + lysozyme. The changes in the curves for strain 18R are presented in Fig. 4.

Lysozyme failed to influence the course of growth (curve 1). After the addition of meticillin the curve continued without alteration for 2 hours, then a gradual lysis occurred. The optical density decreased until the 22nd hour of incubation, then remained at the same level (curve 2). After the addition of meticillin + lysozyme the growth curve showed a regular course for 1 1/2 hours, then the optical density highly decreased; the lysis lasted for 8 hours (curve 3).

The effect of lysozyme, meticillin and meticillin + lysozyme added to logarithmic phase cultures on the survival of staphylococci is summarized

in Table II. Colony-forming units were counted in the 22nd—24th hour. The figures show average values obtained in 3 experiments.

The number of surviving cells of strain 5814/R was not appreciably influenced by 500 μg lysozyme, 800 μg meticillin and 800 μg meticillin + 500 μg lysozyme/ml. No significant change was caused by 1200 μg meticillin without lysozyme. Even meticillin + lysozyme induced only a one exponent decrease.

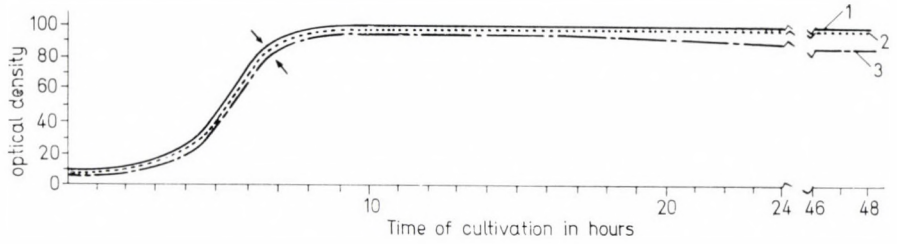

Fig. 3. Changes in growth curves for *Staph. aureus* strain 5814/R as an effect of 500 μg lysozyme/ml (1), 1200 μg meticillin/ml (2) and 1200 μg meticillin/ml + 500 μg lysozyme/ml (3) added in the logarithmic phase

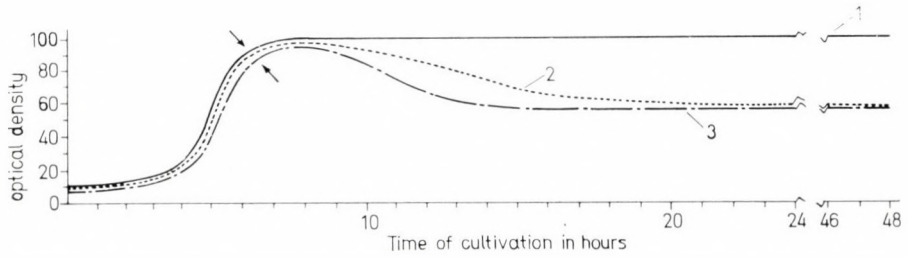

Fig. 4. Changes in growth curves for *Staph. aureus* strain 18R as an effect of 500 μg meticillin/ml (1), 64 μg meticillin/ml (2) and 64 μg meticillin + 500 μg lysozyme/ml (3) added in the logarithmic phase

The survival of strain 18R was not influenced by lysozyme; meticillin decreased the number of surviving cells by 5, meticillin + lysozyme by 6 exponents. For strain 100 lysozyme was ineffective, meticillin caused a 5 exponent, meticillin + lysozyme a 6 exponent decrease. Penicillinase-producing strain 80/81 was more resistant: the decrease in viable cell counts amounted to 3 exponents for meticillin and 5 exponents for meticillin + lysozyme.

These experiments showed significant differences between naturally resistant, adaptively resistant and sensitive staphylococci. Sublethal concentrations of meticillin added to logarithmic phase cultures exerted no effect on naturally resistant cells during 20 hours incubation. The same dose of meticillin caused a marked lysis in adaptively resistant and sensitive cultures.

Table II

Effect of lysozyme, meticillin and meticillin + lysozyme on survival of Staph. aureus strains

Strains	Meticillin added to logarithmic phase cultures, μg/ml	Number of viable cells per ml			
		Control	Lysozyme	Meticillin	Meticillin + lysozyme
5814/R	800	1.2×10^{9}	1.2×10^{9}	6.6×10^{8}	4.1×10^{8}
5814/R	1200	1.2×10^{9}	1.1×10^{9}	6.2×10^{8}	1.6×10^{8}
18R	64	1.2×10^{9}	1×10^{9}	1.4×10^{4}	5×10^{3}
100	1	1.3×10^{9}	1.2×10^{9}	2.5×10^{4}	3.4×10^{3}
80/81	1	1.3×10^{9}	1.1×10^{9}	4.4×10^{6}	4.2×10^{4}

Lysozyme concentration, 500 μg/ml. Determination of viable counts was made after 22—24 hours cultivation.

As an effect of sublethal concentrations of meticillin + lysozyme optical density of the naturally resistant culture showed a slight decrease while there was a rapid considerable lysis in cultures of the two other organisms. Meticillin caused no important decrease in surviving cells of the naturally resistant strain, but in adaptively resistant and sensitive cultures it decreased the viable cell count by 3—5 exponents. Under the effect of a sublethal dose of meticillin the number of surviving bacteria decreased in the naturally resistant culture only by 1 exponent while in the two other cultures by 5—6 exponents.

Summing up, the naturally meticillin resistant culture was insusceptible and the adaptively resistant or sensitive culture was susceptible to the cell wall-damaging effect of meticillin + lysozyme.

Discussion

There are few data in the literature on the basis of which the behaviour of naturally meticillin resistant, adaptively meticillin resistant and meticillin sensitive staphylococci could be explained. No such experiments have been performed with meticillin resistant strains. As to strains sensitive to semisynthetic penicillins, the following data were available.

It is known from the reviews of MORSE [11] and STOLP and STARR [20] that the intact *Staph. aureus* cell and cell wall are resistant to lysozyme. If theicholic acid is extracted from the cell wall with trichloracetic acid, the organism becomes sensitive to lysozyme [11]. The substrate for lysozyme is murein (mucopeptide); lysozyme exerts a specific activity on the beta (1,4)-glycoside bonds of amino sugars present in murein. Theicholic acid, polysaccharides and O-acetyl groups linked to murein protect the molecule against lysozyme-type

intracellular enzymes present in all bacterial cells. Pretreatment with meticillin induces lysozyme sensitivity in meticillin sensitive, lysozyme resistant *Staph. aureus* [20]. According to ROGERS and JELJASZEVICZ [12] meticillin inhibits murein synthesis in the cell wall of meticillin sensitive *Staph. aureus*. WARREN and GRAY [22—24] showed that subinhibitory concentrations of meticillin caused structural disorders and disorganization in the cell wall of sensitive *Staph. aureus* in consequence of which an intracellular polysaccharide accumulation occurred. The structure of the cell wall shows alterations and increased permeability under the effect of semisynthetic penicillins. BARBER [1] showed that in sensitive staphylococci cell wall synthesis was inhibited by meticillin at concentrations allowing multiplication. The inhibitory effect was partial as cell division was not influenced.

Our results obtained with meticillin sensitive and adaptively meticillin resistant *Staph. aureus* strains are in agreement with data in the literature [1, 9, 12—24]. When multiplying in the presence of meticillin, these bacteria are unable to synthetize the complete murein polymer in their cell wall and thus intracellular autolytic enzymes lyse their own cells and even other bacteria. This hypothesis is supported by the finding of WARREN and GRAY [24] that the lysis of naphcillin-treated naphcillin sensitive staphylococcal cells is considerably decreased by heating of the culture.

If adaptively meticillin resistant and meticillin sensitive bacteria are cultured in meticillin + lysozyme, the cells fail to develop a perfect cell wall so that this will be penetrated and easily lysed by lysozyme. This explains the fact that in meticillin + lysozyme (depending on the concentration of the antibiotic) the lag phase increases several times. During this period, cells sensitive to the given meticillin + lysozyme concentration are completely destroyed and, therefore, such lysis and secondary multiplication which was observed when the medium contained only meticillin, does not occur.

The lysozyme resistance of naturally meticillin resistant staphylococci cannot be eliminated with meticillin. If such organisms are cultured in meticillin + lysozyme from the beginning, lysozyme promotes multiplication instead of inhibiting it and increases slightly the degree of meticillin resistance. This effect cannot be explained at present.

A partial decrease in lysozyme resistance can be induced in naturally resistant staphylococci if cultivation is begun in antibiotic and enzyme-free medium, and meticillin and lysozyme are added in the logarithmic phase. The partial decrease of lysozyme resistance highly depends on the concentration of meticillin, but it is not considerable even in the presence of sublethal amounts of the antibiotic. For example, lysozyme resistance of strain 5814/R was not appreciably influenced by 800 μg/ml of meticillin and even 1200 μg/ml of meticillin caused only a slight decrease (see Fig. 3 and Table II).

The results indicate that in the presence of meticillin, naturally meticillin resistant bacteria produce a complete cell wall and damaged cells occur unfrequently, in other words the majority of cells cannot be penetrated by lysozyme, the substrate is protected and the cells remain resistant to lysozyme. It may be concluded that naturally meticillin resistant staphylococci differ from adaptively meticillin resistant and meticillin sensitive *Staph. aureus* strains in cell wall synthesis and/or composition.

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PROPERTIES OF ACETOHYDROXY ACID SYNTHETASE IN STREPTOMYCES RIMOSUS

By

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Summary. Acetohydroxy acid synthetase in *Streptomyces rimosus* requires thiamine pyrophosphate and flavine adenine dinucleotide. The finding that although valine is inhibitory at pH 7.4 and 28°C, it exerts a considerable activating effect at higher pH values or temperatures has been explained by its marked stabilizing effect on the enzyme.

The proportion of α-acetolactic acid and α-aceto-α-hydroxybutyric acid synthesis was studied by determining the effect of the concentrations of the corresponding substrates (pyruvic acid and α-ketobutyric acid). As compared to the enzyme in other microorganisms, the affinity of α-ketobutyric acid to *S. rimosus* acetohydroxyacid synthetase was higher and, accordingly, isoleucine precursor synthetase was higher and, accordingly, isoleucine precursor synthesis in the latter was promoted. This finding may be explained by the derepressing effect of α-ketobutyric acid.

Streptomyces rimosus produces isoleucine in the presence of threonine or α-ketobutyric acid [1, 2]. In a study of this process we have shown that common enzymes involved in valine and isoleucine biosynthesis are derepressed in cells grown in media containing threonine or α-ketobutyric acid. The derepression can be inhibited with valine and leucine [3]. On the basis of these experiments we explained the reason for derepression as follows. It is known that acetohydroxy acid synthetase, the first common enzyme of valine and isoleucine synthesis, acts on two different substrates, *viz.* pyruvic acid and α-ketobutyric acid [4]. It may be assumed that the affinity of α-ketobutyric acid to the enzyme is higher in *S. rimosus* than in other microorganisms [5, 6] and, accordingly, this substance inhibits the synthesis of valine (and isoleucine) precursor. In the absence of valine the enzyme system is derepressed. In order to confirm this hypothesis we have performed study on the properties of acetohydroxy acid synthetase in *S. rimosus*.

Materials and methods

Growth of organism. S. rimosus variant BS 21/76 was grown in glycine-glucose medium described previously [7]. Addition of 5 *mM* α-ketobutyric acid to the medium resulted in a derepression of enzymes involved in isoleucine and valine biosynthesis [3].

Preparation of cell-free extract and partial purification and concentration of the protein solution were performed as described in studies on *Mycobacterium pellegrino* [6], except that in every step the solutions were supplemented with 0.2 *mM*-L-valine in order to improve the enzyme's stability. The protein solution concentrated by ammonium sulphate precipitation was stored not longer than a few hours after preparation; before use it was gel filtered through Sephadex G-25 column.

Assay of acetohydroxy acid synthetase was performed as described previously [6]. α-Aceto-α-hydroxybutyric acid was assayed by the microbiological method of LEAWITT and UMBARGER [8]. Specific activity was expressed as μmole α-acetohydroxy acid produced by 1 mg protein in 1 hour. Protein was determined as described by LOWRY *et al.* [9]. Analytical grade commercial preparations were used.

Abbreviations: TPP = thiamine pyrophosphate; FAD = flavine adenine dinucleotide; ALA = α-acetolactic acid; AHBA = α-aceto-α-hydroxybutyric acid.

Results

Cofactor requirement. This was determined with crude extract gel filtered through a Sephadex G-25 column equilibrated with 0.1 *M* phosphate buffer (pH 7.4). From Table I it is evident that TPP is needed for the activity of the enzyme and FAD exerts an activating effect. Similarly as in other microorganisms, the enzyme requires phosphate at high concentration and the presence of magnesium ions.

Effect of pH on enzyme activity. The reaction rate was examined in 0.1 *M* phosphate buffer in the range of pH 6.5—8.5. Activity was highest at pH 7.4. When the same system had been supplemented with 0.5 *mM* valine (which was definitely inhibitory under pH 7.4), the pH optimum fell between 7.4—8.0, that is, above pH 7.5 valine seemed to exert an activating effect (Fig. 1).

Effect of temperature on reaction rate. The experiments were performed in the absence and in the presence of 1 *mM* L-valine (Fig. 2). Without valine the optimum temperature was 30°C; with valine, it was 35°C. Accordingly, above 30°C valine was markedly activating the enzyme.

Table I

Acetohydroxy acid synthetase activity in reaction mixtures of different composition

	α-Acetolactic acid, μmole
Complete reaction mixture	0.32
Without phosphate buffer*	0.23
Without magnesium	0.28
Without TPP	0.06
Without FAD	0.26
Complete reaction mixture + 1 *mM* L-valine	0.21

The figures indicate μmole acetolactic acid produced in 30 minutes. Complete reaction mixture: phosphate buffer (pH 7.4), 0.1 *M*; magnesium chloride, 0.01 *M*; TPP 0.1 *mM*; FAD, 0.01 *mM*; pyruvic acid, 40 *mM*; protein, 1.3 mg/ml.

* In this experiment the reaction mixture contained a small amount (0.01 *M*) of phosphate, as prior to the reaction the protein solution was filtered through Sephadex G—25 column equilibrated with 0.1 *M* phosphate buffer.

Influence of pH and temperature on the effect of valine. The inhibitory and the above-described activating effect of valine was highly dependent on the pH and the temperature (Fig. 3). At lower temperatures an increase of the pH decreased the sensitivity to valine; the activating effect of higher temperatures became more marked when the pH had been increased.

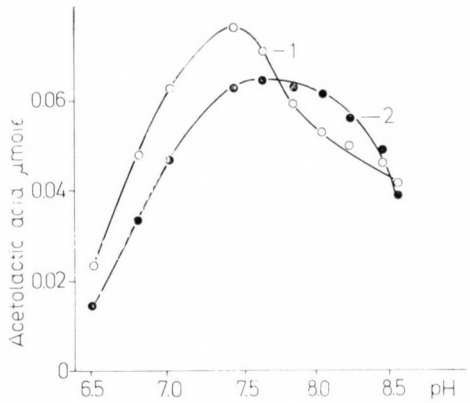

Fig. 1. Acetohydroxy acid synthetase activity *vs.* pH. Reaction mixture: phosphate buffer 0.1 *M*; magnesium chloride, 0.01 *M*; TPP, 0.1 *mM*; FAD, 0.01 *mM*; pyruvic acid, 40 *mM*; protein, 0.5 mg/ml. Reaction time, 20 minutes at 28°C. The pH was adjusted to 6.5—8.5 with a Cambridge pH meter. 1 = without valine; 2 = with 0.5 *mM* valine

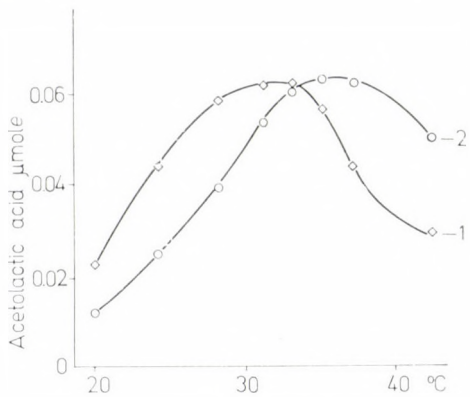

Fig. 2. Acetohydroxy acid synthetase activity *vs.* temperature. Reaction mixture: phosphate buffer, 0.1 *M*; magnesium chloride, 0.01 *M*; TPP, 0.1 *mM*; FAD, 0.01 *mM*; pyruvic acid, 40 *mM*; protein, 0.5 mg/ml. Reaction time, 20 minutes at 20—42°C. 1 = without valine; 2 = with 1 *mM* valine

Effect of substrate concentration on reaction rate. When plotted according to Lineweaver—Burk, the reaction rate showed a linear relationship to pyruvic acid concentration in the range 4—100 *mM* (Fig. 4). The K_M value for this part was 8.7 *mM*. At lower substrate concentrations a slightly sigmoid sub-

strate saturation curve was obtained. In the presence of valine the shape of the curve tended to become straight; at low substrate concentrations valine exerted a strong activating effect (Fig. 5 shows the degree of valine sensitivity versus substrate concentration). In contrast to enzymes extracted from other microorganisms showing no apparent substrate inhibition [15, 16], in the present experiments higher substrate concentrations (10—100 *mM*) failed to influence valine sensitivity.

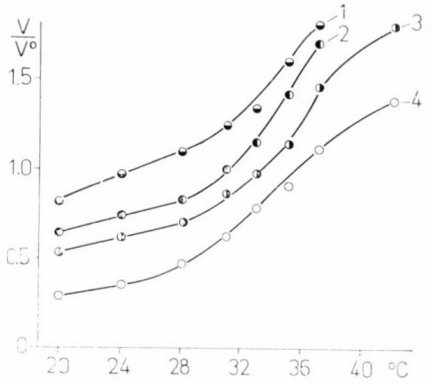

Fig. 3. Effect of valine on acetohydroxy acid synthetase activity at various temperatures and pH values. Reaction mixture: phosphate buffer, 0.1 *M*; magnesium chloride, 0.01 *M*; TPP, 0.1 *mM*; FAD, 0.01 *mM*; pyruvic acid, 40 *mM*; protein, 0.5 mg/ml. The pH was adjusted with a Cambridge pH meter. The temperature ranged between 20—42°C. 1 = pH 8; 2 = pH 7.7; 3 = pH 7.4; 4 = pH 7.0

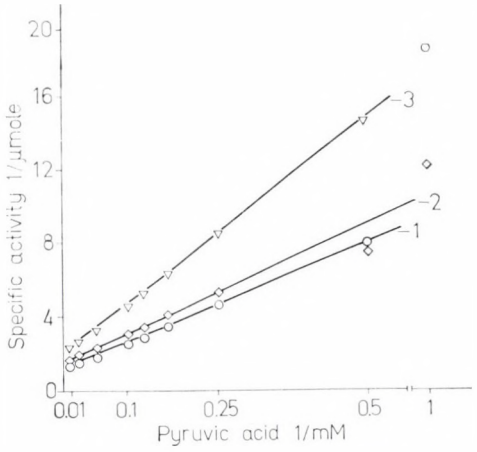

Fig. 4. Relationship between acetohydroxy acid synthetase activity and substrate concentration plotted according to Lineweaver-Burk. Reaction mixture: phosphate buffer, 0.1 *M*; magnesium chloride, 0.01 *M*; TPP, 0.1 *mM*; FAD, 0.01 *M*, protein, 0.56 mg/ml. Substrate concentration ranged between 1—100 *mM*. Reaction time, 20 minutes at 28°C. Reaction rates: 1 = without valine; 2 = with 0.5 *mM* L-valine; 3 = with 2 *mM* L-valine

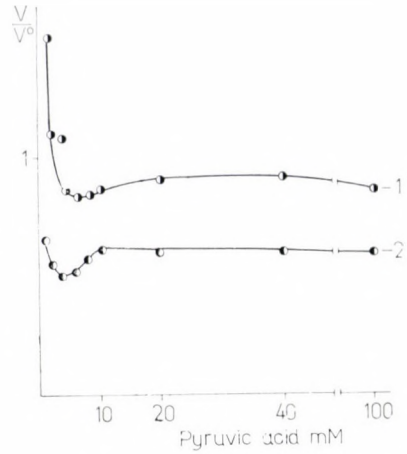

Fig. 5. Valine sensitivity of acetohydroxy acid synthetase at various substrate concentrations. Reaction mixture: phosphate buffer (pH 7.4), 0.1 *M*; magnesium chloride, 0.01 *M*; TPP, 0.1 *mM*; FAD, 0.01 *mM*; protein, 0.56 mg/ml. Pyruvic acid concentration ranged between 1—100 *mM*. Reaction time, 20 minutes at 28°C. 1 = with 0.5 *mM* L-valine; 2 = with 2 *mM* L-valine

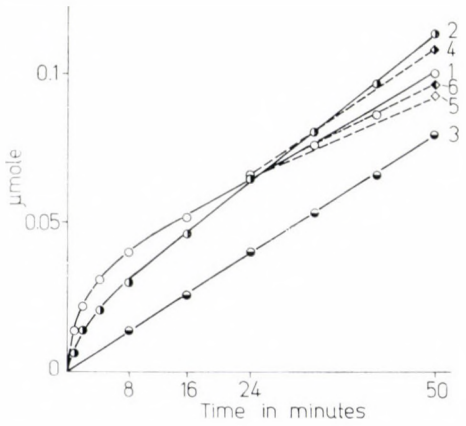

Fig. 6. Acetohydroxy acid synthetase activity in the presence and in the absence of valine. Reaction mixture: phosphate buffer (pH 7.4), 0.1 *M*; magnesium chloride, 0.01 *M*; TPP, 0.1 *mM*; FAD, 0.01 *mM*; protein, 0.4 mg/ml. Reaction temperature, 28°C. The reaction was started in the absence and in the presence of 0.2 *mM* and 1 *mM* L-valine. After 24 minutes all three reaction mixtures were supplemented with L-valine. 1 = reaction mixture without valine; 2 = with 0.2 *mM* valine; 3 = with 1 *mM* valine; 4 = effect of 0.2 *mM* valine added after 24 minutes to valine-free reaction mixture; 5 = effect of 1 *mM* valine added after 24 minutes to valine-free reaction mixture; 6 = effect of 1 *mM* valine added after 24 minutes to reaction mixture containing 0.2 *mM* valine

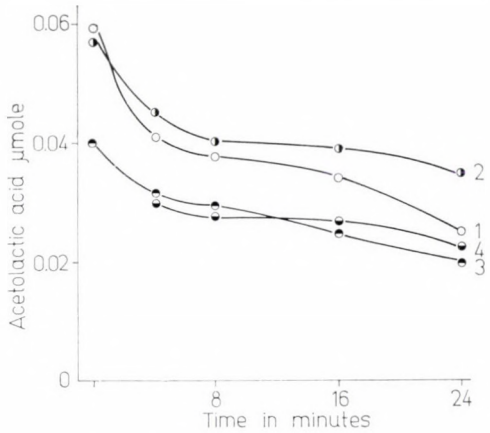

Fig. 7. Acetohydroxy acid synthetase activity in substrate-free reaction mixture at 28°C. Reaction mixture for preincubation: phosphate buffer (pH 7.4), 0.1 *M*; magnesium chloride, 0.01 *M*; TPP, 0.1 *mM*; FAD, 0.01 *mM*; protein, 0.5 mg/ml. The reaction was started by the addition of 40 *mM* pyruvic acid, at the time indicated on the graph. The figures indicate *µ*mole acetolactic acid produced in 20 minutes at 28°C. 1 = enzyme preincubated without valine; 2 = enzyme preincubated with 0.2 *mM* valine; 3 = enzyme preincubated without valine — when starting the reaction 1 *mM* valine was added together with pyruvic acid; 4 = enzyme preincubated with 0.2 *mM* valine — when starting the reaction 1 *mM* valine was added together with pyruvic acid

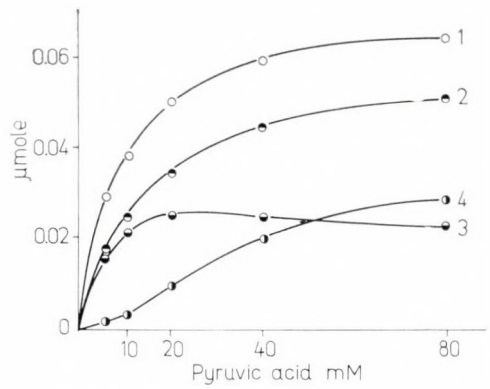

Fig. 8. Proportion of acetolactic acid and acetohydroxybutyric acid produced by *Streptomyces rimosus* acetohydroxy acid synthetase. Reaction mixture: phosphate buffer (pH 8), 0.1 *M*; magnesium chloride, 0.01 *M*; TPP, 0.1 *mM*; FAD, 0.01 *mM*; protein, 0.4 mg/ml. Reaction time, 20 minutes at 28°C. Pyruvic acid concentration ranged between 6—80 *mM* in the absence and in the presence of 1 *mM* ketobutyric acid, 1 = amount of α-acetolactic acid produced in the presence of pyruvic acid, as determined by WESTERFELD's method; 2 = total amount of acetohydroxy acids produced in the presence of α-ketobutyric acid and pyruvic acid, as determined by WESTERFELD's method; 3 = amount of α-acetohydroxybutyric acid assayed by agar diffusion method; 4 = amount of α-acetolactic acid calculated by subtracting α-aceto--α-hydroxybutyric acid from total acetohydroxy acid content

Alteration of enzyme activity during the reaction. The above experiments were performed with enzyme solutions stored in the presence of 0.2 *mM* valine and gel filtered before use. The solution was diluted 1 : 10 in the reaction mixture. If gel filtration had been carried out without adding valine previously, during the first 10 minutes of the reaction a high activity was observed which thereafter decreased gradually. In the presence of 0.2 *mM* valine the initial increase in activity was less definite, but the enzyme retained a higher activity during the 50 minutes reaction time as compared to the valine-free control (Fig. 6). The relationship was completely linear if the reaction rate in the presence of 1 *mM* valine was plotted against time.

If 0.2 *mM* valine was added to the valine-free control in the 24th minute, activation was slight; 1 *mM* valine under the same condition exerted a slight inhibitory effect. If valine was added to a system containing 0.2 *mM* valine, 36% inhibition was demonstrated. Accordingly, the inhibition was more marked than that found in the valine-free control (20%). The time course of the reaction rate clearly indicates the stabilizing effect of valine.

Enzyme activity decreased by nearly 50% in the absence of pyruvic acid if the reaction mixture contained protein at a low concentration and was incubated at 28°C (Fig. 7). After 6—8 minutes incubation, enzyme activity decreased considerably and inactivation could hardly be influenced with 0.2 *mM* valine. The part of the curve associated with rapid inactivation resembled the time course of the decrease in activity during the reaction, but it was steeper than the latter. If the degree of inactivation was examined in the presence of valine, there was no change in valine sensitivity.

Effect of α-ketobutyric acid on acetohydroxy acid production. If the reaction rate was studied by determining the total acetohydroxy acid content in the presence of 1 *mM* α-ketobutyric acid and at different pyruvic acid concentrations, similarly to results with other microorganisms [5, 6], a competitive inhibition was demonstrated (Fig. 8). If, however, the proportion of α-aceto-α--hydroxybutyric acid and α-acetolactic acid was determined by assaying the former microbiologically, it was evident that at 10 *mM* pyruvic acid concentration acetohydroxybutyric acid was produced practically by itself; the formation of valine precursor (acetolactic acid) was observed only at higher pyruvic acid concentrations.

Discussion

As mentioned in the introduction, the properties of *S. rimosus* acetohydroxy acid synthetase were studied in order to explain the derepressing effect of α-ketobutyric acid. For this purpose it was necessary to elucidate certain characteristics of the enzyme. Accordingly, before discussing the main

Table II

Properties of acetohydroxy acid synthetases isolated from various microorganisms

Microorganism and reference	Specific activity	K_M pyruvic acid *mM*	K_M TPP *μM*	K_M FAD *μM*	Valine *mM* inhibiting 50%	Optimum pH	Temperature °C
Escherichia coli K—12 [16, 17]	0.6	4.0	50.0	0.25	0.25	8.0	37
Aerobacter aerogenes [11]	3.3				0.03	7.5	37
Salmonella typhimurium [17]	1.6	6.25		0.25	1.0	8.0	37
Pseudomonas aeruginosa [18]	3.0	9.3	34.7	0.117	2.0	7.6	20
Mycobacterium pellegrino [6]	0.61	1.61	50.0	0.2	10.0	7.8	20
Mycobacterium tuberculosis [19]	0.72				0.5	6.2	25
Streptomyces rimosus	0.36	8.7	50.0	0.2	5.0	7.4	28

purpose of the study, we compare the general properties of acetohydroxy acid synthetases in different microorganisms.

On the basis of the literature these enzymes can be divided into 3 groups. In *Aerobacter aerogenes*, the corresponding enzyme of which was first studied by JUNI [10], UMBARGER *et al.* demonstrated that the enzyme had an optimum pH of 6 [11], played a catabolic role and was involved in acetoin production. The enzyme then was purified by STØRMER [12]; it was shown to differ in properties from other bacterial biosynthetic enzymes.

The second group comprises enzymes isolated from *Neurospora crassa* [13, 14] and *Phaseolus radiatus* [15]. These enzymes have their optimum under pH 7 and are presumably biosynthetic agents; their valine sensitivity is not unanimously accepted.

The third group contains biosynthetic enzymes isolated from different bacteria. Acetohydroxy acid synthetase in *S. rimosus* also belongs to this group. For the optimum activity of this enzyme, TPP and FAD coenzymes are needed (the role of the latter has not been elucidated). Data for biosynthetic enzymes are summarized in Table II.

The characteristics in which *S. rimosus* acetohydroxy acid synthetase differed from the corresponding enzyme in other bacteria were as follows. The enzyme is very sensitive: its stability can be ensured only in the presence of low valine concentrations. This extraordinary sensitivity is clearly indicated by the dependence of the reaction rate on pH and temperature. As compared to other enzymes, its pH optimum is lower. The low temperature optimum is obviously due to high sensitivity, as in the presence of stabilizing valine the optimum pH shifts to the alkaline side and the optimum temperature falls at higher values. Valine exerts opposite effects: it inhibits the enzyme used, owing to its stabilizing property, it apparently acts as an activator.

On the enzymes examined so far, the stabilizing effect of valine is the most definite in *S. rimosus;* as to the effect of the substrate, it also differs from other enzymes.

Other enzymes show a certain degree of activation at low substrate concentrations; in *S. rimosus* this effect was demonstrable in the presence of valine only. In *Pseudomonas aeruginosa* and in *Mycobacterium pellegrino* an apparent substrate inhibition was caused by higher substrate concentrations owing to the inactivation of the enzyme. No such substrate inhibition was observed in *S. rimosus;* in contrast, the substrate exerted a certain stabilizing effect.

Before discussing the main purpose of this study, we present the steps of acetohydroxy acid synthetase activity (Fig. 9).

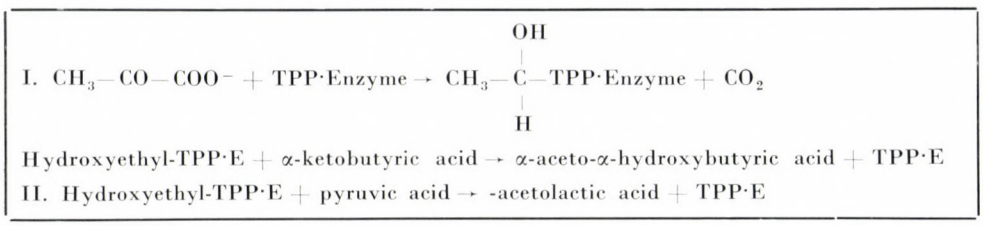

Fig. 9. Steps of acetohydroxy acid synthetase activity

As shown by kinetic experiments, the enzyme reaction involves two steps. First, pyruvic acid reacts with the prosthetic group of the enzyme and oxyethylthiamine pyrophosphate is produced. In the second step the latter compound is condensed with a pyruvic acid or α-ketobutyric acid molecule.

If the total acetohydroxy acid produced in the presence of α-ketobutyric acid is considered, it is evident that the latter compound inhibits the reaction competitively. In this respect *S. rimosus* is similar to other microorganisms. As an explanation of the finding it may be assumed that in the first step only pyruvic acid reacts with the enzyme and α-ketobutyric acid is competitively inhibiting the reaction. The second step is more complex, as shown by the following data when studying the proportion of the two acetohydroxy acids it was evident in all microorganisms that at low pyruvic acid concentrations practically only acetohydroxybutyric acid (isoleucine precursor) is produced. The synthesis of valine precursor becomes significant at higher pyruvic acid concentrations. A minimal isoleucine precursor synthesis, however, takes place even at high pyruvic acid concentrations [5, 6]. This process plays an important metabolic role, since it ensures isoleucine synthesis in the presence of α-ketobutyric acid at low concentrations. These findings throw light on the results of WAGNER *et al.* [20] who examined the ratio of valine: isoleucine syn-

Table III

Amount of isoleucine and valine precursors produced at various pyruvic acid concentrations in cell-free extracts of Pseudomonas aeruginosa, Mycobacterium pellegrino and Streptomyces rimosus

Pyruvic acid μM	*Ps. aeruginosa*		*M. pellegrino*		*S. rimosus*	
	AHBA	ALA	AHBA	ALA	AHBA	ALA
1	0.08	—	0.06	traces	0.05	—
2	0.25	traces	0.10	0.02	0.08	traces
5	0.38	0.2	0.19	0.05	0.15	0.025
10	0.38	0.5	0.23	0.12	0.21	0.04
20	0.30	0.80	0.25	0.16	0.25	0.10
40	0.30	1.05	0.25	0.20	0.25	0.20

The figures indicate the μmoles of isoleucine and valine precursors produced in 1 hour by 1 mg protein at 1 *mM* α-ketobutyric acid and 1—40 *mM* pyruvic acid concentration.

thesis in a cell-free system and showed that α-ketobutyric acid inhibited the synthesis of valine.

The proportion of valine and isoleucine precursors was essentially similar for two different microorganisms [5, 6]. In *S. rimosus*, however, considerably higher pyruvic acid concentrations were needed for valine synthesis (Table III). In view of the characteristics of the enzymes, the derepression due to α-ketobutyric acid may be explained by a valine and leucine deficiency caused by the substance even at high pyruvic acid concentrations. This hypothesis is supported by the finding of a maximum repression of the enzyme when α-ketobutyric acid, leucine and valine are present together [3].

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SUCROSE AND RAFFINOSE BREAKDOWN BY ESCHERICHIA COLI

By

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Summary. Testing of sucrose and raffinose assimilation by 56 laboratory and freshly isolated *E. coli* strains indicated that the majority of the cultures either failed to attack these sugars or utilized both of them. Cultures assimilating only sucrose comprised about 1/5, those assimilating only raffinose about 1/20 of the examined strains. Experiments with one representative of each of the four groups showed that an adaptive enzyme ("bacterial invertase") was responsible for the breakdown of both raffinose and sucrose. The enzyme was inducible by both sugars. An originally sucrose negative strain could be adapted in a suitable medium to a low degree of sucrose-splitting activity. This sucrose negative, raffinose positive strain acted upon raffinose primarily by melibiase. A strain assimilating sucrose on screening examination produced only alpha-glucosidase after adaptation to sucrose. Strains failing to utilize sucrose and raffinose could also be induced to produce the corresponding enzymes, however, at low activity.

Unlike in other microorganisms, invertase and melibiase are localized intracellularly in bacteria. Similarly to lactose breakdown, which is the main diagnostic feature of *Escherichia*, splitting of sucrose and raffinose was inducible, in contrast to the constitutive trehalose and maltose breakdown.

In interpreting diagnostic carbohydrate assimilation spectra of bacteria one should consider the fact that identical results may be brought about by different mechanisms. (Assimilation means in this paper the growth on a carbohydrate as sole source of carbon and energy.) The first steps of assimilation (oligosaccharide splitting, transport processes) may be different, while further steps of the intermediary metabolism (glycolysis, Entner—Doudoroff pathway, etc.) are generally uniform within the same taxonomic unit.

The exact knowledge of the first steps of sugar assimilation may be useful for *in vitro* studies on antibiotics and chemotherapeutics. For example, the effect of an antibiotic may increase if the medium contains a carbohydrate which is attacked by adaptive enzyme(s) of the tested bacterium, because the antibiotic may inhibit the synthesis of the respective adaptive enzyme(s) [7, 14, 17].

Knowledge of the problem is defective, since only some representative species have been examined in this respect. No systematic studies comparing various species have so far been performed. The present experiments were inspired by results obtained in comparative studies on the carbohydrate metabolism of yeasts [28, 29].

Sucrose breakdown is a good example to point out the importance of such studies. In different organisms sucrose is attacked from the fructose-side

by invertase (beta-h-fructosidase), inulinase (beta-fructosidase) and levansaccharase, while from the glucose-side by "saccharase", maltase (alpha-glucosidase), dextransaccharase, amylosaccharase and sucrosephosphorylase [3, 4, 6, 8, 9, 11, 12, 25, 28]. Theoretically each particular process could be recognized by examining whether the organism utilizes raffinose, inulin or maltose in addition to sucrose. Transport problems, however, make the situation more complicated. On this basis another difference can be made, namely, whether the cleavage of the oligosaccharide occurs extracellularly or intracellularly, in other words whether the intact substrate or its decomposition products are transported.

Materials and methods

In preliminary experiments screening tests were carried out for carbohydrate assimilation by 56 *E. coli* strains. Forty-six strains were obtained from the Hungarian National Collection of Medical Bacteria (HNCMB), the remaining 10 strains were isolated in the Central Laboratory Péterfy Hospital. The former strains corresponded to type strains for O antigens 1, 2, 6, 7, 8, 13, 14, 17, 18, 26, 27, 30, 32, 33, 34, 35, 42, 49, 50, 114 and 115, K antigens 1L, 2L, 5L, 6L, 7L, 8L, 11L(O13), 11L(O125), 12L, 14L, 15L, 16L, 20L, 35A and 36A, H antigens 1, 2, 4, 5, 10, 11, 19, 20, 27 and 28.

Carbohydrate assimilation spectrum was determined on desoxycholate-phenol-red (DP) medium for 22 substrates: adonitol, D-arabinose, L-arabinose, D-ribose, D-xylose, dulcitol, D-mannitol, D-sorbitol, D-glucose, D-fructose, D-mannose, D-galactose, L-sorbose, L-rhamnose, lactose, cellobiose, trehalose, maltose, sucrose, raffinose, p-arbutin and m-inositol. The substances were impregnated into filter paper strips. DP plates were seeded with 18 hour broth cultures then the paper strips were placed onto the media [1]. Unlike in routine bacteriological examinations where acid production is considered, improved growth around the strips was regarded as positive reaction. The diagnostic importance of the results will not be dealt with in this paper.

Assimilation of sucrose and raffinose and, as a comparison, of lactose was examined in detail with strains E 40 (HNCMB 30047), P 11a (HNCMB 30056), G 3404/41 O antigen series (HNCMB 30023), G 3403/41K antigen series (HNCMB 30163). The behaviour of the 4 strains on DP medium is shown in Table I (in the following the HNCMB designation will be used).

The cultures were grown on simple agar in Roux flasks at 37°C for 18 hours. For adaptation, the corresponding carbohydrate was added to the medium at 2%. The cells were harvested and washed three times by centrifugation in saline. Then living (intact) cells, acetonized cells and cell-free extracts were prepared and examined as described previously [26] except that all of them were used in 1 : 10 dilution. Quantitative estimation of sucrose and raffinose-

Table I

Assimilation of various substrates on DP agar

Strain	Adonitol	D-Arabinose	L-Arabinose	D-Ribose	D-Xylose	Dulcitol	D-Mannitol	D-Sorbitol	D-Glucose	D-Fructose	D-Mannose	D-Galactose	L-Sorbose	L-Rhamnose	Lactose	Cellobiose	Trehalose	Maltose	Sucrose	Raffinose	p-Arbutin	m-Inositol
30047	−	−	+	+	+	−	+	+	+	+	+	+	−	±	+	−	+	+	+	+	−	−
30056	+	−	+	+	+	−	+	+	+	+	+	+	−	+	+	−	+	+	−	+	−	−
30023	−	−	+	+	+	−	+	+	+	+	+	+	−	+	+	−	+	+	+	−	−	−
30163	−	−	+	+	+	−	+	+	+	+	+	+	±	−	+	−	+	+	−	−	−	−

splitting activity was made by BENEDICT's picronitric acid method [16] as modified by us [2]. To the mixture of 0.1 ml sample and 1.9 ml distilled water, 3 ml 0.4% picronitric acid solution were added which, due to its deproteinizing effect, stopped the reaction. The mixture was centrifuged if necessary, then 2 ml 20% sodium hydroxide were added to the supernatant and the mixture was kept in boiling water for 15 minutes. After cooling the resulting red colour was determined photometrically at 520 mμ. The mixture was sampled at hourly intervals during incubation lasting for 6 hours.

Results

On DP medium the strains differed in sucrose and raffinose assimilation. The two characters are compared in Table II.

Table II

Comparison of 56 E. coli strains for sucrose and raffinose decomposition

		Raffinose	
		+	−
Sucrose	+	20	10
	−	3	23

Table III

Sucrose, raffinose and lactose decomposition by various preparations from 4 E. coli strains adapted and not adapted to sugars

Cultivation (adaptation)	Substrate (for splitting tests)	30047* (S+ R+)			30056* (S− R+)			30023* (S+ R−)			30163* (S− R−)		
		L**	A	H	L	A	H	L	A	H	L	A	H
Simple agar	Sucrose	−	−	−	−	−	−	−	−	−	−	−	−
	Raffinose	−	−	−	−	−	−	−	−	−	−	−	−
	Lactose	−	−	−	−	−	−	−	−	−	−	−	−
Simple agar + sucrose	Sucrose	−	+	+	−	+	+	−	+	+	−	+	+
	Raffinose	−	+	+	−	+	+	−	−	−	−	−	−
	Lactose	−	−	−	−	−	−	−	−	−	−	−	−
Simple agar + raffinose	Sucrose	−	+	+	−	+	+	−	−	−	−	(+)	(+)
	Raffinose	−	+	+	−	+	+	−	−	−	−	+	+
	Lactose	−	+	+	.	+	.	−	−	−	−	−	−
Simple agar + lactose	Sucrose	−	−	−	−	−	−	−	−	−	−	−	−
	Raffinose	−	−	−	−	−	−	−	−	−	−	−	−
	Lactose	−	+	+	−	+	+	−	+	+	−	+	+

Incubation for 6 hours. Chromatographic results for monosaccharides indicating substrate splitting: − = spots absent, (+) = spots weakly visible, + = spots well visible, . = not tested.

* Designation of strain: sucrose and raffinose assimilation on DP agar.

** L = living cells, A = acetonized cells, H = cell-free homogenate.

The contingency table indicates that in the majority of cultures there was a two-way correlation between sucrose and raffinose breakdown (20 strains positive and 23 strains negative for both characters). Ten strains split sucrose but not raffinose; only 3 strains gave positive raffinose and negative sucrose reaction. As the last variation is very unfrequent not only in *E. coli* but also in yeasts [30, 31], in the investigation of raffinose splitting one representative (strain 30056) was included in addition to the strains representing the three other variations. The results are summarized in Table III.

Table III shows that all four strains exerted the corresponding splitting activity only after adaptation. It is also clear that with living cells neither a decrease in the amount of sugar nor an appearance of decomposition products

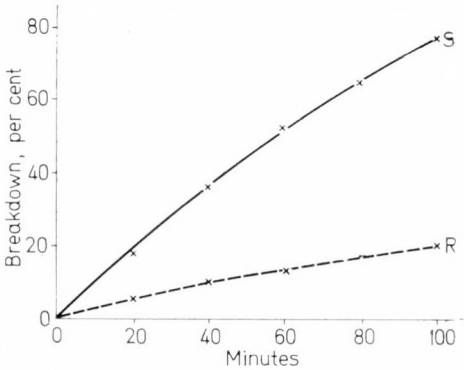

Fig. 1. Splitting of sucrose (S) and raffinose (R) by acetonized cells of *E. coli* 30047 adapted to sucrose

was observed (sugar assimilation was negligible owing to the small amount of bacteria). When the cultures were grown in the presence of the corresponding oligosaccharides (adaptation), certain splitting activities were demonstrable in the samples. No sign of splitting to monosaccharides by living cells was evident in the 6 hour or the earlier samples. With treated cells, however, a progressive increase in sugar breakdown was observed.

The sucrose-splitting activity of strain 30047 (sucrose and raffinose positive on DP agar) was 4 times higher than its raffinose-splitting activity if acetonized cells were used (Fig. 1). As this preparation exerted a beta-h-fructosidase-type activity (see later, Fig. 4) against both sugars, the higher sucrose-splitting activity is explained by the fact that by this enzyme raffinose is less readily attacked than sucrose [12, 15]. The inductive character of sucrose-splitting activity (Table III) is confirmed by data in Fig. 2, since cell-free extracts of cultures grown in sucrose-free medium failed to act upon sucrose; in contrast, when the culture had been adapted to sucrose the cell-free extract showed a considerable activity.

Acetonized cells of strain 30056 (raffinose positive, sucrose negative on DP agar) when adapted to raffinose split not only this sugar but weakly also

sucrose. After cultivation in the presence of sucrose, acetonized cells of the same strain failed to attack either sugar during 100 minutes incubation (Fig. 3). As this strain exerted also an alpha-galactosidase-type activity after cultivation on raffinose (see later, Fig. 4), the results shown in Fig. 3 indicate that

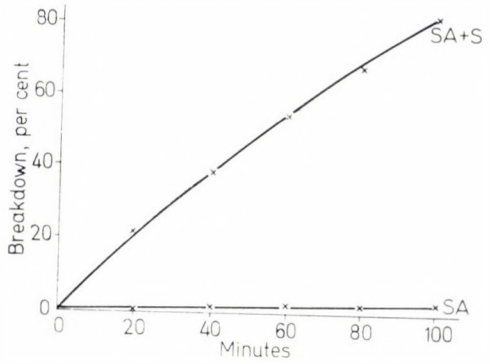

Fig. 2. Sucrose-splitting activity of cell-free extract of *E. coli* 30047 cultured on simple agar in the presence (SA + S) and in the absence (SA) of sucrose

under this condition alpha-galactosidase activity appears first and sucrose-splitting activity is induced later as an effect of sucrose accumulation in the medium. For the latter process the organism utilizes as substrate the galactose released from raffinose. When cultured directly with sucrose, the absence of galactose may be the cause of the failure of induction of sucrose-splitting activity.

Fig. 4 summarizes the results of the examination of the representatives of four variants presented in the contingency table on the basis of chromatograms of samples taken after 6 hours incubation.

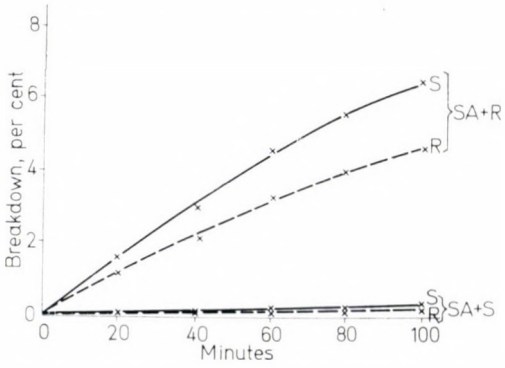

Fig. 3. Splitting of sucrose (S) and raffinose (R) by acetonized cells of *E. coli* 30056 cultured on simple agar in the presence of raffinose (SA + R) and sucrose (SA + S)

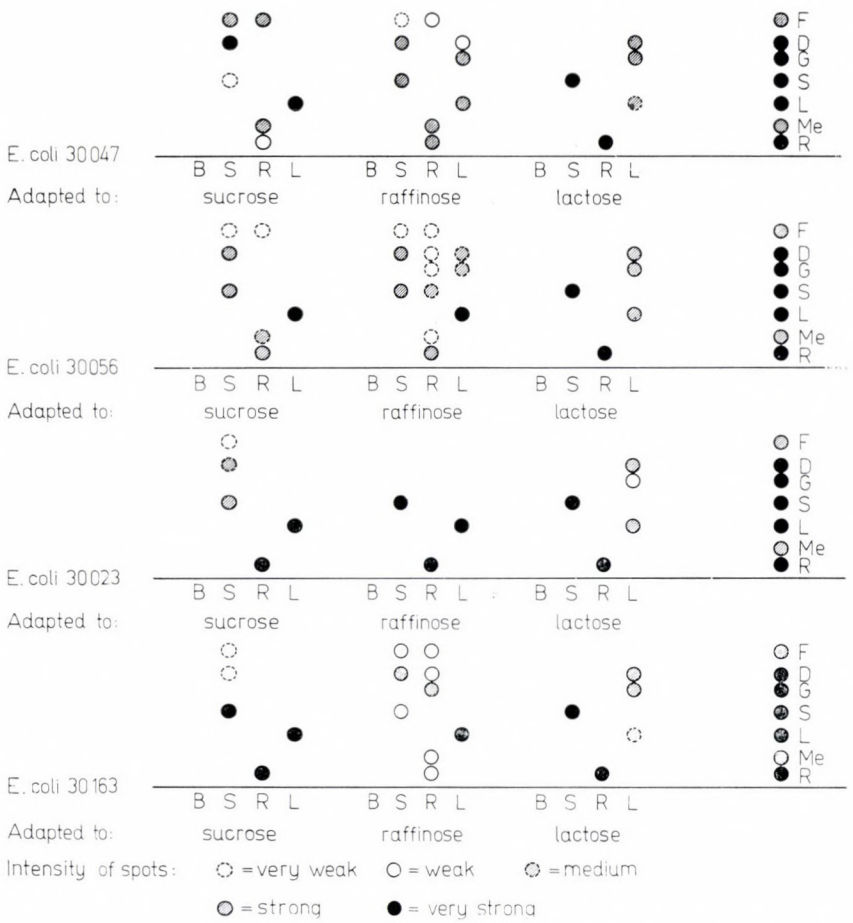

Fig. 4. Sucrose, raffinose and lactose breakdown by acetonized cells of *E. coli* strains representing four variants differing in sugar assimilation spectrum. The organisms were cultured on simple agar containing sucrose, raffinose or lactose. Chromatography was performed after 6 hours incubation without substrate (B) and in the presence of 2% sucrose (S), 2% raffinose (R) and 2% lactose (L). Controls: F = fructose, D = glucose, G = galactose, S = sucrose, L = lactose, Me = melibiose, R = raffinose

It should be noted that none of the various preparations made from the four strains showed endogeneous release of monosaccharides (see blanks).

Acetonized cells of strain 30047 (sucrose and raffinose positive on DP agar) showed beta-h-fructosidase-type activity after culturing with sucrose, *i.e.* sucrose was inverted and raffinose was split into fructose and melibiose. Lactose breakdown was not observed. When the organism was grown with raffinose, the above type of activity was less marked and a weak splitting of lactose occurred. After culturing with lactose, only this sugar was attacked, and more readily than by cells grown with raffinose.

Acetonized cells of strain 30056 (sucrose negative, raffinose positive on DP agar) grown with sucrose showed very weak beta-h-fructosidase activity in respect to both sucrose and raffinose breakdown. After growing with raffinose the above activity and a higher degree of alpha-galactosidase activity appeared. Raffinose breakdown may occur in two different manners. Beta-h--fructosidase releases first fructose and melibiose, then alpha-galactosidase splits melibiose into glucose and galactose; or, else, alpha-galactosidase produces first galactose and sucrose then beta-h-fructosidase splits sucrose into glucose and fructose. In both kinds of pathway, intact raffinose and all intermediary and end products (melibiose, sucrose, glucose, galactose and fructose) can be demonstrated. Culturing with raffinose induced in strain 30056 a weak activity against lactose. When grown with lactose, the organism split only lactose.

Strain 30023 (sucrose positive, raffinose negative on DP agar) after adaptation to sucrose acted upon this sugar but not upon raffinose, *i.e.* it exerted an alpha-glucosidase-type activity. Culturing with raffinose induced neither alpha-glucosidase nor beta-h-fructosidase activity and probably this complete inadequacy for attacking raffinose was responsible for the lack of lactose breakdown. However, lactose induced a lactose-splitting activity in this culture.

Strain 30163 (sucrose and raffinose negative on DP agar) exerted traces of sucrose-splitting activity and completely failed to attack raffinose and lactose after cultivation with sucrose. Induction by raffinose caused partly the appearance of alpha-galactosidase activity (on the chromatogram there were weak raffinose and melibiose spots and marked glucose and galactose spots), partly a sucrose-splitting activity stronger than that exerted by the culture adapted to sucrose. This was indicated also by the absence of the sucrose spot after the breakdown of raffinose. This finding may be explained by an indirect induction, in which galactose released by alpha-galactosidase of the raffinose-adapted culture was the initial substrate for induction by sucrose. Sucrose breakdown seems to be a beta-h-fructosidase-type reaction as the chromatogram for raffinose-splitting showed the presence of melibiose. Raffinose-induction failed to bring about lactose-splitting activity. Culturing with lactose, as usually, resulted in the development of lactose-splitting activity.

It should be noted that acetonized cells and, to a lower degree even extracts, showed an activity against trehalose and maltose without induction.

Discussion

As shown in the contingency table, screening tests for sucrose and raffinose assimilation gave three variations in addition to the uniformly negative results. These variations may be interpreted as follows. (i) Sucrose and raffi-

nose positive strains produce invertase; (ii) raffinose positive, sucrose negative strains produce melibiase; (iii) sucrose positive, raffinose negative strains produce maltase (or other alpha-glucosidase) type enzymes.

Comparison of the four variations yielded the following results. Strain 30047 assimilating both sucrose and raffinose on DP agar was, as expected, exerting fructosidase activity (though in an adaptive form). Beside the inductive character, this enzyme differed from the invertases of yeasts and molds [32, 33] in localization and optimum pH since in living cells it failed to exert extracellular activity and its optimum pH was at 6.5 instead of the usual 4.5 in fungi. Invertases are attributed mainly to yeasts and molds [12, 14]. Bacterial invertases were described by DOUDOROFF [5], SCHOCHER [35] and NEGORO [18—25]. These data indicate that "invertase activity" in *Pseudomonas saccharophila* is a weak intracellular process. In some clostridia invertase is an inductive enzyme. Invertase was isolated from *Bacillus subtilis* var. *saccharophila*. Accordingly, until more extensive data are available, the sucrose and raffinose-splitting enzyme in strain 30047 may be termed "bacterial invertase".

Raffinose and sucrose breakdown by strain 30056 as compared to that by the above strain may be defined as follows.

1. Although strain 30056 fails to assimilate sucrose on DP medium, if it is cultured on simple agar in the presence of sucrose or raffinose, a sucrose-splitting activity is induced. This induction does not occur on DP agar where the organism probably uses other nutrient substances for growth.

2. The assimilation of raffinose without sucrose breakdown on DP medium probably involves a melibiase activity. This is shown by the presence of sucrose (and galactose) spots on chromatograms made with acetonized cells and extracts of cultures grown with raffinose.

3. When grown on simple agar containing raffinose, the parallel appearance of sucrose breakdown is induced by sucrose released from raffinose as a result of melibiase activity.

4. The chromatograms clearly show that in raffinose breakdown by acetonized cells and extracts of cultures adapted to raffinose not only melibiase but also a sucrose-splitting enzyme is involved, as both melibiose and fructose are present. The latter enzyme seems to be of an invertase type, as in raffinose breakdown small amounts of melibiose are detected.

5. The data indicate that in view of the induction of sucrose breakdown, DP agar is less suitable than simple agar as otherwise sucrose assimilation would be evident also on the former medium.

Accordingly, strain 30056 produces mainly melibiase, but under suitable conditions it may elaborate another enzyme resembling the above-mentioned invertase. The activity, or more probably the amount, of the latter is lower than in strain 30047.

The localization of melibiase, as well as that of invertase, differs in *E. coli* and in the yeasts. In the yeasts the enzyme acts extracellularly or, at least, outside the cell wall barrier [26, 27]. DOUDOROFF [5] has shown that melibiase in *Ps. saccharophila* is an endoenzyme exerting higher activity and being present in larger amounts than invertase.

As expected, strain 30023, which, on DP medium, assimilated sucrose but not raffinose, showed alpha-glucosidase ("specific saccharase") activity. This enzyme appeared also only after induction. No other types of enzyme were detected in this strain. It was interesting that raffinose markedly inhibited its growth on DP medium.

The raffinose and sucrose negative strain 30163 exerted very weak sucrose-splitting activity when adapted to this sugar. Its activity upon sucrose became more marked if the strain had been cultured with raffinose. On the basis of the weak sucrose-splitting activity and the absence of raffinose breakdown, the type of the enzyme involved cannot be determined, as inactivity against raffinose may be a result of not only the alpha-glucosidase type of enzyme but may also be due to the fact that invertase activity is considerably weaker against raffinose than it is against sucrose. The appearance of raffinose breakdown after adaptation to raffinose indicates the presence of both invertase and melibiase, since on the chromatograms melibiose, fructose, glucose and galactose were all present. Accordingly, in the effective sucrose breakdown induced by raffinose an intermediary sucrose adaptation is involved, the energy-yielding substance of which is galactose released from raffinose by melibiase together with sucrose.

Not only the above processes but also lactose breakdown, the most characteristic feature in *E. coli*, proved to be of inductive character in all the 4 strains. In strains 30047 and 30056, lactose breakdown could be induced even with raffinose. This finding, termed by HESTRIN and LINDEGREN [10] heterologous induction, was not surprising. HOLMES *et al.* [13] described that in bacteria raffinose belongs to a substrate group the members of which are capable of inducing both alpha-galactosidase (melibiase) and beta-galactosidase (lactase).

The present study revealed important differences in strains representing four groups of *E. coli* strains differing in assimilation spectrum. It may be assumed that similar experiments with other bacteria may yield valuable data for different metabolic pathways in otherwise homogeneous bacterial groups, serotypes and species.

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ACTION OF CATION TRANSFER ATPase INHIBITORS ON EFFICIENCY OF INFECTION WITH POLIOVIRUS

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Summary. In a system consisting of poliovirus adsorbed to suspended monkey kidney cells, the reduction of K^+ ion content to, or below, 0.91 mEq/l for at least 1 hour caused a 4fold increase of the efficiency of infection as measured by the cycle's final yield. The effect was abolished when K^+ concentration had been restored to normal by the end of the cycle's first 30 minutes. Absence of Mg^{++} ions during the cycle's first hour caused a 70% reduction of the final yield. Considering the known effects of K^+ or Mg^{++} ions on membrane ATPase, these results appear to provide a further indirect evidence for the possible correlation between ATPase conformation and efficiency of the virion's irreversible attachment.

We have recently published some data on the effect of different heart glycosides on the efficiency of infection by poliovirus of suspended tissue culture cells [1, 2, 3]. It seemed that compounds having high affinity to the cation transfer membrane ATPase stimulated virion uptake at concentrations between 6×10^5—6×10^6 molecules per cell. This indirect evidence of the possible involvement of the membrane ATPase in this process has initiated the experiments to be reported.

Materials and methods

The Mahoney strain of type 1 poliovirus and the PMK III/1 permanent monkey kidney cell line were used throughout. For details concerning materials and methods, the first pape of this series should be consulted [1].

Experimental

As demonstrated by Holland and McLaren [4] adsorption of poliovirus to susceptible cells required the presence of cations, while the phase designated by them as eclipse turned out to be insensitive to the presence or absence of cations. In our experiments, adsorption was always performed in Hanks' balanced salt solution with glucose (HBS). The effect of cations was examined exclusively in the postadsorption period of the cycle. The specificity of the cation effect observed was proven by omitting only a single cation from HBS.

After adsorption of virus to the cells at a multiplicity 1 for 10 minutes at 20°C and for 5 minutes at 37°C, the unadsorbed virus was removed by re-

peated washing in HBS. One step growth curves were obtained by incubating 10^6 virus-treated cells suspended in 10 ml medium at 37°C under gentle shaking in siliconed bottles in a 5% CO_2 atmosphere. Maximum virion yield was regularly obtained by the end of the 6th hour of incubation and this value did not change significantly on further prolongation of the incubation by 1—2 hours. Therefore, we designated the yield in the 6th hour as final yield (FY_6). All results presented in this paper are given as *relative* FY_6. This means that yields were always referred to that obtained simultaneously in the control system in complete HBS.

Effect of reduction of K^+ *ion content.* The total K^+ ion content of HBS is 3.1 mEq/l. Of this, 2.5 mEq/l is present in the form of KCl and 0.6 mEq/l in that of KH_2PO_4. The latter was always present and only the KCl content varied. The effect of the reduced K^+ ion content on the relative FY_6 is given in Table I. Apparently, there is a critical minimum concentration of K^+ ion between 0.91 and 1.22 mEq/l. Reduction of the K^+ ion content from 3.10 to 1.22 mEq/l did not significantly affect the yield while at both 0.91 and 0.60 mEq/l there was an about 4fold increase of the yield.

Table I

Effect of K^+ *depletion on relative final virion yield*

HBS containing KCL (mM/l)	Total K^+ mEq/l	Relative FY_6
5.00 (control)	3.10	1.00
2.50	1.85	0.85
1.25	1.22	1.24
0.62	0.91	4.11
0.00	0.60	4.41

Table II

Effect of Mg^{++} *ion and of total ion depletion on relative final virion yield*

Medium	Relative FY_6
HBS	1.00
HBS — Mg^{++}*	0.33
Tris buffer + sucrose**	0.015
Tris buffer + sucrose + Mg^{++}	0.005
Tris buffer + sucrose + HBS (v/v = 1/1)	1.00

* Mg^{++} 0.10 mEq/l
** Tris buffer 10 mM/l, sucrose 250 mM/l

Effect of Mg^{++} ion deficiency. Mg^{++} ion has been known to be essential for the activity of membrane ATPase [5]. The HBS contains 0.10 mEq/l of Mg^{++} ion in the form of $MgSO_4.7\ H_2O$ (2 mM/l). One step growth curves were obtained in HBS lacking Mg^{++} ions. Simultaneously control experiments were set up in a tris buffer—sucrose (10 mM/l—250 mM/l) medium with and without Mg^{++} (0.10 mEq/l) and in complete HBS. Results are shown in Table II.

The total absence of external Mg^{++} ions reduced the yield by about 70%. In tris buffer—sucrose the reduction of yield was 98.5 and 99.5% in the absence and presence of Mg^{++} ions, respectively. Thus Mg^{++}, as the sole ion, did not allow for a full yield. The presence of half the amount of the total ion content of HBS was, however, satisfactory for the production of a full FY_6 if the solution was rendered isotonic by adding enough tris buffer—saccharose mixture.

Table III

Effect of the ion depletion's duration on the relative final virion yield

Cation	Duration of depletion	Relative FY_6
K^+	none	1.00
	0—15 min	0.85
	0—30 min	1.20
	0—60 min	3.00
	0— 6 hours	3.30
Mg^{++}	0—30 min	1.0
	0—60 min	0.40
	0— 6 hours	0.33

Table IV

Effect of some substances on ATPase activity and on virion production

Substance	Inhibition of ATPase activity at the level of		Relative FY_6	Phase of cycle affected
	formation of enzyme-ATP complex	hydrolysis of enzyme-ATP complex		
Digitalis [1]*	—	+	increase	0—60 min.
K^+ deprivation	—	+	increase	0—60 min.
Mg^{++} deprivation	+	—	reduction	0—60 min.

* reference.

Effect on the FY_6 of the duration of cation depletion. In order to ascertain which phase of the viral cycle was affected by the absence of K^+ or Mg^{++} ions, the medium was completed at various points of time during the cycle. Results are shown in Table III.

K^+ ion depletion had to be maintained during the first hour to obtain a 3fold increase of yield. If the K^+ content had been adjusted to normal in the first 30 minutes, no increase of yield was observed. The absence of Mg^{++} ions from the system had to be maintained during the cycle's first hour, to achieve a full yield-reducing effect. Restoration in the 30th minute abolished the yield reducing effect. The effects of some membrane ATPase inhibitors and activators on the relative FY_6 of the viral cycle are shown in Table IV.

Discussion

The membrane ATPase inhibitory action of heart glycosides consists of the inhibition of hydrolysis of the Mg^{++}—K^+-ATP-enzyme complex [5]. Heart glycosides have been shown to increase the efficiency of infection in a poliovirus—monkey kidney cell system [1, 2, 3]. The effect was observed after adsorption of the virus in the first hour of the cycle. As one of the possible explanations we have proposed that the infection efficiency increasing effect of glycosides was a result of demasking or activating additional virus receptor sites by "locking" the ATPase complex in one of its possible conformations. In this case, other agents having the same effect on ATPase should similarly increase final virus yield. Absence of K^+ ions from a medium containing both Mg^{++} and Na^+ ions has been known to inhibit ATP hydrolysis in the plasma membrane [6]. The same inhibitory effect is produced by ouabaine which competes with K^+ [5].

As shown by the present studies, the final virion yield increased 4fold when the K^+ ion content of the medium had been reduced for at least 1 hour below a certain critical concentration. This effect failed to appear when the K^+ ion content had been restored to normal in the first 30 minutes of the cycle. Thus, the absence of K^+ ions affected a process taking place between the 30th and 60th minute of the viral cycle, and the deprivation affected the system in a way essentially similar to that observed with heart glycosides at certain concentrations.

The presence of Mg^{++} ions is required for the formation of an enzyme-ATP-K^+ ion complex [5]. Thus, in the absence of Mg^{++} ions from the medium the supposedly favourable conformation of the plasma membrane ATPase cannot develop. Under these conditions the final virion yield was found to be reduced by 70%. Restoration to normal of the Mg^{++} content in the first 30 minutes abolished the yield reducing effect. After 60 minutes, however, the effect was no longer reversible.

Examinations of the effects of K^+ and Mg^{++} ions on the post-adsorption interactions of poliovirus and its host cell yielded additional indirect evidence in favour of the theory that the conformation of K^+, Na^+, Mg^{++} membrane ATPase affected the efficiency of infection.

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AUXOTROPHIC MUTATION ASSOCIATED WITH LOW STREPTOMYCIN RESISTANCE AND SLOW GROWTH IN BACILLUS SUBTILIS

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Summary. Nutrient agar plates containing 100—200 μg streptomycin/ml were heavily inoculated with *Bacillus subtilis* strain 168 ind$^-$. Small or medium sized colonies developed in a proportion of 10^{-6} after a few days incubation at 37°C. About one fourth of the isolates produced minute colonies when transferred to blood agar. Isolates showing normal colony size on blood agar exhibited certain new nutritional requirements allowing the classification of the mutants into 5 groups. The majority of the mutants multiplied well on complete media as yeast peptone agar but failed to grow under defined nutritional conditions. Approximately one tenth of the mutants showed a deficiency in the pathway of tetraporphyrin (protohaem) synthesis. A few isolates with a dependence on aromatic amino acids were also encountered. Although the isolates were obtained at high streptomycin concentrations, they exhibited a low tolerance to this antibiotic.

Slow growth was another feature of the mutants. Auxotrophy, slow growth and moderately increased streptomycin tolerance apparently resulted from mutation at one single locus since reversion of auxotrophy to prototrophy restored their sensitivity and normal growth. It has been concluded that a direct action of streptomycin on chromosomal replication might be involved in the genesis of these peculiar mutants.

ANDERSON and IVÁNOVICS [1] have recently observed that various effects, including treatment with copper sulphate or nitrosoguanidine, induced haemin dependent mutants in *Bacillus subtilis*. In preliminary experiments on streptomycin-containing medium they obtained δ-aminolaevulinic acid (ALA) dependent isolates. In examining these mutants we were able to study an unfrequent type of streptomycin resistance characterized by low antibiotic tolerance, increased nutritional requirement and slow growth. The increased nutritional requirement was characterized by a deficiency in protohaem synthesis. Since all these three features were associated in both appearance and reversion, it seemed justified to regard these mutants as a separate group of auxotrophs.

Materials and methods

Bacterial strains. B. subtilis strain 168 and a haemin dependent isolate [1] were used. The genotype of the latter (RI 5, ALA dependent) was expressed as *hem Al.*

Culture media. Medium *GGM* containing glutamate and glycerol was prepared as described previously [1]. If necessary, the medium was supplemented with tryptophan (20 μg/ml). Yeast extract peptone medium *(YP)* was described in reference [2.] Medium *YT:* yeast extract (Oxoid), 2.5 g; Trypton (Oxoid), 10 g; K_2HPO_4, 1 g; NaCl, 5 g; tap water, 1000 ml; pH 7.5.

Medium *HAC:* GGM was supplemented with haemin (2.5—5 μg/ml), cysteine (20 μg/ml) and fraction *V* bovine serum albumin (1—2 mg/ml). *Blood agar:* to YP agar 5% citrated human blood was added.

Inoculum. Precultivation was performed overnight at 37°C in YP broth without shaking. Then the culture was diluted 1 : 20 with the same medium and shaken gently at 37°C until an optical density of 0.5—1.0 had been reached. Density measurements were carried out in a Bausch and Lomb Spectronic "20" spectrophotometer. The culture was centrifuged and the bacteria were resuspended in 1/10 volume. This suspension was seeded undiluted or after dilution in 0.1 ml aliquots onto streptomycin-containing agar plates. The number of inoculated colony formers varied between 10^7 and 10^8.

Ultraviolet-irradiated inoculum was prepared as described in reference [1].

Determination of catalase activity. The bacteria were grown on YP or heated blood agar. After 20 hours incubation the cells were harvested in phosphate buffer (0.1 *M*, pH 6.8) and the suspension was adjusted to o. d. = 0.5. To 2 ml suspensions, 5 ml 0.015—0.017 *M* H_2O_2 were added. After 5 minutes standing at 37°C the amount of decomposed H_2O_2 was determined iodometrically [3].

Minimal nutritional requirement. The isolates were first grown on blood agar then transferred to YP and blood agar. If the isolate seemed to be porphyrin dependent, it was inoculated onto GGM—ALA (2 μg/ml) and HAC media.

If the isolate grew on YP agar but not on GGM agar, its specific requirement was tested for all amino acids, nuclein bases and 11 water-soluble vitamins.

Streptomycin resistance. In orientation experiments a slightly opalescent suspension was prepared from the colony tested, then agar plates containing streptomycin at various concentrations were seeded with loopful amounts of bacteria (10^4—10^5 colony formers). In order to obtain more precise data, in quantitative experiments $1—2\times10^3$ colony formers were streaked on a series of blood agar plates with gradually increasing streptomycin concentrations. The degree of tolerance was expressed as the decrease in the number of colony formers. Resistance to the bactericidal effect of streptomycin will be presented in the experimental part of this paper.

Results

Isolation and nutritional requirement of mutants. As in our previous experiments, Hartley agar plates containing streptomycin (200 μg/ml) were seeded with 10 to 100×10^6 colony formers and the media were incubated at 37°C. After 48 hours few colonies were visible. Their number increased on further incubation. After 96 hours, varying with the size of inocula and with individual experiments, 10 to 200 colonies were observed per plate. Similar numbers of colonies were obtained on other complete media (YP, YT or blood agar). On GGM or HAC medium containing streptomycin (200 μg/ml) there was no growth in several repeated experiments. Irradiation of the inoculum did not affect the results obtained with inoculum without ultraviolet irradiation.

After 96 hours incubation on streptomycin agar the size of colonies varied considerably. About 3/4 of colonies was small (0.5—1 mm). Large colonies, 3 mm or more in diameter, occurred in about 10%. The size of the remaining colonies varied between these values.

The following results reflect findings obtained in 5 different experiments. Colonies grown on antibiotic agar plates were transferred to blood agar. About 1/5 of the isolates failed to develop into colonies, *i.e.* they consisted of dead bacteria. On blood agar 72 out of the 266 transferred isolates formed colonies 0.5 mm or less in diameter. These "minute" colonies were highly unstable in

further subcultures: they often lost viability or segregated colonies resembling those of the parent cultures. In phenotype (nutritional requirement) 85 out of 194 isolates obtained on blood agar were identical with the parent strain. Part of these isolates had probably originated from a contamination of the inoculum. More than one hundred colonies showed an alteration in nutritional requirements; 57 isolates were examined in detail. A deficiency in porphyrin synthesis was observed in 14 cultures. Eight isolates required aromatic amino acids. Further 19 isolates failed to grow on GGM agar even when the medium had been supplemented with all amino acids, nucleic acid bases and water-soluble vitamins. All of them grew, however, abundantly on YP and on other complete media. The remaining 6 isolates multiplied only on blood agar.

In subsequent experiments a decrease in the concentration of streptomycin (lowest value, 50 μg/ml) resulted in an increase in the number of colonies. At low concentrations mainly "minute" colonies or auxotrophs with undefinable requirement developed. In the presence of 200 μg streptomycin/ml the proportion of colony formers was $0.2 \times 10^{-6} - 10^{-6}$. At lower concentrations this value increased to 10^{-5}.

In this study about one thousand isolates were examined for defined characters. Undefinable auxotrophs occurred frequently; these were preceded in number only by "minute" mutants. The proportion of mutants deficient in porphyrin synthesis varied greatly in the individual experiments. Sometimes 20% of the colony formers required haemin or ALA, while in other experiments

Table I

Relevant phenotypes of mutants isolated from strain 168 ind⁻

Class of mutants	Growth on different media					
	GGM	GGM + ALA[1]	HAC[2]	GGM + aro[3]	YP	YP + blood
Parent strain 168 ind⁻	+	+	+	+	+	+
"Minute"	−	−	−	−	±	+
nda	−	−	−	−	+	+
nda-b	−	−	−	−	−	+
aro	−	−	−	+	+	+
hemA	−	+	+	−	−	+
hem	−	−	+	−	−	+

[1] GGM with δ-aminolaevulinic acid (2 μg/ml)
[2] See methods
[3] GGM with tyrosine and phenylalanine (10 μg/ml each)

Remarks to the designation of isolates. The nomenclature of DEMEREC *et al.* [4] was adopted. Mutants lacking δ-aminolaevulinic acid synthetase are denoted as *hemA*. Haemin dependent mutants are designated by their phenotypes as *hem* isolates.

these mutants were entirely absent. The lowest frequency was found with *aro* mutants; in two individual experiments only 8 isolates were found.

On the basis of phenotype the mutants were classified into groups, as shown in Table I.

Among other results the following are worth mentioning. We were unable to characterize the nutritional requirement of *nda* (non-defined auxotrophs). In the presence of a great number of various metabolites some of them exhibited a minimal growth on GGM agar. Apart from this difference in phenotype all isolates seemed to be uniform; occasional traces of growth were attributed to the "leaky" character of the culture. Mutants *nda-b* (*nda* with blood requirement) grew poorly on GGM blood agar but abundantly on YP blood agar. Members of group *aro* required aromatic amino acids (tyrosine and phenylalanine). These isolates were probably deficient in the synthesis of prepheic acid or its precursor.

Some strains representing all kinds of isolates were examined for catalase activity. On the average, 2 ml suspension of the parent strain decomposed 3 ml 0.01 *M* H_2O_2. The reaction was inhibited by sodium azide. Within the limits of experimental error, identical results were given by all representatives in that mutation was never associated with an alteration in catalase activity. Bacteria deficient in the haematin pathway were, of course, cultured in the presence of exogeneous porphyrin.

Mutants with changed nutritional requirements were obtained not only with inocula containing vegetative bacteria, but also with spores.

Streptomycin tolerance of the isolates. Isolates obtained on plates containing 100—200 μg streptomycin/ml showed a very low tolerance to the antibiotic. For example, in one experiment 49 colonies were examined by preparing slightly opalescent suspensions from blood agar subcultures and transferring 10^4—10^5 bacteria to blood agar containing streptomycin. Table II shows the number of isolates giving confluent growth in the presence of streptomycin at different concentrations.

Quantitative streptomycin tolerance tests for defined mutants are presented in Table III.

Table II

Antibiotic tolerance of colonies selected at random from growth on YP agar with 200 μg streptomycin/ml

Streptomycin, μg/ml	5	20	50	100	"Dependent"
No. of isolates	9	26	4	5	5

In nutritional requirement 15 out of the 49 isolates tested were identical with the parent strain. These isolates tolerated 100 μg streptomycin/ml. The remaining isolates appeared to be *nda* in phenotype. "Dependent" isolates tolerated streptomycin at low concentrations (20 μg/ml or less) and grew somewhat better in the presence of the antibiotic.

Table III

Percentage of colony formers of individual isolates with defined nutritional requirements on blood agar plates containing streptomycin at different concentrations

Strain	Size of inoculum	Per cent of survivors at μg streptomycin/ml				
		5	10	20	50	100
168 ind⁻	1200	90	7	—	—	—
hemA1	1300	95	100	100	—	—
hemA3	1350	100	38	2	—	—
hemA5	1500	95	97	100	100	—
hem-8	2300	100	90	80	40	—
hem-10	2100	96	72	90	80	—
hem-11	2500	90	55	50	5	—
hem-12	2500	95	80	62	9	—
hem-13	2800	50	50	60	35	—
hem-15	2400	70	40	46	—	—
aro-1	1800	100	96	90	—	—
aro-2	2400	2	—	—	—	—
aro-8	4000	34	32	10	—	—

Streptomycin sensitivity of strain 168 ind⁻ was tested also at very low concentrations. All bacteria of the inoculum consisting of 1500 colony formers produced colonies at 2, 3 and 4 μg streptomycin/ml. Survival rate was 90% at 5 μg/ml, 15% at 8 μg/ml, and 4% at 10 μg/ml.

Five further isolates with *aro* character were also included in these tests; all but one *(aro-5)* exhibited a high sensitivity to streptomycin similar to that of *aro-2*. Dash indicates no growth.

It is seen that all isolates examined were heterogeneous in streptomycin sensitivity. The parent strain (168 ind⁻) was highly sensitive. Mutants *hemA* and *hem* differed slightly but significantly from the parent culture. Only 3 *aro* isolates were slightly resistant, the remaining 4 strains behaved like the parent culture. Similar experiments will be presented later in this paper.

It should be noted that, corresponding to the original technique, media containing 100—200 μg streptomycin/ml were seeded with large inocula (10^7 bacteria) of some isolates. The applied complete media were supplemented with metabolites (*e.g.* ALA) when necessary. Depending on the streptomycin concentration and composition of the medium sometimes a thin film of confluent growth developed. Mostly, however, even these large inocula gave rise to few colonies.

Stability of isolates. Mutants *hem* and *hemA* showed a remarkably weak sporulating capacity. When subcultured frequently, *hem* mutants split off revertants. Mutants *hemA* and *aro* were highly stable. Isolates *nda* and *nda-b* were unstable and had therefore to be maintained by freeze-drying.

Isolates showing nutritional requirements different from those of the parent strain reverted spontaneously to the original phenotype. In order to

Table IV

Change in streptomycin tolerance associated with reversion of auxotrophy to the parent phenotype
(The inoculum varied between 970 to 2400 bacteria)

Isolate and its revertant	Per cent of survivors at μg streptomycin/ml					
	5	10	15	20	50	100
hemA6[1]	100	85	50	45	15	0
hemA6 revertant	0	0	0	0	0	0
hemA6[1]	60	30	30	30	0	0
hemA6 revertant	5	0	0	0	0	0
hemA11	45	2	1	0	0	0
hemA11 revertant	0	0	0	0	0	0
hem-8	50	50	30	30	0	0
hem-8 revertant	5	0	0	0	0	0
hem-10	30	5	2	0	0	0
hem-10 revertant	0	0	0	0	0	0
hem-13	80	9	3	0	0	0
hem-13 revertant	40	0	0	0	0	0
hem-14	100	10	1	1	0	0
hem-14 revertant	3	0	0	0	0	0
hem-15	100	100	100	100	0	0
hem-15 revertant	20	0	0	0	0	0
hem-16	100	100	80	80	0	0
hem-16 revertant	60	0	0	0	0	0
aro-2[2]	5	0	0	0	0	0
aro-2 revertant	2	0	0	0	0	0
aro-5	100	100	100	90	0	0
aro-5 revertant	90	1	0	0	0	0
nda-3	100	100	100	100	90	0
nda-3 revertant	5	1	1	1	0	0
nda-15	100	100	100	90	0	0
nda-15 revertant	13	0	0	0	0	0
nda-17	100	29	20	5	1	0
nda-17 revertant	7	0	0	0	0	0
nda-b-6	100	100	100	100	50	1
nda-b-6 revertant[3]	55	5	0	0	0	0

[1] Individual experiments with two revertants isolated independently.
[2] Similar experiments with three further *aro* isolates exhibited also a low rate of tolerance
[3] To parent type strain. Intermediate revertant *nda* showed an increased tolerance et streptomycin.

determine the frequency of reversion, bacteria grown in sufficiently supplemented media were centrifuged and washed in GGM. The suspension was seeded on GGM and sometimes on YP plates. The proportion of reversion for 9 *hem* isolates was 0.5—2.8 per 10^7 bacteria. A lower frequency reversion was observed with *hemA* isolates: sometimes even 2×10^8 organisms failed to yield revertants. Reversion was more frequent in *nda* isolates. Revertants always

occurred in populations of 10^8 bacteria; the frequency of reversion reached a maximum of $100/10^7$ bacteria. Reversion of *nda-b* strains occurred in two steps: first *nda* (independent from blood) then the parent phenotype appeared. Unlike with *aro* isolates, in size and structure of colonies the revertants and the parent culture were identical. When revertants were sought on GGM medium two different kinds of colonies were observed: a small proportion of the colonies resembled to the parent culture, but the majority comprised slowly-growing small colonies (about 2 mm) even after 48 hours incubation. In the latter the reversion was probably due to a suppressor mutation.

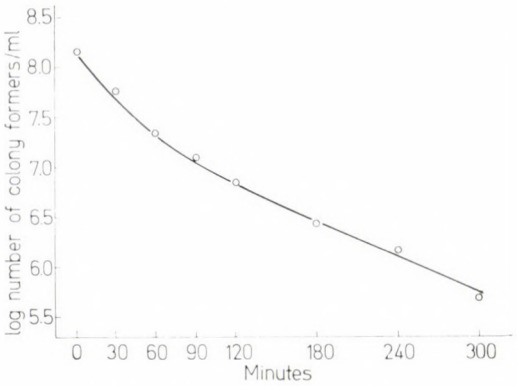

Fig. 1. The killing action of streptomycin on the parent strain (*B. subtilis* 168 ind$^-$). A 1.9 m sample of exponential phase culture in YP broth was mixed with 0.1 ml 1% streptomycin sulphate. The suspension was incubated as a thin layer at 37°C and bacterial counts were made at intervals

Comparative examination of auxotrophic mutants and their revertants. Isolates reverted from mutants with new requirements showed a decreased streptomycin tolerance similar to the tolerance of the parent strain (Table IV).

It would appear that the revertants were somewhat more sensitive to streptomycin than was the parent strain (see Table III). Because of the low tolerance of *aro* cultures only *aro-5* could be compared to the corresponding auxotrophic mutant. Reversion of this mutant was also associated with a decrease in streptomycin tolerance.

Streptomycin at high concentrations was bactericidal to cells of the parent strain. The kinetics of this effect are presented in Fig. 1.

Table V shows the bactericidal effect of the antibiotic to several isolates and to their revertants.

The sensitivity of the parent strain varied within reasonable limits in different experiments. The auxotrophic isolates were more resistant than was the parent culture. Reversion decreased their resistance to the bactericidal action of streptomycin. Strain *aro-7* was an exception as after reversion it showed no change in sensitivity. It should be noted that this culture showed

Table V

The killing action of 500 μg streptomycin/ml on bacteria after 1 hour exposure in broth

Strain	Survivors, per cent
Parent (168 ind⁻) exp. 1	6.4
Parent (168 ind⁻) exp. 2	12.8
Parent (168 ind⁻) exp. 3	8.2
hemA6	48.8
hemA6 revertant	20.9
hemA8	85.8
hemA8 revertant	13.3
hem-9	47.2
hem-9 revertant	18.6
aro-7	21.7
aro-7 revertant	22.3
nda-3	96.2
nda-3 revertant	10.5
nda-15	45.9
nda-15 revertant	2.4

Bacteria were grown in YP medium containing 2 μg ALA/ml or 2.5 μg haematin + 0.2% bovine albumin/ml. Simple YP broth was used for porphyrin independent mutants. When the o.d. value reached 0.5, 1.9 ml aliquots of the cultures were mixed with 0.1 ml 1% streptomycin solution and incubated in a water bath of 37°C for 60 minutes. Bacterial counts were made on blood agar plates.

no difference from the parent strain in the bacteriostatic effect of the antibiotic.

The results of an experiment carried out later with *aro-5* and its revertant has been omitted from Table V. In this experiment the degree of sensitivity of the parent strain (tested for comparison) was strikingly high. The proportion of survivors was only 1% for the parent culture, 15.5% for *aro-5*, and 3% for the revertant. The difference may be explained by the fact that in these experiments a new batch of medium was used.

The auxotrophs showed increased resistance to both the bacteriostatic and the bactericidal effect of streptomycin. The *aro* mutants behaved irregularly in this respect as well as in the type of reversion. In contrast to the finding of GORINI and KATAJA [5], who described some conditionally streptomycin dependent (CSD) *E. coli* strains, the new nutritional requirement of our typical auxotrophs could not be supplemented by the addition of streptomycin. Many of our isolates were examined as to whether their requirement for ALA, haemin or complete medium could be supplemented by streptomycin incorporated in minimal medium (GGM). In the range of 0.5—5 μg streptomycin/ml, however, no such effect was demonstrated.

Table VI

Colony size of isolates and their nutritional revertants on different media after 24 and 48 hours incubation at 37 °C

Strain	Size of colonies in mm on media							
	YP		YP+ALA or haematin[1]		Blood agar		Heated blood agar	
	24	48	24	48	24	48	24	48
168 ind⁻	5.9	6.9			6.8	8.5	3.9	5.6
hemA1			1.0	2.8	1.2	6.0	1.8	5.0
hemA8			0	2.0	0.6	5.0	1.0	5.1
hemA8 revertant			6.8	7.6	6.0	8.0	4.6	7.3
hem-8			0.5	1.0	1.9	4.6	3.8	5.9
hem-8 revertant-1			5.3	7.1	5.4	5.2	4.3	5.2
hem-8 revertant-2			10.6	15.8	7.5	8.2	7.9	12.1
nda-3	1.0	2.9			1.0	1.9		
nda-3 revertant	4.6	7.1			5.0	5.9		
nda-15	1.8	3.9			0.8	1.6		
nda-15 revertant	8.0	9.9			5.0	5.0		
aro-2	6.7	9.1			4.9	5.6		
aro-2 revertant	6.5	7.8			5.9	6.4		
aro-7	3.1	5.0			3.0	4.0		
aro-7 revertant	4.4	6.2			4.2	5.3		

[1] Supplemented either with ALA for *hemA* isolates or with haematin + bovine albumin for *hem* mutants. 0 = Not visible by the naked eye.

Appropriate dilutions of exponential growth phase liquid culture were plated. Inocula consisted of 15 to 20 colony formers. The values represent the average size of 30 to 50 colonies. Variation in the size of individual colonies was less than ± 1 mm.

Slow growth was another striking characteristic of our isolates. For technical reasons instead of determining the generation time, the time needed for the development of colonies was examined. As shown in Table VI there was a striking difference in the size of colonies between *hem*, *hemA* and *nda* isolates and the parent culture. The difference was especially great after 24 hours incubation but was still marked after 48 hours.

Reversion of the mutants restored the original rapid growth or resulted in an increased degree of multiplication. In this respect *aro* auxotrophs behaved irregularly. Their growth decreased slightly and reversion exerted little effect. Some *aro* revertants grew very slowly; these restored their original phenotype probably by suppressor mutation.

When heavy inocula of streptomycin sensitive bacteria of *B. subtilis* 168 were plated on nutrient agar media containing high concentration of streptomycin, the observations made could be summarized as follows. It was characteristic that about 80% of the colony formers differed in phenotype from the inoculated culture. The formed auxotrophs showed no wide range of vari-

ation: on examination of about 1000 colonies all isolates could be classified into 5 groups. Three out of the 5 groups were characterized by a deficiency in protohaem synthesis. The requirement of the most frequently isolated auxotroph *(nda)* could not be defined; it seemed to revert to its original phenotype in one step. It was characteristic that auxotrophy was accompanied by low streptomycin resistance and slow growth. Reversion from auxotrophy resulted in a loss of increased streptomycin tolerance and in regaining normal growth. The behaviour of some *aro* isolates was variable and less characteristic than that of the other mutants.

Discussion

Colonies developing on nutrient agar plate containing aminoglycoside antibiotics may differ from the parent strain in colony morphology and minimal nutritional requirement. JENSEN and THOFERN [6] described streptomycin resistant mutants of *Staphylococcus aureus* the growth of which was highly enhanced by haemin. Haemin dependent mutants were isolated from *Escherichia coli* by serial passages on streptomycin-haemin medium [7]. On neomycin or streptomycin agar salmonellae were described to split off resistant mutants characterized by dwarf colonies [8]; these consisted partly of bacteria dependent on one or more metabolites (cysteine, serine, niacin). A well-characterized δ-aminolaevulinic acid (ALA) deficiency in the porphyrin pathway was first demonstrated in a *B. subtilis* mutant obtained by the use of streptomycin. Shortly thereafter a similar mutant was isolated from *E. coli* with neomycin [9a]. YEGIAN *et al.* in 1959 [9b] isolated haemin dependent mutants from *Staph. aureus* with kanamycin. Recently, TIEN and WHITE [10a] applied kanamycin for isolating large numbers of mutants from the same organisms in order to map the genetic control of the biochemical pathway of protohaem synthesis. It was striking that the mutants failed to show kanamycin resistance.

The pleiotropic effect of streptomycin has long been known; here only some examples are mentioned. High streptomycin resistance in *Salmonella typhimurium* is accompanied by methionine (GOLDSCHMIDT *et al.* [10b]) or thiamine and niacin (DEMEREC and LAHR [10c]) requirement. WATANABE and WATANABE [11, 12] stressed the importance of the degree of streptomycin resistance. They showed that in *S. typhimurium* high (> 500 μg/ml) and moderate streptomycin resistance is associated with different loci. Mutation to low resistance was characterized by slow growth and, in part of the isolates, associated with thiamine and niacin requirement. These mutants could be transduced in a single step to the wild-type phenotype; the transductant became streptomycin sensitive, grew rapidly and turned prototrophic in nutritional requirement. It has, therefore, been concluded that all these markers were controlled by one single locus.

In phenotype, our *B. subtilis* mutants seem to be similar to those described by WATANABE and WATANABE [11, 12]. The great variability in the properties of streptomycin resistant phenotypes justified their classification into well-defined groups. Accordingly, it is recommended that this peculiar type of streptomycin resistance should be designated with the symbol "ALSAS" (associated low streptomycin resistance, auxotrophy and slow growth).

In phenotype there are similarities between our mutants and the *E. coli* strains of GORINI and KATAJA [13] isolated from conditional streptomycin dependent mutants of *E. coli*. The phenotype of their revertants was associated with the appearance of a suppressor functioning also in the absence of streptomycin.

The question arises as to how ALSAS mutants survive the high streptomycin concentration used for their isolation and how is it possible to obtain auxotrophs when the required metabolite is absent from the medium. For example, the ALA required for growth by *hem A* mutants is not contained by the usual nutrient media. The strict association between streptomycin tolerance and environment (constituents of the medium) is well known. According to the conclusion of GYŐRFFY and KÁLLAY [14] the survival of bacteria at a given streptomycin concentration "may be controlled either physiologically, *i.e.* by a favourable microenvironment or by temporary changes at the cellular level . . .; or the survival may be controlled genetically by heritable resistance". Components released from disintegrating bacteria of the heavy inoculum interfere with the effect of streptomycin only slightly, but yield metabolites lacking from the medium.

Mutants isolated by the use of aminoglycoside antibiotics behave peculiarly as they frequently show a deficiency in porphyrin synthesis. Streptomycin acts similarly on eukaryotes: it suspends chlorophyll synthesis irreversibly [15, 16]. As an effect of this antibiotic *Euglena gracilis* cells lose their pigmentation but retain their plastid structure [15, 18]. If ultraviolet irradiation affects in an isolated manner only the cytoplasm of *Euglena*, pigment production ceases. Irradiation of the nucleus fails to exert this effect [19, 20]. Of the papers published since 1962, that of RAY and HANAWALT [21] must be mentioned. These authors assumed that the chloroplast possesses its own DNA responsible for information on chlorophyll production and that this nucleic acid is different from that present in the nucleus. The chloroplast ribosomes are also specific and different from those of the nucleus [22].

According to the model of SCHIFF and ZELDIN [23] streptomycin induces a deficient DNA polymerase on plastid m-RNA and ribosome complex. This defective DNA polymerase would eventually lead to plastid loss associated with permanent bleaching of *Euglena*. Although this alteration cannot be considered a mutation in the usual sense, its result is identical with that of the latter. Despite the fact that several authors described the mutagenic effect of

streptomycin, this antibiotic has not been included in the list of mutagenic agents. Streptomycin has been shown to be mutagenic for the non-chromosomal determinant of *Chlamydomonas* [24], and also for the host range marker of T_2 phage [25]. The discoloration of *Euglena* induced by streptomycin has been regarded as mutation [26] as well as the disappearance of chlorophyll from the sprouting onion, where the antibiotic caused morphologically demonstrable chromosomal aberrations [27].

Streptomycin attacks primarily the cytoplasmic membrane [28]. LANDMAN and BURCHARD [29] assumed that the membrane contains an extranuclear factor taking part in membrane synthesis. Streptomycin-depleted dependent mutants fail to produce a normal cytochrome system [30]. Streptomycin resistant *Salmonella* L-forms do not yield bacterial revertants in the absence of the antibiotic. Streptomycin depletion causes a hereditary alteration in cell wall production [31]. Under physiological conditions, approximately 1/4 of all bacterial ribosomes integrate to the membrane where, bound to m-RNA, they exert a specific template function [32]. The model elaborated by JACOB *et al.* [33] reflects the association of bacterial chromosome and membrane. This association has been proven partly by electron microscopic studies [34] partly by biochemical examinations indicating that DNA replication took place in the membrane. The membrane fraction contained 1/4 of the DNA polymerase [35].

Does the bacterial membrane contain an integrated chromosomal segment more or less independent from nuclear DNA and does it supply information for the synthesis of the membrane and its enzymes? The question is far from being irrational. Part of the DNA is closely connected with the cell wall. BISHOP *et al.* [36] showed the presence of deoxyribonuclease resistant DNA in carefully isolated cell wall preparations of *B. subtilis*. It would be inconsistent with the economy of nature if enzymes associated with the membrane including the porphyrin-containing cytochrome system, were not produced locally. In *B. subtilis* there was a difference in frequency between membrane-associated and cellular DNA-transformable markers. The proportion of origin and terminal markers was higher in the membrane fraction than in cellular DNA [37]. This finding is in agreement with the fact that chromosomal replication begins in the membrane-associated DNA [38].

Streptomycin exerts its action first on the bacterial membrane where it causes an increase in the efflux of K ions and a transient stimulation of net RNA synthesis [39]. In the light of the above observation it is tempting to speculate on the possible mutagenic action of streptomycin. It seems conceivable that streptomycin, a cationic agent, may react with the strongly anionic DNA attached to the membrane. This reaction may result in failures in the replication of the corresponding segment of chromosome.

The mutants described in this report are being subjected to genetic investigation.

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FAST SEDIMENTING FRACTIONS CONTAINING DNA FROM STREPTOMYCES GRISEUS*

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Summary. The sedimentability of DNA from the mycelium of *Streptomyces griseus* strain No. 52—1 after disintegration was examined under different conditions. A considerable amount of fast sedimenting fraction was obtained after lysozyme digestion in a medium containing polyethylene glycol 300 at 6000 *g* and after mechanical disruption in TMK medium at 25,000—38,000 *g* (39 and 30% respectively).

The amount of the fast sedimenting DNA fraction depended on the *Streptomyces* strain used, the age of the culture, and the medium in which washing, disruption and sedimentation had taken place.

No fast sedimenting fractions from *Escherichia coli* and *Bacillus cereus* were obtained under the same conditions which were optimal for *S. griseus*.

The DNA containing fraction was purified by density gradient centrifugation and these fractions were analysed for DNA, RNA, protein, and polysaccharide contents. The protein to DNA ratio of some fractions yielded by density gradient centrifugation was lower than 1.0.

In investigations into the regulation of gene activity it is important to study the nature of the supramolecular structures in which DNA occurs within the cell. A number of authors reported the isolation of such structures both from procaryotic and eucaryotic organisms [4], and in the case of *Bacillus megaterium* [14, 3, 1] and *Bacillus subtilis* [10] these DNA containing structures sedimented faster than free molecular DNA. There are, however, no reports on the isolation of such structures from other bacteria and we failed in our attempts to isolate them from *Escherichia coli* and *Bacillus cereus*.

During the life cycle of *Streptomyces griseus* marked morphological changes occur in the nuclear core [6, 16]. Light microscopic examination of stained preparations revealed that in the young hyphae the nucleoid is diffuse and pale and on aging or at the reproductive differentiation of hyphae, discrete intensely staining chromatin bodies appear. These variations in the appearance of the nuclear material, which can be observed along the same hypha of a 72 or a 96 hours old mycelium, may be important in the differentiation observed in *S. griseus*. This paper will deal with the isolation and partial characterization of fast sedimenting DNA containing structures from *S. griseus*.

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Materials and methods

Organisms and growth conditions. The organisms used were *Streptomyces griseus* strain No. 52—1, *Escherichia coli* B and *Bacillus cereus* strain No. 902.

S. griseus was grown in soybean cornsteep medium [15] in 500 ml Erlenmeyer flasks or 17 ml ampoules (5.2 × 2.4 cm) containing 100 ml and 3 ml medium, respectively. The flasks were incubated at 27°C on a horizontal rotating shaker at 230 r.p.m. with a stroke length of 50 mm.

E. coli was grown in a medium containing Difco beef extract, 0.3%; Difco bactopeptone, 0.5%; NaCl, 0.5%; glucose 0.1%; 0.005 *M* Tris malate buffer pH 7.6. Overnight stationary phase cultures were diluted 1 : 4 with fresh medium of the same composition at 37°C and the incubation was continued until the optical density of the suspension had reached 1.75 at 560 mμ, as determined by a Spektromom 360 spectrophotometer.

B. cereus was grown from a spore inoculum in a medium containing meat extract, 0.5%; Difco bactopeptone, 0.5%; $Na_2HPO_4.7\,H_2O$, 1%; KH_2PO_4, 0.1%; glucose, 2% w/v. The constituents of the medium were dissolved in tap water. The final pH value was 7.3. The bacteria were incubated on a rotary shaker at 27°C and harvested during the early sporulating phase after 12 hour incubation.

At the end of growth the bacteria were harvested by centrifugation and resuspended in one of the following media.

(a) PEG: polyethylene glycol 300 (Fluka, Switzerland) 14% v/v in distilled water.

(b) TMK: 0.01 *M* Tris-HCl, pH 7.8, containing 0.01 *M* magnesium acetate, 0.06 *M* KCl, and 0.006 *M* mercaptoethanol [11].

(c) SP: 0.3 *M* sucrose, 0.005 *M* $MgCl_2$, 0.05 *M* KH_2PO_4. The pH was adjusted to 7.3 with NaOH [5].

Cell breakage and fractionation. The bacterial suspensions were disrupted by lysozyme (final concentration 0.5 mg/ml) at 37°C. The process was followed microscopically and was found to be complete within 45 minutes. A modification of this method was used in other experiments. Here the bacterial suspensions were shaken in a Mickle disintegrator [9] with glass beads (Ballotini No. 12) at 0—8°C, in the presence of lysozyme (1 mg/ml). The cylindrical vessel was filled half with beads and the density of the mycelial suspension was 10 to 15 mg dry wt/ml. Cell breakage was carried out in 1 hour and subsequently the beads were removed by filtration through a G2 sintered glass filter and washed three times with the appropriate suspending medium.

Unbroken cells were removed by centrifugation at 1550 *g* for 20 minutes. The supernatant was then fractionated by centrifugation at 4000—6000 *g* 30 minutes followed by centrifugation at 25,000—38,000 *g* for 30 minutes (as given in Results) in a MSE Highspeed 18 centrifuge in rotor No. 6918. Further fractions were obtained by centrifugation at 78,000 *g* for 1 hour and 200,000 *g* for 3 hours (MSE Superspeed 50, rotor No. 2410).

Separation of components within these fractions was achieved by sucrose density gradient centrifugation using a 10 to 40% w/v linear gradient of sucrose and centrifuging for 30 minutes at 13,000 *r.p.m.* in the No. 2417 swing out rotor in a MSE Superspeed 50 centrifuge. Fractions were sucked off from the surface. After the fractions had been collected the pellets remaining at the bottom of the centrifuge tubes were resuspended in the remaining small amount of gradient fluid and diluted with TMK if necessary.

Unless otherwise stated, all operations were carried out between 0° and 4°C.

Chemical assays. The protein and nucleic acids present in the various fractions were precipitated with 0.5 *M* perchloric acid (PCA). The precipitates were washed three times with cold 0.5 *M* PCA to remove contaminating materials which could interfere with the subsequent assays, before extracting the nucleic acids. These were extracted from the precipitates by heating with 0.5 *M* PCA at 70°C; two extractions for 15 minutes were sufficient for removing all the nucleic acids. DNA and RNA were estimated by the methods described by SEIBERT [13] and BROWN [2], respectively. Calf thymus DNA sodium salt (BDH, England) and yeast RNA (Mann Research Laboratories Inc., USA) were used as standards. Residual protein and carbohydrate were estimated by the methods of LOWRY *et al.* [8] and ROE [12]. As protein standard, a protein fraction from *S. griseus* was used and a standard curve was obtained by plotting the extinctions against mg protein, obtained by multiplying by 6.25 the Kjeldahl figures. A solution of 470 μg protein/ml gave an extinction of 1.25 at 750 mμ.

Morphological examination. The fractions obtained by gradient centrifugation were fixed with glutardialdehyde (50 ml of 25% v/v glutardialdehyde; 263 ml of *M*/15 phosphate buffer, pH 7.2; 7% w/v of sucrose). After standing for 24 hours at 0°C the preparations were washed three times with distilled water and pelleted by centrifugation at 31,000 *g* for 30 minutes. Smears made from the final pellets were stained with methyl green pyronine [7] and examined using a Reichert Zetopan microscope with a Pl Apo Oel 100/1.32 objective.

Results

Initially, breakage of the suspensions of *Streptomyces griseus* was carried out using lysozyme at 37°C in either distilled water or in PEG medium. The DNA contents of the various cellular fractions obtained from these preparations are shown in Table I. There were marked differences in the DNA contents of the various fractions resulting from the different suspending media used. In PEG approximately 40% of the DNA sedimented at 6000 *g* and only 39% remained in solution after sedimentation at 38,000 *g*. When the cells were disrupted in distilled water, 74% of the total DNA remained in the supernatant after centrifugation at 38,000 g. However, this method of cell breakage was abandoned since according to some authors [3] the particles obtained after incubation at 37°C are damaged and no longer suitable for metabolic experiments. In the course of our own studies we were unable to detect RNA polymerase activity in fractions obtained from *S. griseus* after lysozyme lysis.

Disruption of *Streptomyces griseus* in TMK by the Mickle disintegrator in the presence of lysozyme resulted in a considerable quantity of a fast sedimenting DNA fraction, but, unlike that obtained by lysozyme digestion at 37°C, this material was in the 38,000 *g* pellet and not in the 6000 *g* one (Table II). If the mycelium was disrupted in the SP medium used by GODSON and BUTLER [5] for isolating nuclear material from *Bacillus megaterium*, the greater part of the DNA failed to sediment up to 31,000 *g*.

In several experiments, after Mickle disruption a centrifugation at 4000 *g* for 30 minutes was found to be sufficient for removing the heavier wall and membrane debris and that centrifugation at 31,000 or even 25,000 *g* for 30

Table I

Distribution of DNA in the lysates of Streptomyces griseus strain No. 52-1 after lysozyme digestion

The strain was cultivated in 500 ml Erlenmeyer flasks in a volume of 100 ml medium for 96 hours. The mycelium was harvested and washed twice in distilled water then digested in the presence of 0.5 mg/ml lysozyme in the indicated media at 37°C. The PEG sample contained 14% v/v of polyethylene glycol 300

Fractions	Suspending medium			
	distilled water		PEG 300	
	DNA μg	per cent	DNA μg	per cent
1,550 *g* supernatant	17,900	100.0	43,400	100.0
6,000 *g* pellet	— *	—	16,900	39.4
38,000 *g* pellet	4,025*	22.5	8,500	18.9
38,000 *g* supernatant	13,250	74.1	17,090	39.4
Recovery	17,275	96.6	42,500	98.0

* The lysate, after centrifugation at 1,550 *g*, was immediately centrifuged at 38,000 *g*.

minutes was sufficient to spin down the fast sedimenting DNA fraction. Fractionation was done under such conditions in the subsequent experiments. The DNA remaining in the supernatant after centrifugation at 31,000 *g* was not sedimented by centrifugation at 78,000 *g* for 1 hour. Centrifugation at 200,000

Table II

Distribution of DNA in the cell fractions of Streptomyces griseus strain No. 52-1 after disruption in Mickle disintegrator

In experiment 1 the mycelium was the same as in Table I. In experiment 2, 40×3 ml cultures of 72 hours were shaken in ampoules of 17 ml. Half of them were treated in TMK and the other half in SP medium. The mycelium was washed twice in the given media and shaken in the Mickle disintegrator for 1 hour in the presence of Ballotini glass beads and 1 mg/ml of lysozyme

Fractions	Experiment	Suspending medium			
		TMK		SP	
		DNA μg	per cent	DNA μg	per cent
1,550 *g* supernatant	1	7,575*	100.0	—	.
	2	5,214	100.0	5,183	100.0
6,000 *g* pellet	1	1,246	16.4	—	.
	2	592	11.3	530	10.2
38,000 *g* pellet	1	2,549	33.6	—	.
	2	1,683**	32.3	747**	14.4
38,000 *g* supernatant	1	3,780	49.9	—	.
	2	2,400**	46.1	3,600**	69.5
Recovery	1	—	100.0	—	.
	2	4,675	89.7	4,877	94.2

* Calculated value ** Centrifuged at 31,000 *g*.

Table III

Distribution of DNA in the cell fractions of Streptomyces griseus strain No. 52-1 depending on the age of the culture

The mycelium was cultured as given in Table I treated in TMK and disrupted in the Mickle disintegrator (see Table II)

Age of culture (hours)	Fractions					
	6,000 *g* pellet		38,000 *g* pellet		38,000 *g* supernatant	
	DNA μg	per cent	DNA μg	per cent	DNA μg	per cent
24	1,735	13.6	1,403	11.0	9,650	75.6
48	2,640	27.6	2,220	13.2	4,700	49.2
72	3,720	27.6	4,790	35.6	4,905	36.5
96	3,380	22.0	5,325	34.7	6,660	43.4

g for 3 hours resulted in the sedimentation of a proportion of DNA similar to that obtained from *Escherichia coli* in the same experiment.

The proportion of DNA sedimenting at 6000 *g* and 38,000 *g* increased with the age of the culture up to 72 hours when it was maximum for both fractions (Table III).

Treatment of *Escherichia coli* and *Bacillus cereus* suspensions under conditions identical with those used for *S. griseus* did not yield an appreciable quantity of DNA sedimenting at 31,000 *g* as was obtained from *S. griseus* (5 and 7% compared to 22%, respectively. Table IV).

Table IV

Comparison of DNA content in cell fractions of Streptomyces griseus strain No. 52-1 of Escherichia coli B and of Bacillus cereus strain No. 902

The *S. griseus* mycelium was the same as in experiment 2 in Table II. Concerning the cultivation of *E. coli* and *B. cereus*, see under Methods. Cultures were treated in TMK with a Mickle disintegrator

Fractions	*S. griseus*		*E. coli*		*B. cereus*	
	DNA μg	per cent	DNA μg	per cent	DNA μg	per cent
1,550 *g* supernatant	9,940	100.0	12,960	100.0	5,330	100.0
6,000 *g* pellet	1,097	11.0	1,070	8.3	556**	10.4
38,000 *g* pellet	2,200*	22.1	658*	5.1	391	7.3
38,000 *g* supernatant	5,010*	50.4	8,580*	66.3	3,670	68.9
Recovery	8,307	83.6	10,308	79.6	4,617	86.7

* 31,000 *g* centrifugation.
** 4,000 *g* centrifugation.

Table V

Composition of cell fractions of Streptomyces griseus strain No 52-1

Mycelium and treatment were the same as in Experiment 2 in Table II. The samples taken for chemical determinations were precipitated then washed three times in 0.5 *M* PCA, followed by the extraction of nucleic acids

Fractions	DNA (μg)	RNA (μg)	Protein (μg)	Polysaccharide (μg)	DNA : RNA : protein : polysaccharide
1,550 *g* supernatant	9,075	39,600	98,250	4,460	1 : 4.36 : 10.83 : 0.491
4,000 *g* pellet	542	4,610	16,600	1,500	1 : 8.52 : 30.60 : 2.770
31,000 *g* pellet	1,269	7,390	19,000	1,540	1 : 5.82 : 15.00 : 1.215
31,000 *g* supernatant	7,010	23,000	61,950	436	1 : 3.28 : 8.82 : 0.062
Recovery	8,821	35,000	97,550	3,476	

Density gradient centrifugation. A mycelial suspension was disrupted and fractionated by the method described in Table II in TMK, and the DNA, RNA, protein and polysaccharide contents of the various fractions were determined. Results are shown in Table V. Attainment of purification of a "nuclear fraction" would result a lower value of protein to DNA ratio, but centrifugation at 31,000 *g* by itself did not cause a purification of the DNA containing particle. The 31,000 *g* pellet obtained in this experiment was resuspended in TMK, layered onto a sucrose density gradient and centrifuged in the No 2417 swinging bucket rotor of the MSE Superspeed 50 centrifuge at 13,000 *r.p.m.* for 30 minutes. Five major fractions were collected (Table VI) and the DNA, RNA, protein and polysaccharide contents of each fraction were determined. The first fraction was almost colourless and transparent, the second was sharply separated from the first and brownish in colour. This brown colour progressively diminished towards the bottom of the tube and the fourth fraction was again almost colourless. The particles present in the various fractions were examined microscopically after methyl green pyronine staining. In the second fraction, small, round, greenish particles 0.3—0.4 μ in diameter could be observed in a strongly pyronine positive background. In the 3rd 4th and pellet fractions similar but smaller particles could be detected. In the heavier fractions, the background staining was shifted from red to violet. The incidence of these particles was consistent with the hypothesis that they represent the DNA containing component.

In a subsequent experiment the fast sedimenting DNA containing fraction was sedimented at 25,000 *g* for 30 minutes before fractionation on a sucrose density gradient (Table VII).

Table VI

Purification of DNA fraction of Streptomyces griseus No. 52-1 sedimented at 31,000 g in sucrose density gradient

Part of the 31,000 g pellet in Table V (2.2 ml, 1150 μg DNA) after resuspension was layered over 36.5 ml sucrose density gradient. Fractions were collected as indicated

No.	Fractions	DNA (μg)	RNA (μg)	Protein (μg)	Polysaccharide (μg)	DNA : RNA : protein : polysaccharide
1.	0.0— 4.9 ml	181	1,833	3,410	279	1 : 10.11 : 18.85 : 1.540
2.	4.9—13.0 ml	214	1,225	6,030	656	1 : 5.75 : 28.20 : 3.070
3.	13.0—22.0 ml	177	394	2,227	90	1 : 2.22 : 12.59 : 0.508
4.	22.0—35.2 ml	102	193	736	20	1 : 1.89 : 7.22 : 0.198
5.	gradient pellet	398	286	3,460	118	1 : 0.72 : 8.70 : 0.277
	Recovery μg	1,072	3,931	15,863	1,164	
	%	93.2	63	83.5	89.3	

It can be seen (Table VI) that the gradient fractions of the 31,000 *g* pellet yielded protein to DNA ratios just lower than that of the 31,000 *g* supernatant (fraction 4 : 7.22; fraction 5 : 8.7; 31,000 *g* supernatant: 8.82). The corresponding ratios in the gradient fractions of the 25,000 *g* pellett (Table VII) were more favourable (fraction 3 : 3.6; fraction 4 : 2.0; and fraction 5 : 3.7). The value of 2.0 for fraction 4 in Table VII was lower than the 3.0 value reported by SPIEGELMAN *et al.* [14] for a similar fraction from *B. megaterium*.

Table VII

Purification of DNA fraction of Streptomyces griseus strain No. 52-1 sedimented at 25,000 g in sucrose density gradient

The mycelium was of the same kind as in Experiment 2 in Table II. After Mickle disintegration in TMK the suspension was sedimented at 1550 *g*, at 4000 *g* then at 25,000 *g*. Part of the 25,000 *g* pellet (1.3 ml, 570 μg DNA) after resuspension was layered over a 36.5 ml density gradient

No.	Fractions	DNA (μg)	RNA (μg)	Protein (μg)	Polysaccharide (μg)	DNA : RNA : protein : polysaccharide
	25,000 *g* pellet	1,150	15,750*	9,360*	773	1 : 13.70 : 8.14 : 0.672
1.	0.0— 6.1 ml	125	283	1,488	60	1 : 2.26 : 11.90 : 0.480
2.	6.1—11.3 ml	57	161	600	110	1 : 2.79 : 10.43 : 1.913
3.	11.3—18.3 ml	75	121	270	29	1 : 1.60 : 3.60 : 0.391
4.	18.3—30.3 ml	75	205	150	17	1 : 2.71 : 2.00 : 0.228
5.	Gradient pellet	126	231	468	49	1 : 1.89 : 3.70 : 0.387
	Recovery μg	458	1001	2,976	265	
	%	80.5	12.8*	58.8*	63.4	

* The 31,000 *g* pellet after precipitation with 0.5 *M* PCA was not washed in PCA.

The quantity of this fast sedimenting fraction of DNA with the different variants of our strain of *S. griseus* unfortunately varied considerably; the mechanism for this could not be explained. In 72 to 96 hours cultures from a particular variant of *S. griseus* disrupted in TMK, more than 30% of the DNA sedimented at 31,000 *g* whereas with the other strains this value was less than 10%. The results reported in Table VI and VII represent the data of pellets which contained less than 20% of the total DNA and a further fractionation was performed on a 25,000 *g* pellet from a 96 hours old culture which in this case contained more than 30% of the total cellular DNA. The results of this fractionation are shown in Fig. 1. The protein to DNA ratio varied in the different fractions and it would appear from this experiment that the ratio for the DNA containing particles was less than 1.0 and that the higher values reported in Table VI and VII could be due to protein contamination of these

fractions. (In Fig. 1 the reciprocal values of this ratio are illustrated.) The age of the culture may also have affected the result since the experiments reported in Table VI and VII were performed with 72 hours cultures whereas those for Fig. 1 with 96 hours ones.

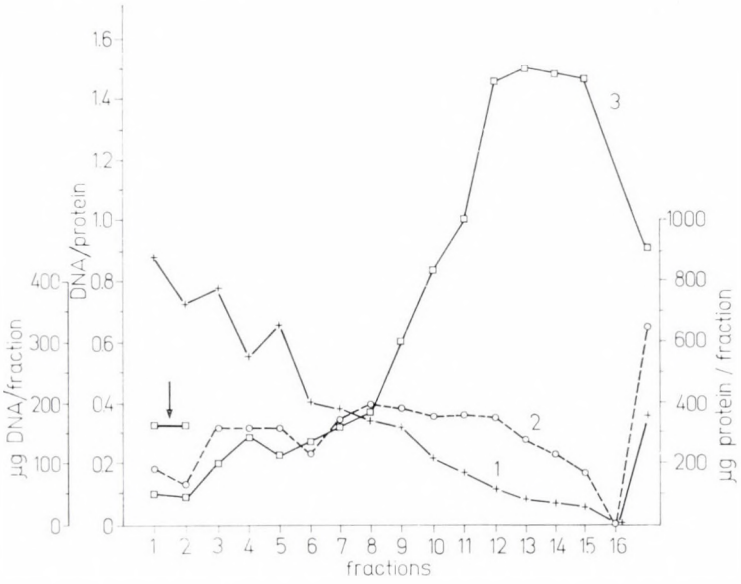

Fig. 1. Sucrose density gradient centrifugation of the fast sedimenting DNA fraction. 96 hours old mycelium of *Streptomyces griseus*, the same as in Table I. As for cell disruption and fractionation, see Table VII. The pellet obtained at 25,000 *g* was layered over 10—40% w/v sucrose gradient and centrifuged at 13,000 r.p.m. for 30 minutes. Fractions of 2 ml were collected. 1 = protein/fraction; 2 = DNA/fraction; 3 = DNA/protein. Fraction 17 represents the gradient pellet. The line marked with arrow shows the DNA: protein ratio of the 25,000 *g* pellet

When the fractions obtained on sucrose density gradient were diluted with an equal volume of TMK and centrifuged at 31,000 *g* for 40 minutes, the DNA again appeared in the pellet fraction suggesting that there was no appreciable dissociation of the DNA containing particles during sucrose density gradient centrifugation.

Discussion

The broken cell preparations obtained by lysozyme digestion at 37°C or Mickle disintegration in the presence of lysozyme at 0—8°C contained considerable quantities of a fast sedimenting DNA fraction. The proportions of the cellular DNA sedimenting under these conditions depended largely on the method of breakage and the suspending medium used. Polyethylene glycol 300 may exert a protective effect on particles containing DNA. It is also prob-

able that the change in the proportion of material in the 6,000 and 31,000 *g* pellets in the different experiments was due to the degradation of the native particle.

The conditions which have been used successfully for the isolation of a fast sedimenting DNA containing fraction from *B. megaterium* [3] could not be applied for *S. griseus* and this difference may well be connected with differences in the ion requirements for the stability of the DNA containing particles from *S. griseus* and *B. megaterium*. A further difference is that in media used for *B. megaterium* [3] a suspension of high viscosity is obtained also from *S. griseus* after the disruption of mycelium, but in TMK medium the viscosity remains low.

The proportion of DNA sedimenting at relatively low speeds reached a maximum value of 58%, considerably lower than the value obtained for *B. megaterium* [3]. A number of possible explanations could be offered for this. It could have been due to sub-optimal experimental conditions or it may have been a definite part of microscopically heterogeneous DNA which was present in this form. The latter explanation was to some extent supported by the finding that the proportion of DNA in these fractions depends not only on the age of the culture but also on the particular strain of *S. griseus*.

The 31,000 *g* pellet is obviously a complex mixture and contained DNA as well as protein, RNA and polysaccharide, and in some instances the ratio of these compounds to DNA was greater than in the mycelium homogenates (Table V). The presence of a considerable proportion of RNA may be important.

The proportion of DNA to other components increased during density gradient centrifugation (Fractions 3, 4 and 5, Table VI and VII). In the gradient fractions, the RNA to DNA ratio was higher than that found by other authors in *B. megaterium e.g.* SPIEGELMAN *et al.*: 1.0 [14]; BUTLER and GODSON: 0.143 [3], but it was not clear whether the changes in the RNA to DNA ratio for the different fractions had resulted from a removal of RNA containing contaminations, or from variations in the composition of a DNA-RNA-protein containing complex.

The polysaccharide measurements were performed to determine the level of contamination in these fractions by materials of the cell envelope, and it is clear from the results that this varied in Fractions 3, 4 and 5, but a definite purification was achieved.

There was also a considerable variation in the protein to DNA ratios and much of this could be accounted for contamination of the DNA-protein particle with other proteins since under some circumstances it was possible to obtain particles containing more DNA than protein (Fig. 1).

The results showed that by centrifugation at 6,000 to 38,000 *g* we succeeded in isolating DNA containing particles stable in TMK. They may have com-

mon features with those reported by TREMBLAY *et al.* [17] but are discernible also under the light microscope and may be identical with the chromatin bodies observed in the mycelium of *S. griseus* and may have a more complex structure. The presence of smaller particles appearing in the various fractions might be explained by a degradation of these particles during cellular breakage.

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SEROLOGICAL AND CHEMICAL STUDIES OF SH. SONNEI, PSEUDOMONAS SHIGELLOIDES AND C27 STRAINS

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Summary. The biochemical behaviour of Bader's *Ps. shigelloides* and Ferguson's C27 strains was characteristic of the genus *Aeromonas*.

The S forms and R mutants of the two strains were serologically identical with each other as well as with *Sh. sonnei*, phases I and II, respectively.

The identity in antigenic structure of *Sh. sonnei* and *Ps. shigelloides* was demonstrated by mouse protection test, too.

The LPS produced from both *Aeromonades* contained sugar constituents qualitatively identical with the LPS of *Sh. sonnei* in respect of either S and R forms, but the *Aeromonades* possessed in addition a serologically inactive uronic acid component and the LPS of C27 a small quantity of galactosamine.

Sh. sonnei and the two strains of *Aeromonas* studied were representing different chemotypes, but even the strains *Ps. shigelloides* and C27 did not belong to the same chemotype.

Flagellated bacteria not belonging to the enteric organisms and having an antigenic structure common with *Sh. sonnei* phase I, were isolated by several authors from faeces of humans suffering of enteritis as well as from the inner organs and faeces of animals. The first report is linked with the names of Ferguson and Henderson [1] who in 1947 described a strain considered an anaerogenic variant of the group *E. coli* and referred to as C27. Bader [2] published a similar report in 1954, classifying the strain in the genus *Pseudomonas* and suggesting the name *Pseudomonas shigelloides*. Since then similar strains were isolated from various sources by several authors [3—13]. Ewing *et al.* [14, 15] identified the C27 and *Ps. shigelloides* strains reffering them as a particular species of the genus *Aeromonas*. Habs and Schubert [16] as well as Eddy and Carpenter [17] suggested to classify them in a new genus, *Plesiomonas*. Sebal and Veron [18] thought also of creating a new genus by the name *Fergusonia*. We have accepted the classification of strains in the genus *Aeromonas*, since in our investigations, too, their biochemical behaviour was characteristic of this genus. The strains studied by us — with the reservation of the previous statement — were designated in this paper for simplification by their original name: *Pseudomonas shigelloides*, and C27.

The group *Aeromonas shigelloides* was shown by several authors to be heterologous serologically and strains inagglutinable by *Sh. sonnei* phase I serum are often encountered. Of the 48 biochemically identical strains of Ewing and Johnson [15] 20, of the 10 strains of Habs and Schubert [16] 5, of 52

strains of ALDOVA [12] 7 were agglutinated by *Sh. sonnei* phase I serum. The 22 strains studied by SAKAZAKI *et. al.* [19] were composed of 5 antigenic groups and 7 strains were serologically identical with *Sh. sonnei* phase I.

Since according to the literature some of the *Aeromonas shigelloides* strains had an antigenic structure identical with that of *Sh. sonnei*, it seemed interesting to study whether this extragenetic relation rested on an identical chemical structure.

In the course of the experiments, first an exact serological analysis of the strains was performed. Then the components of the lipopolysaccharide (LPS) antigens of these strains were investigated comparing them with the components of *Sh. sonnei* determined by us earlier [20].

Materials and methods

Strains. Sh. sonnei, phases I and II, studied previously [21] as well as the original strains C27 of FERGUSON and HENDERSON [1] and the original *Ps. shigelloides* of BADER [2] were studied. From the last named two strains spontaneous R mutants were isolated and included in the studies, as well as a R mutant isolated from *Sh. sonnei*, phase II [21]. The clone differing from phase II in antigenic structure may be considered an advanced phase of the R mutation, since the phase II should be regarded as the first step of R mutation [21].

Agglutinating and precipitating sera. Rabbits were immunized with suspension killed by acetone and subsequently boiled for 2 hours. The R strains were suspended in distilled water to avoid sedimentation. The titres of sera were 1 : 1600—3200.

Bacterial agglutination. Tube agglutination was carried out in volumes of 0.5 ml by addition of 0.05 ml of boiled suspensions of 2×10^9 density.

Absorption was performed by suspending 0.1 g of acetone killed bacteria in 5 ml of serum diluted 1 : 20. The mixture of serum and bacteria incubated at 37°C for 2 hours and then at 5°C overnight was absorbed repeatedly if needed, according to the results of control agglutinations.

Haemagglutination was done by using sheep RBC preserved in Alsever's solution and sensitized by heat activated Westphal antigen, by the Takátsy's [22] microtechnique.

Absorption for haemagglutination was carried out by the addition of 5 mg of Westphal antigen to 5 ml serum 1 : 20. Incubation at 37°C for 2 hours then at room temperature overnight was followed by centrifugation and the procedure had been repeated.

Agar-gel precipitation was performed by OUCHTERLONY's method [23]. By aid of a metal model in the purified agar-gel containing merthiolate wells 5 mm in diameter were formed at distances of 10 mm. Into the central well 0.1 ml of concentrated serum, while into the peripheral ones Westphal antigens at a concentration of 5 mg/ml were measured. Reversed experiments were also carried out, placing the sera around the antigen measured into the central well. Incubation was done in a wet chamber at room temperature, and the result was evaluated after 72 hours.

Active immunization. Groups of 4 mice each were immunized orally using a metal bougie on five occasions at 3 day intervals, with decreasing cell counts of *Sh. sonnei* I and *Ps. shigelloides* killed at 60°C. Five days after the last immunizing dose the animals were intraperitoneally challenged by 50 LD_{50} (approximately 2×10^3 organisms) of a 24 hour culture of *Sh. sonnei* phase I, suspended in sterile 5% mucin. ED_{50} was calculated according to KÄRBER [24].

Chemical studies. LPS antigens were produced by the hot phenol/water method of WESTPHAL *et. al.* [25] from bacterial mass culture killed by acetone. In the experiments substances purified by ultracentrifugation at 100,000 *g* were used.

The qualitative and quantitative determination of sugar components was carried out as described earlier [20]. Galacturonic acid was identified by high voltage paper electrophoresis on Whatman's No 1. paper in a pyridine : acetic acid : water 100 : 40 : 860 buffer at pH 5.3, 3000 V, 120 mA, for 2 hours. The spots were developed with naphthoresorcin. For the quantitative determination of uronic acid a modified method with carbazole was applied [26]. Galacturonic acid was identified by chromatography on Sephadex column and by microchemical methods.

Results

I. Biochemical reactions. The biochemical reactions of the two strains were investigated by EWING *et al.* [14, 15]. In our studies, the cytochromoxydase reaction characteristic of the genus *Aeromonas* was given by both strains. Strain C27 was ONGP positive, fermenting lactose without gas production within 24 hours, while *Ps. shigelloides* lacked this ability. EWING *et al.* studied 50 strains and found only 4 lactose positive ones [15]. WINTON's strain was ONPG positive [13]. The 22 strains of SAKAZAKI *et. al.* [19] fermented lactose slowly. CASELITZ [27] found among 31 strains of *Aeromonas* a single lactose positive one. Otherwise the strains investigated by us corresponded in every respect to the biochemical characteristics described by EWING *et al.*, thus, their belonging to the genus *Aeromonas* was doubtless.

II. Antigenic structure. (a) *Cross absorption.* The titres of immune sera and the values for cross agglutination were determined by bacterial agglutination and haemagglutination. The titres were lying so close that average values could be considered. Then the sera were serially absorbed by te studied strains and by LPS prepared from them. The results of bacterial agglutination and haemagglutination of the absorbed sera also permitted the use of average values.

As regards the antigenic structure of S strains, conclusions can be drawn from Table I. The strains *Sh. sonnei*, I, *Ps. shigelloides* and C27 gave cross-agglu-

Table I

Antigenic structure of S strains

Serum		*Average* titre in bacterial agglutination and in haemagglutination against RBC sensitized with strains and LPS		
Designation	Absorbed by strains and LPS	*Sh. sonnei* I	*Ps. shigelloides*	C27
Sh. sonnei I	∅	2560	2560	1280
	Ps. shigelloides	—	—	—
	C27	—	—	—
Ps. shigelloides	∅	1280	1280	640
	Sh. sonnei I	—	—	—
	C27	—	—	—
C27	∅	2560	2560	2560
	Sh. sonnei I	—	—	—
	Ps. shigelloides	—	—	—

Table II

Antigenic structure of R strains

Serum		*Average* titre in bacterial agglutination and in haemagglutination against RBC sensitized with strains and LPS			
Designation	Absorbed by strains and LPS	*Sh. sonnei* II	*Ps. shigelloides* R	C27 R	*Sh. sonnei* R
Sh. sonnei II	∅	2560	2560	1280	320
	Ps. shigelloides R	—	—	—	—
	C27 R	—	—	—	—
	Sh. sonnei R	640	640	320	—
Ps. shigelloides R	∅	1280	1280	1280	160
	C27 R	—	—	—	—
	Sh. sonnei II	—	—	—	—
	Sh. sonnei R	640	640	320	—
C27 R	∅	1280	1280	1280	160
	Ps. shigelloides R	—	—	—	—
	Sh. sonnei II	—	—	—	—
	Sh. sonnei R	640	640	320	—
Sh. sonnei R	∅	320	160	320	1280
	Ps. shigelloides R	—	—	—	640
	C27 R	—	—	—	320
	Sh. sonnei II	—	—	—	640

tination up to the end titres and absorbed the sera mutually. In the light of these results the S antigenic structure of the 3 strains is identical.

From Table II the antigenic structure of R strains may be established. The agglutinating sera produced by R mutants of strains *Sh. sonnei* phase II, *Ps. shigelloides* and C27 mutually agglutinated both the bacterial suspensions and the sensitized RBC close up to the end titres. The sera were mutually absorbable by bacterial suspensions as well as by LPS. Hence the antigenic structure of strains *Sh. sonnei* phase II, *Ps. shigelloides* R and C27 R is identical.

According to our previous observations [21] the antigenic structure of R strains isolated from *Sh. sonnei* phase II is, however, different. The cross-agglutinability of the mutant and that of the immune-serum prepared against it is partly of lower grade than that of the homologous system, partly the mutant is incapable to absorb the immune-serum prepared against the 3 other strains and even in the homologous serum a significant amount of agglutinin remained after cross-absorption. Thus, the R mutant shows an antigenic rela-

tionship of lower grade with the strains *Sh. sonnei* II, *Ps. shigelloides* R and C27 R, and it possesses a particular antigen, too.

(b) *Agar-gel precipitation.* The antigenic structure of S forms and R mutants of *Ps. shigelloides* and C27 as well as of *Sh. sonnei* phases I and II was then studied by agar-gel precipitation, using Westphal antigens and sera produced against the strains listed. Two arrangements were applied. First the interrelationship of serum measured into the central well and of solutions of antigens measured into the peripheral wells were studied, then the experiment was reversed. Results are illustrated in Figs 1 and 2.

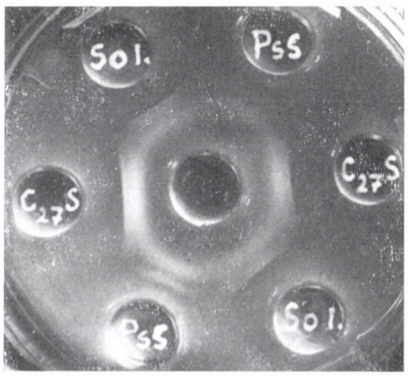

Fig. 1. Agar-gel precipitation of *Sh. sonnei* phase I serum. The central well contains *Sh. sonnei* phase I serum, the peripheral wells contain the antigens

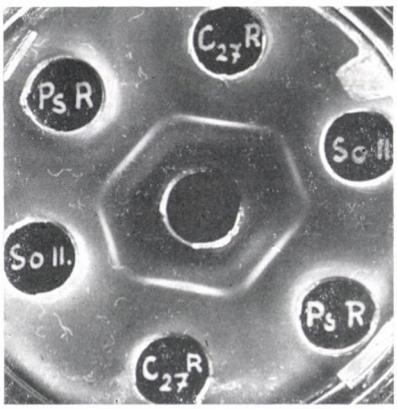

Fig. 2. Agar-gel precipitation of *Sh. sonnei* phase II serum. The central well contains *Sh. sonnei* phase II serum, the peripheral wells contain the antigens

Fig. 1 demonstrates that on encountering *Sh. sonnei* phase I serum and S antigens a multiple band had formed. The individual bands were giving a continuous line confirming the identity of the antigens.

From Fig. 2 it can be seen that *Sh. sonnei* phase II serum and the antigens of *Ps. shigelloides* R and C27 R gave an inner indistinct and an outer distinct band. Both bands formed a continuous line, indicating the identity of the antigens.

III. Mouse protection test. A decisive proof of the identity of antigens was provided by the mutual protective ability of the strains. The protection in mice afforded by oral administration of suspensions of *Sh. sonnei* phase I and the S forms of *Ps. shigelloides* killed at 60°C was investigated against challenge with *Sh. sonnei* I.

In the experiment shown in Table III, 50% of mice were protected by 8×10^9 germs against homologous challenge with *Sh. sonnei*. The ED_{50} of

Table III

Protection of mice immunized orally by Sh. sonnei I and Ps. shigelloides cultures killed at 60°C, against challenge with Sh. sonnei

(Infective dose 50 LD_{50} of *Sh. sonnei* I)

Immunization dose (No. of bacteria)	Indicators of immunity	
	Sh. sonnei	*Ps. shigelloides*
2.5×10^{11}	4/4	4/4
2.5×10^{10}	3/4	2/4
2.5×10^{9}	1/4	1/4
2.5×10^{8}	0/4	0/4
ED_{50} (No. of bacteria)	8×10^{9}	2.5×10^{10}

Denominator indicates the number of infected mice, numerator shows that of the surviving ones.

Ps. shigelloides proved to be 2.5×10^{10} germs. The difference was not significant, and indicated the antigenic identity of the two strains.

IV. *Chemical studies.* Results are summarized in Table IV.

Table IV shows the main components of S and R lipopolysaccharides. As compared to *Sh. sonnei*, the important sugar components were qualitatively iden-

Table IV

Percentage of the main components comprising the LPS of S forms and R mutants of Ps. shigelloides and C27 strains

	Ps. shigelloides		C27	
	S	R	S	R
KDO	13.7	21.0	9.6	28.6
Heptose	11.9	18.3	10.2	18.8
Glucosamine	6.8	10.1	6.4	7.9
Galactosamine	—	—	1.1	—
Glucose	5.8	10.10	2.9	10.10
Galactose	5.1	5.5	3.0	5.9
Galacturonic acid	2.7	4.1	3.0	2.7
N-acetyl	3.8	0.5	6.7	1.2
O-acetyl	0.3	0.6	4.2	1.2
Ester (in form of palmitic acid methylester)	9.0	11.0	6.7	8.3
Phosphorus	2.0	2.7	1.0	3.5
Nitrogen	4.3	2.2	6.7	3.2

tical in *Sh. sonnei* and *Aeromonas*. Expressing in per cent the ratio of sugar components, in the serologically identical mutants of *Aeromonas* and *Shigella* very different rates were obtained. Investigating, however, the molar ratio of sugar components related to each other, the corresponding LPS of *Aeromonas* and *Shigella* showed a high similarity [20]. An uncommon qualitative difference was indicated by the presence of galacturonic acid in the LPS of S and R forms of *Ps. shigelloides* and C27. In case of LPS of C27 also galactosamine should be added. Regarding the quantitative composition of LPS of C27 even the low content of galactose and galactosamine is of special interest. Galactosamine was identified by means of ninhydrin; the heptose proved to be d-glycero-d-mannoheptose in all the four LPS.

Discussion

Numerous papers [3—13] have dealt with the serological inter-relationship of *Sh. sonnei* and of the strains C27 [1] and *Pseudomonas shigelloides* [2]. While their taxonomic position seems to be clarified [14, 15] a single report only was analyzing their serological relationship to *Sh. sonnei*, by means of agar-gel precipitation [28]. As to the chemical structure, again a single report was only found on the relationship between the S antigens of *Sh. sonnei* phase I and *Ps. shigelloides*, studying the CF-inhibition by sugars [29]. In the present investigation of the S and R mutants of the original strains C27 of FERGUSON, of BADER's *Ps. shigelloides* and of *Sh. sonnei* phases I and II and R mutants, we have tried to approach the question from various sides.

1. According to our biochemical studies, *Ps. shigelloides* and C27 have the characteristics of the genus *Aeromonas*, and their taxonomic position is indisputable. Strain C27 was ONPG positive while *Ps. shigelloides* did not ferment lactose and it was probably due to this behaviour that FERGUSON and HENDERSON had classified the strain in the *E. coli* group. Now it is clear that with *Aeromonas* strains, ONPG positivity and lactose fermentation are exceptional features [13, 15, 19, 20].

2. Our experiments concerning the determination of the antigenic structure were carried out with both S and R mutants, including in the case of *Sh. sonnei*, beside the strains of phases I and II, even the R mutant derived from the latter. Cross-agglutinations were distinct and the absorptions with bacteria and with Westphal antigens yielded identical results.

The agglutinating titres of absorbed sera were closely similar in bacterial agglutination and haemagglutination tests. The S forms of C27 and *Ps. shigelloides* were consequently identical and their antigenic structure corresponded to that of *Sh. sonnei* phase I. In antigenic structure the R mutants of the two strains were identical and proved identical also with *Sh. sonnei* phase II. However, *Sh. sonnei* phase II, as well as the R mutants of C27 and *Ps. shi-*

gelloides did not show a complete serological identity with the R mutant of *Sh. sonnei*. To explain this it was assumed that *Sh. sonnei* phase II contained R antigen already in traces and hence R agglutinins were present in the phase II serum. The serological relation of *Ps. shigelloides* and C27 R and of the R mutants of *Sh. sonnei* might be explained in the same way. The successive degradation of R mutants is well-known from the studies of LÜDERITZ *et al.* [30] concerning the changes in serological specificity occurring parallel with a change in the constituents of the R mutants of Salmonellae. SAKAZAKI *et al.* [19] also isolated R mutants from *Aeromonas shigelloides* strains, but these strains failed to agglutinate in *Sh. sonnei* phase II serum. These R mutants might have been in a more advanced phase of mutation than the strains investigated by us.

3. To identify the antigenic structure, agar-gel precipitation was also carried out with Westphal antigens prepared from S strains of *Ps. shigelloides*, C27 and *Sh. sonnei* phase I, from the R mutants of these strains, as well as from phase II. The precipitation bands were formed of continuous lines indicating the complete identity of S and R antigens. MARTIN *et al.* [28] investigated by agar-gel precipitation the interaction of sera and antigens produced at various temperatures to compare *Sh. sonnei* phase I and a particular strain of *Aeromonas* C27. The antigenic relationship between *Sh. sonnei* phase I and the investigated *Aeromonas* C27 strain was found to be maintained by heat labile antigens. In our studies we applied Westphal antigens, and sera of rabbits immunized by boiled cultures to exclude the disturbing effect of flagellar antigens and of eventual heat labile antigens. The heat stable antigens and the corresponding sera produced such distinct reactions (Figs 1 and 2) which pointed to the leading role of heat stable antigens in the antigenic relationship.

4. As a conclusive method of identification of the antigenic structure, the immune reactions in the macroorganism were studied. It was found that a suspension of *Sh. sonnei* phase I and *Ps. shigelloides* treated at 60°C was protecting the mice against challenge with 50 LD_{50} of *Sh. sonnei* I. The difference in the ED_{50} between homologous and heterologous strains was not significant. The protection against each other afforded by the two strains again confirmed the identity of the antigens. In these experiments oral immunization was applied so that the effect of eventual minor antigens could not disturb the efficacy of antigens determining the specificity. Hence the result was regarded as highly specific.

5. According to the chemical analysis, the components of LPS of S and R forms were qualitatively identical with the LPS of *Sh. sonnei* phases I and II, except that the LPS of both *Ps. shigelloides* and C27 contained d-galacturonic acid. The result was unexpected, because so far only the LPS of *P. mirabilis* [31], *P. vulgaris* and *Xanthomonas* [32] were known to contain uronic acid. In the S strain C27, even galactosamine was demonstrated.

Though the percentage of the common sugar components was different from that of the LPS of *Sh. sonnei*, the ratio of components in relation to each other corresponded exactly with the ratios found in the LPS of *Sh. sonnei*.

It was expected that the serological identity was due to the exactly identical structure of the LPS, on the basis of the close relationship of the serotypes in the *Salmonella* group [33]. The sugar components of the LPS were exactly identical also in the case of a serological identity of species belonging to different genera [34]. However, there was no complete chemical identity between *Sh. sonnei* and the studied strains of *Aeromonas* as discussed earlier. The uronic acid present in the *Aeromonas* strains did not seem to have a role in serological specificity, due to the fact, that this component does not indicate the presence of a new antigenic factor compared to *Sh. sonnei*. The galactosamine found in the LPS of C27 was also considered to have no role in the formation of serological specificity, because the antigenic structure of *Sh. sonnei* II, lacking this component was identical with that of *Ps. shigelloides*. Due to these chemical differences — despite of their serological identity — the three strains cannot be classified in the same chemotype and actually even the strains *Ps. shigelloides* and C27 are representing two chemotypes of the same genus.

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ISOLATION OF HFR DERIVATIVE BY THE USE OF SHIGELLA FLEXNERI 4b-MODIFIED F FACTOR

By

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Summary. By transferring the *hsp* gene of *Shigella flexneri* 4b to F+ *Escherichia coli* K-12 a *Sh. flexneri* 4b-modified F factor was produced. Transfer of this factor to another mutant of the *Shigella* host resulted in an F+ culture yielding a stable *Hfr* derivative.

The F integration site of *Sh. flexneri* 4b *Hfr* derivative falls between the *Mtl* and *Ilv* markers, and if projected to the linkage map of *E. coli* K-12, appears approximately at 72 minutes. The transfer order followed a clockwise direction: O-*ilv-met-thr-trp*.

In previous studies we have shown that the in many respects irregular behaviour of K-12 factor is a consequence of host-specific modification and restriction. The finding that F+ *Shigella flexneri* strains failed to yield stable *Hfr* derivatives also appeared to be associated with restriction.

The present paper gives an account of experiments in which, on the basis of this finding, we prepared *Sh. flexneri* 4b host cell-modified F factor and successfully isolated a *Hfr* derivative from an F+ strain carrying this F factor.

Materials and methods

Organisms. Bacterial strains and their characteristics are presented in Table I.

Phages and phage titration. The modification—restriction relationship was examined by the use of T5 phage, on the basis of earlier studies [2].

For preliminary studies on *hsp* specificity of recombinants the quick method of COLSON *et al.* [3] was used.

For phage titration the phages were diluted with LOEB's solution [4]. The number of plaques obtained for various strains was expressed in relative values (efficiency of plating = = e. o. p.) by taking the number of plaques produced by the phage propagated and tested on a given strain as 1.

F transfer. The technique described in reference [1] was used.

Acriflavine treatment. As in previous studies [2] the original method of HIROTA [6] was applied.

Conjugation technique. The membrane filter method of MATNEY *et al.* [7] as modified by FERGUSON was used. Interrupted conjugation was performed as described previously [2]. Chilled 4 hour cultures of the donor and recipient were mixed (1 : 10), then 1 ml of the mixture was passed through a Millipore HAWP 047 00 membrane filter. After washing with chilled saline the membrane was kept on chilled minimal medium until conjugation had been started. Conjugation began after placing the membranes onto prewarmed (37°C) 1.6% agar plates. Conjugation was stopped by placing the membrane into 10 ml chilled saline. Mating pairs were separated by treatment for 1 minute in a vortex-type apparatus (ČSAV 5001/H). The 1/1000 or higher dilutions of the original suspension were immediately seeded onto selective media.

Isolation of Hfr derivative was performed by the method of TAYLOR and ADELBERG [9]. For purification purposes the procedure was repeated first with, then without, U. V. irradiation.

Culture media. All media including the minimal medium of FALKOW *et al.* [10] with glucose at double concentration were prepared as described in references 1 and 2.

Nomenclature. According to the recommendations of DEMEREC *et al.* [11].

Table I

Parent strains used and isolates obtained in the present studies

Designation	Sexual polarity	Markers
W1177	F^-	*thi*$^-$ *thr*$^-$ *leu*$^-$ *lac*$^-$
W6	F^+	*met*$^-$
W1177/50	F^+	*thi*$^-$ *thr*$^-$ *leu*$^-$ *lac*$^-$
W1177/54	F^+	*thi*$^-$ *(thr*$^+$ *hsp · 4b) leu*$^-$ *lac*$^-$
D41012	F^+	*Nia*$^-$
UP40097	*F*$^-$	*Nia*$^-$
D4122	F^+	*Nia*$^-$ *(F · 4b)*
UP4204	Hfr	*Nia*$^-$
UP3009	F^-	*His*$^-$*-1, Ilv*$^-$*-1*
UP3042	F^-	*His*$^-$*-1, Thi*$^-$*-1, Thr*$^-$*-3, Trp*$^-$*-2, Met*$^-$*-9, Mal*$^-$*-1*
UP3075	F^-	*His*$^-$*-1, Thi*$^-$*-1, Thr*$^-$*-3, Trp*$^-$*-2, Met*$^-$*-9, Mal*$^-$*-1, Mtl*$^-$*-1*

Strains denoted with "W" corresponded to *E. coli* K-12 cultures originating from Dr. J. Lederberg and to their derivatives produced in our laboratory. Strains isolated and prepared in the WHO Escherichia Centre, State Serum Institute, Copenhagen, are designated with "D", those isolated and prepared in our laboratory are designated with "UP". The latter are *Sh. flexneri* strains; the first figure refers to their serotype (4 = 4b, 3 = 3).

Results

1. *Isolation of Hfr derivatives.* As mentioned in the introduction, our previous experiments with T5 phages and observations on the reciprocal transfer of *hsp* genes in *E. coli* K-12 and *Sh. flexneri* 4b [2] have indicated that the failure to isolate stable *Hfr* derivatives is due to a specific restriction of the host cell. In view of BOYER's experiment [12] solving an analogous problem in relation to *E. coli* K-12 and B, the method of this author was adapted for the preparation of the modified F factor.

Because of its *thr* dependence, *E. coli* K-12 F^- W1177 seemed suitable for the isolation of recombinants which have acquired *Sh. flexneri* 4b *hsp* gene with the assumption that *Shigella hsp* gene shows a close linkage with the *thr* locus. Thus F factor was transferred from strain W6 F^+ into this culture (W1177/50) then its F^- phenocopy was prepared by culturing on minimal agar for 48 hours. This F^- phenocopy was crossed with an F^+ *Sh. flexneri* 4b

(D41012) strain obtained in previous experiments. Double recombinants having a pattern of *Sh. flexneri* 4b with T5.K and T5.4b modified phages were sought among *thr*$^+$ recombinants obtained in the conjugation. One of such strains obtained by COLSON's quick method [3] was examined for restriction and modification characters (Table II).

Table II

Examination of E. coli K-12 hybrid W1177/54 assumed to carry Sh. flexneri 4b hsp gene by modified T5 phage lysate

Strains	Relative e.o.p. value*		
	T5.K**	T5.4b	T5.W1177/54
E. coli K-12 W1177	1	5.1×10^{-3}	7.8×10^{-3}
Sh. flexneri 4b D41012	2.3×10^{-2}	1	1
E. coli K-12 hybrid W1177/54	1.1×10^{-2}	1	1

* The number of plaques obtained was expressed in relative values by taking as 1 the number of plaques produced by the phage propagated as well as tested on the same strain.
** Abbreviation of modified phages. *E.g.* T5.K means that T5 phage was propagated on strain K-12.

Table II shows that, in respect to the restriction and modification of phage T5, hybrid W1177/54 behaves as *Sh. flexneri* 4b, in other words, it carries probably the *hsp* gene of the latter. It has been assumed that, due to this alteration, its F factor also acquired a Shigella-specific modification. In this manner the F factor of hybrid W1177/54 was transferred to another, F$^-$, mutant (UP40097) of *Sh. flexneri* 4b. In this experiment instead of the usual 0.5—1% F-transfer frequency [1] the value was as high as 20%. The F$^+$ derivative carrying a modified F factor (F.4b) was designated as D4122.

From strain D4122 F$^+$ it was easy to isolate several *Hfr* derivatives by the method of TAYLOR and ADELBERG [9]. One of these (UP4204) was exam-

Table III

Characteristics of Sh. flexneri 4b F$^+$ and Hfr derivatives carrying modified F factor

Characteristics		D4122 F$^+$	UP4204 *Hfr*
Effectiveness of acriflavine deletion		80%	< 0.5%
F-transfer frequency		38%	< 0.5%
Chromosomal transfer frequency	*met*$^+$		1.8×10^{-2}
	thr$^+$	10^{-5}	4.5×10^{-3}
	trp$^+$		2.1×10^{-4}

ined more thoroughly. In Table III the F and chromosome transfer capacity of *Hfr* UP4204 and the effect of acriflavine treatment are summarized. F^+ derivative D4122 served as a control.

The data indicate that the *Hfr* character of derivative UP4204: F transfer was absent in contrast to the 38% frequency shown by F^+ strain D4122 carrying the modified F factor. Acriflavine resistance referring to an integrate F factor and the result of conjugation experiments showing not only a higher but an oriented chromosome transfer also supported the *Hfr* character of the derivative UP4204.

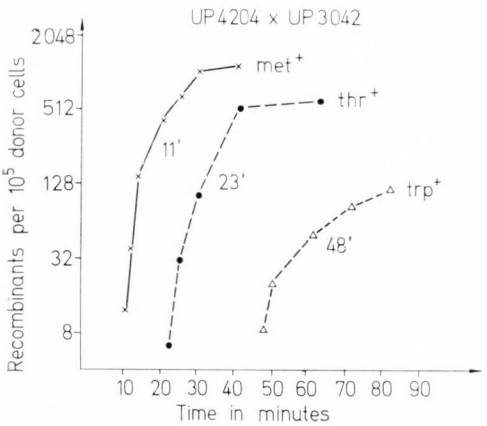

Fig. 1. Interrupted conjugation of *Hfr Sh. flexneri* 4b and F^- *Sh. flexneri* 3 strains. Time of entry and recombination kinetics of different markers

2. *Characterization of the Hfr derivative UP4204.* Before starting these experiments it was assumed that the length of chromosomes and the rate of conjugation are similar in *Sh. flexneri* and in the K-12 system and that the *Sh. flexneri* genes are allelic with K-12 genes identical in phenotype.

The lack of biochemical characterization of mutants isolated from our *Sh. flexneri* strain presented another difficulty. Accordingly, we chose markers the informations of which were localized in one cluster *(ilv, thr, trp)*. The most suitable mutants were derived from *Sh. flexneri* 3. This strain was used on the basis of the observation that it exerted no restriction to T5 phage propagated on strain 4b [2].

Fig. 1 presents the results of interrupted conjugation experiments with strains *Hfr* UP4204 and UP3042 in respect to 3 markers: *Thr*, *Met* and *Trp*. The curves were plotted from average values obtained in 3—5 different experiments. It is evident that as to recombination kinetics the results were the same as in the K-12 system. The time of entry for the 3 markers was: Met^+ at 11 minutes, Thr^+ at 23 minutes and Trp^+ at 48 minutes. The maximum difference in individual experiments amounted to ± 1 minute. Several attempts

have failed to transfer the *Mal*$^+$ marker into *Mal*$^-$ recipient UP3042. The transfer of *Mtl*$^+$ into *Mtl*$^-$ mutant UP3075 was also unsuccessful: in the examined *Met*$^+$ recombinant class neither of these markers could be detected. The time of entry of the *Ilv*$^+$ marker was at 7 minutes as determined by the use of mutant of UP3009.

The data obtained were drawn in a provisional chromosome map ranging from 0 to 90 minutes. As for the K-12 chromosome, the *thr* locus was placed arbitrarily at minute 90/0 (Fig. 2).

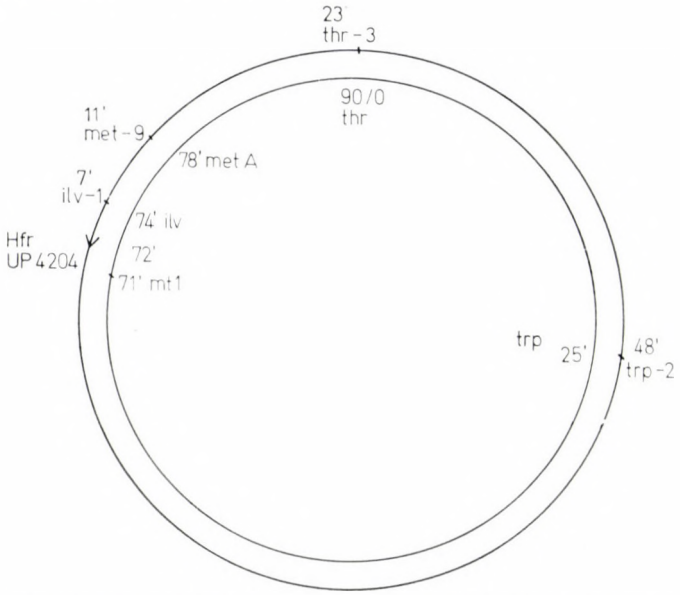

Fig. 2. Integration site and transfer direction for UP4204 *Hfr* strain and provisional chromosome map of *Sh. flexneri* on the basis of a few markers. The outer circle represents the provisional *Sh. flexneri* chromosome with localized markers. The inner circle depicts the localization of allelic markers in *E. coli* K-12 chromosome. The arrow shows the integration point of F factor in the *Hfr* strain and the direction of transfer

Fig. 2 shows the times of entry for the *Hfr* strain and the localization of identical phenotype markers on the map of K-12 chromosome. *Sh. flexneri ilv, thr* and *trp* genes are allelic with the corresponding K-12 genes. At the position of our *Met* marker (at 78 minutes) the K-12 chromosome shows the presence of the *metA* locus. In view of the standard error of the technique, no precise identification can be offered since the 77 minute point is occupied by *metB* and *metF* loci [13]. In view of the negative result for *Mtl* marker, the F integration point of the *Hfr* derivative may be placed between the *mtl* (71 minutes) and *ilv* (74 minutes) loci. On the basis of the 5 minute latency period elapsing until the appearance of the first marker in the K-12 system, the integration point can more precisely be determined as being at 72 minutes. The transfer order follows a clockwise direction: 0-*ilv-met-thr-trp*.

Discussion

In previous studies [2] we attempted to isolate a *Hfr* derivative from a *Sh. flexneri* 4b F^- strain carrying the K-12 F factor. This strain was shown to be a chromosome donor at a level of 10^{-7} (this value was 100 times lower than the frequency observed for the K-12 system). Attempts to isolate *Hfr* strain were at that time unsuccessful, although a temporarily increased transfer frequency was observed and conclusions could be drawn as to the variable integration of the F factor. This increased chromosomal transfer was unstable and accompanied by a detectable F transfer. Subsequent investigations into host-cell specific modification and restriction yielded positive results in relation with *E. coli* K-12 and *Shigella*. ARBER [14] has pointed out that reduction in phage titre in a foreign host cell can be estimated only qualitatively; nevertheless, it should be noted that the lowest degree of phage titre reduction was observed in *Sh. flexneri* 4b and that this very strain was the one to show most definitely the presence of F factor (phage sensitivity, antigenicity) and the ability to act as a chromosome donor. In the experiment *Sh. flexneri* 3 and *E. coli* other than K-12 were used as recipients; with these cultures restriction of phage T5 propagated on the donor was very slight or nil. These strains, chosen with more chance than the cultures used in the classical experiments of LURIA [15], allowed to study the donor properties of F^+ shigellae and of the possibility of isolating *Hfr* derivatives.

It is not clear why an F factor modification does not occur in the F^+ cell, why is it necessary to transfer by BOYER's method [12] the *hsp* gene from the foreign host cell to the K-12 F^+ cell, and why is the F plasmid modified in this case. The problem may probably be clarified by the experience that only a small proportion of phages propagated on foreign host-specific cells attain a new specific modification. For example, CHRISTENSEN [16] estimated for P2 phage the proportion of new modified phages at not more than 3%. However, as demonstrated in the present experiments, in this manner stable *Hfr* derivatives can successfully be isolated. Our isolate conforms in all respects to the criteria of *Hfr* strains (acriflavine resistance, absence of F transfer and oriented transfer of chromosomal markers). The supposed integration point of F factor on the provisional map at 72 minutes is a predilection site also in the K-12 chromosome [17]. With an identical clockwise transfer order the F factor in *Hfr* strains G10, J6 and R1 (K-12 derivatives) is integrated at this point.

The purpose of our further examinations will be the isolation of *Sh. flexneri Hfr* derivatives with integration sites different from that revealed in this study, to elucidate the possibility of integration at other positions in the *Sh. flexneri* chromosome. It may be assumed that positive results of these experiments would confirm the value of our method, which already seems to have

several advantages over the method using terminal selection for obtaining hybrid *Hfr* strains [18, 19].

Our data do not suffice for conclusions as to the chromosome map of *Sh. flexneri*. The few localized markers appear to be allelic with K-12 markers of identical phenotype. Conjugation frequency and recombination kinetics were identical in *Sh. flexneri* and in K-12 systems and the identity of the length of chromosomes may also be supposed. These data are not striking, as *S. typhi murium*, which is less closely related to *Escherichia* than is *Shigella*, shows minor chromosomal differences from K-12 The virtually longer chromosome in the former is due merely to technical causes [20].

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IMMUNOLOGICAL AND BIOCHEMICAL ACTIVITY OF ANTI-RIBOSOME IMMUNE SERUM*

By

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Summary. Immune sera against $MgCl_2$-treated guinea-pig liver ribosomal fraction were produced in rabbits and rats. The presence of anti-ribosome antibodies was demonstrated by liquid and gel precipitation as well as by ribosome-latex agglutination. Impurities were controlled by the immunodiffusion study of the immune sera produced against ribosomes and liver homogenate. Normal sera had also some precipitation activity. The precipitins were contained by the IgG fraction. The isolated anti-ribosome IgG fraction inhibited the functional activity of ribosomes in the *in vitro* amino acid incorporating system.

It has been known for years that the production of nucleic acid-specific antibodies may be induced by immunization with bacterial or mammalian ribosomes. Although the polynucleotides represent only non-immunogenic haptens, the ribosomal fraction is still immunogenic, since its RNA and protein form a natural complex. This seems to explain why antibodies are produced not only against the carrier ribosomal proteins but also against the ribosomal RNA [9, 22].

The produced anti-nucleoprotein antibodies are, however, heterogeneous both as to structure and specificity [9, 13, 14]. Accordingly, the present investigations were designed to elucidate the problems (a) whether the immunogenicity of the ribosomal fraction may be increased by purification; (b) whether there is a parallelism between the antibody activities demonstrable by various methods; and, (c) whether there is any demonstrable activity of anti-ribosome antibody in the *in vitro* amino acid incorporation system.

Materials and methods

1. *Preparation of guinea-pig liver homogenate and isolation of the ribosomal fraction.* Guinea-pigs weighing 300 to 400 g were killed, their liver was removed and homogenized in TMSK solution (0.25 *M* sucrose, 5 *mM* $MgCl_2$, 50 *mM* 2-mercaptoethanol, 0.02 *M* tris buffer, pH 7.8) in a glass homogenizer fitted with a Teflon pestle. The nucleus and cell debris were sedimented at 1,000 *g*. Part of this centrifuged supernatant was stored at —20°C and used as homogenate in the experiments. The major part was centrifuged at 15,000 *g* for 15 minutes. The pellet was discarded and the supernatant used for isolating microsomes. These were sedimented at 105,000 *g* for 60 minutes and treated with 1% sodium deoxycholate; the ribosomes were centrifuged at 105,000 *g* for 3 hours. For further purification, precipitation with $MgCl_2$

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was used. The appropriate $MgCl_2$ concentration was adjusted by dialysis. The ribosomal fraction was stored at −20°C.

2. *Preparation of immune sera.* Chinchilla rabbits weighing 2.5 to 3 kg were immunized with guinea-pig liver homogenate (with 40 mg of protein per dose) or with 12 mg of ribosomes from guinea-pig liver, 4 times in all, at intervals of 14 days. The antigen was injected into the four paws and into the muscle at two sites. Seven rats were immunized with 6 mg of guinea-pig liver ribosomes according to the same schedule. Two rats were immunized with the washed precipitate of anti-ribosome sera produced in rabbits and of ribosomes (0.2 mg protein + 0.05 mg RNA), three times in all. All the antigens were mixed into complete Freund adjuvant. Ten days after the last injection the animals were exsanguinated and the sera were stored at −20°C.

3. *Quantitative precipitation of immune sera.* PANIJEL's [18] method was adopted, with a few modifications. 0.5 ml quantities of sera of various dilutions were mixed with an equal part of ribosomes diluted to various extents. The precipitates formed during incubation at +4°C for 17 hours were centrifuged, washed once with TMSK, and twice with TSK solution (TMSK solution without sucrose). The sucrose-free precipitate was reprecipitated with 25% TCA, left to stand at +4°C for 15 minutes and washed three times with 10% TCA. The precipitate was hydrolysed with 0.3 *N* KOH for 60 minutes at 37°C. Finally, the hydrolysate was used for the determination of the protein and RNA contents.

The protein content was determined according to LOWRY *et al.* [12].

RNA determination. The hydrolysate was adjusted to pH 2 by adding PCA. After having kept it in thawing ice for 15 minutes, the precipitate was centrifuged and washed twice with 0.2 *N* PCA. The extinction of the combined supernatants was measured at 260 mμ. On the basis of the ribose content determined by the orcinol colour reaction [4] and on that of the extinction, the multiplying factor was determined in an informative experiment. Accordingly, multiplying the optical density by 28.3, the RNA content of the various supernatants is obtained in μg.

4. *In immunodiffusion and immunoelectrophoretic studies* OUCHTERLONY's [17] linear double diffusion method and SCHEIDEGGER's [25] immunoelectrophoresis micromethod were adopted.

5. *Preparation of rabbit IgG fraction.* IgG fraction was prepared by DEAE cellulose column chromatography both from normal and from anti-ribosome immune rabbit sera [27].

6. *Ribosome-latex agglutination.* Latex suspensions of various concentrations were prepared (3.5% veronal buffer, pH 7.0) and various amounts of ribosomes were added to the dilutions. The dilution consisting of equal amounts of 1% latex and of ribosomes, with 4 mg of protein/ml, furnished the best reagent. This suspension was stabilized by 1/20 vol. 2% HSA-solution after incubation at +4°C for 3 hours and could be used for a week. An equal number of drops of the immune serum dilutions and of the ribosome-latex suspension were mixed on slides, placed into a wet chamber and the reaction was evaluated after 20 minutes.

7. *Histological study.* Sections from the liver, lung and kidneys of rabbits immunized with ribosomes were stained with haematoxylin-eosin.

8. *In vitro amino acid incorporation system.* The ribosomes required for the cell-free system were prepared according to KORNER [10], the pH 5 fraction according to HOAGLAND *et al.* [8] (for details see the paper by HIDVÉGI *et al.* [7]). The system comprised 2 mg RNP/ml of ribosomes and pH 5 fraction with 1.7 mg/ml of protein. Uniformly labelled ^{14}C-valine (91.5 mCi/*mM*, UVVVR, Prague) at a concentration of 2 μCi/ml was used for incorporation. The ribosomes were preincubated with the appropriate IgG fraction at 0°C for 45 minutes. After completing the system it was incubated at 37°C for a further 60 minutes. The tubes were precipitated with TCA. To compare the specific radioactivity of labelled proteins, the same amount of IgG protein was added to the control systems, which contained buffer instead of IgG during the incubation. The labelled protein fraction was isolated according to SIEKEVITZ [26] and dissolved in formic acid for protein content determination [12]. Aliquots were transferred to aluminium planchets, dried, and the radioactivity of the samples was determined in a Friesecke—Hoepfner 2π gas flow counter.

Results

1. *The importance of purity in the immunogenicity of ribosomes.* Rabbits were immunized with guinea-pig liver homogenate, with crude ribosomes and with ribosomes precipitated three times with 50 *mM* $MgCl_2$. The precipitin

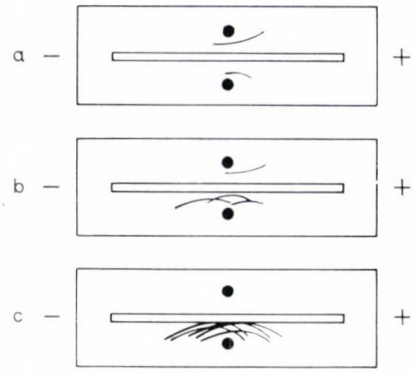

Fig. 1. Immunoelectrophoretic pattern of anti-ribosome immune sera. Key: on each slide the upper reservoir contained ribosomal fraction; the bottom reservoir, liver homogenate. a + b = = rabbit immune sera to guinea-pig liver ribosomes treated with $MgCl_2$; c = rabbit immune serum to guinea-pig liver homogenate

Table I

Effect of precipitation with $MgCl_2$ on the immunogenic property of guinea-pig liver ribosomes

Immunizing antigen	Immune serum	Number of precipitation bands	
		with ribosomes	with liver-homogenate
Guinea-pig liver homogenate	12	—	5—9
Guinea-pig liver ribosomal fraction	2	—	2
	1	—	3
	2		4
	1		5
Ribosomal fraction precipitated three times with $MgCl_2$	1	1	1
	1	1	2
	1	1	3
	2	—	2

content of the immune sera was investigated in agar-gel by double diffusion. The number of precipitation bands obtained with guinea-pig liver homogenate was studied. As it appears from Table I, immunization with guinea-pig liver homogenate did not elicit a demonstrable anti-ribosome antibody production. Immunization with crude ribosomal fraction also failed to induce the production of anti-ribosome precipitin. On the other hand, ribosome precipitins were found in the majority of rabbits immunized with $MgCl_2$-treated ribosomes. However, even these latter immune sera gave more precipitation arches with liver homogenate. There was but a single immune serum which gave only the ribosome precipitation arch with liver homogenate. (This serum

was used in most of the experiments.) The reaction of specific antibodies with ribosomes or liver homogenate could be demonstrated also by immunoelectrophoresis (Fig. 1).

2. *Quantitative precipitation of ribosomes and ribosome-specific immune serum.* The diffusion of ribosomes was slight in agar. Accordingly, a fluid me-

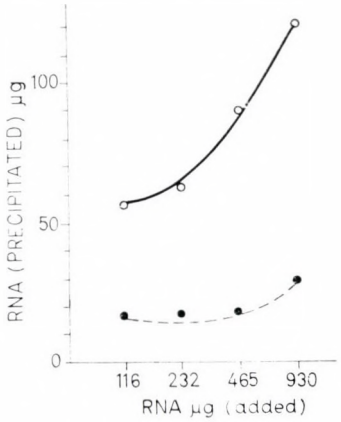

Fig. 2. Quantitative precipitation of guinea-pig liver microsomes. RNA content of ribosomes added to the tubes is plotted on the abscissa; RNA content of precipitated ribosomes, on the ordinate. Key: full line = precipitation with anti-ribosome rabbit serum; dotted line = reaction with normal rabbit serum

dium appeared to be more convenient for precipitation tests (Fig. 2). Normal sera formed hardly any, while immunized rabbit sera gave rise to definite precipitates. When increasing the amount of antigen, the amount of precipitated ribosomes also increased.

Of the factors which may influence the precipitation reaction, the role of Mg^{++} concentration was studied (Table II). While under the conventional 5 *mM* Mg^{++} concentration 1 ml of an immune rabbit serum precipitated 125 μg ribosomal RNA, the same serum precipitated 110 μg RNA from ribosomes dialysed against Mg-free TSK solution. Accordingly, the precipitation system was hardly affected by changes in the concentration of Mg^{++}.

Table II

Influence of Mg^{++} concentration on the precipitation of guinea-pig liver ribosomal fraction and of specific immune serum

	Mg^{++} concentration	RNA precipitated by 1 ml serum
Anti-ribosome	5.0 *mM*	125 μg
Rabbit serum "B"	< 0.5 *mM*	110 μg

The role of heat-labile factors in precipitation was also studied. The precipitation activity of immune sera stored at −20°C, before and after inactivation at +56°C for 30 minutes, was compared (Fig. 3). Inactivated serum proved less effective, as it precipitated less ribosomes than serum not subjected to heat treatment.

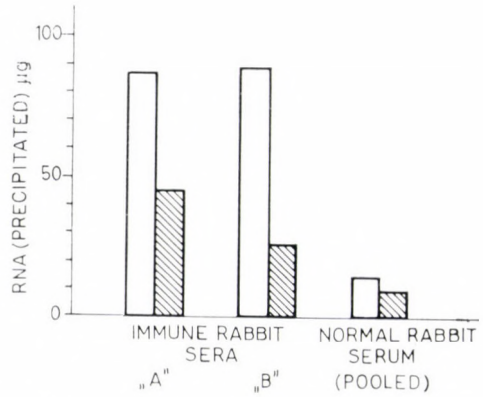

Fig. 3. Effect of heat treatment (56°C for 30 minutes) on the precipitation activity of anti-ribosome immune sera. Open bars = before treatment; hatched bars = after treatment

Table III

Comparison of anti-ribosome immune sera by ribosome-latex agglutination and precipitation reaction in agar-gel

Serum	Ribosome-latex agglutination	Ribosome precipitation in agar-gel
aR horse serum	+	−
normal horse serum	−	−
aR rabbit serum "A"	−	+
aR rabbit serum "B"	−	+
aR rabbit serum 1	−	−
aR rabbit serum 2	+	−
aR rabbit serum 3	+	+
normal rabbit serum	−	−
aR rat serum "O"	−	+
aR rat serum "1"	+	+
aR rat serum 3	+	−
aR rat serum 4	−	−
aR rat serum 5	−	−
aR rat serum 6	+	−
aR rat serum 7	+	−
normal rat serum	−	−

Key: aR = anti-ribosome.

3. *Correlation between activity in ribosome-latex agglutination and precipitin content.* The ribosome-latex and anti-ribosome precipitin activities in the various rat and rabbit immune sera were compared (Table III): the actions did not show any strict parallelism. Certain immune sera proved to be effective in both systems, others only according to one of the methods. Antibacterial ribosome horse serum agglutinated ribosome-latex, but did not precipitate guinea-pig liver ribosomes to a measurable extent.

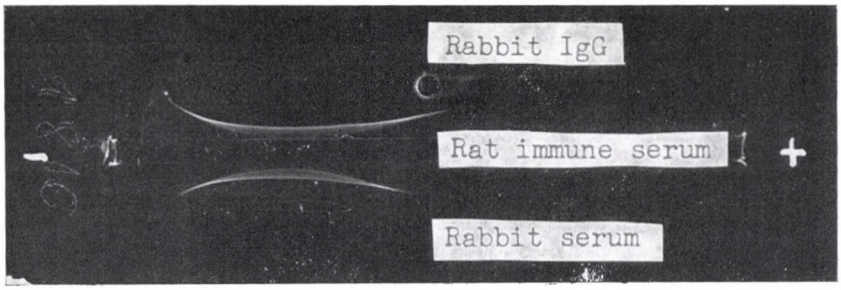

Fig. 4. Immunoelectrophoretic picture of serum from rats immunized with the washed complex of guinea-pig liver ribosomes + anti-ribosome rabbit serum

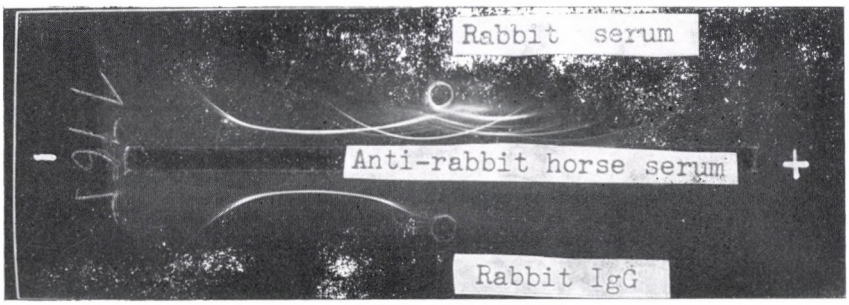

Fig. 5. Immunoelectrophoretic control of the purity of isolated rabbit IgG. (Horse serum used: an immune serum to rabbit whole serum, HUMAN)

4. *Determination of the serum protein responsible for ribosome precipitation.* Sera from rats immunized with washed precipitate of inactivated immune serum and of ribosomes were studied to reveal which rabbit serum protein was responsible for the precipitation of ribosomes. As seen in Fig. 4, this immune serum gave a single precipitation arch with whole rabbit serum, and corresponded to the arch of IgG.

5. *Histological study of the organs of immunized animals.* Some vacuolar degeneration was revealed in the liver of nearly every animal and an accumulation of eosinophilic cells could be observed in the kidneys and lungs.

6. *Effect of anti-ribosome IgG fraction on protein synthesis in vitro.* After having established the role of IgG immune sera in the precipitation of ribosomes, IgG fraction was isolated by DEAE cellulose column chromatography. Its purity was checked by immunoelectrophoresis (Fig. 5). To the *in vitro*

amino acid incorporation system, 1.29 mg/ml IgG fraction (final concentration) was added. As established in an informative experiment, this amount did not induce precipitate formation during 17 hours. Normal rabbit IgG also inhibited amino acid incorporation to a certain extent. However, the inhibitory effect of immune IgG appreciably exceeded that of normal IgG. Accordingly, ribosome-antibodies are able to inhibit the function of ribosomes.

Table IV

Effect of normal and anti-ribosome rabbit IgG fraction on ^{14}C-valine incorporation by guinea-pig liver microsomes in vitro

	IgG-free control	In the presence of normal IgG (1.29 mg/ml)	IgG-free control	In the presence of immune IgG (1.29 mg/ml)
I	3360	2960	3200	1560
II	3340	2880	3420	1486
Mean	3350	2920	3310	1523
Rate of inhibition		−13%		−46%

Discussion

It has been shown that the purification of the ribosomal fraction plays a decisive role in obtaining satisfactorily immunizing antigens. Precipitation with Mg seemed to be a suitable method for removing impurities. Experiments had failed to decide whether the contaminating components consisted of proteins synthesized on or adsorbed onto the surface of ribosomes or were contaminations deriving from imperfect isolation. Precipitation with anti-liver homogenate immune serum is a generally accepted method to check the immunochemical purity of the subcellular fractions [15]. We are, however, of the opinion that the precipitation of the anti-ribosome immune serum with liver-homogenate is a more reliable procedure checking the purity of the ribosomal fraction and it allows to demonstrate much smaller amounts of impurities. The antibodies produced against these impurities may be decisive when studying biochemical systems [16]. The immune serum that precipitated only ribosomes led to a demonstrable precipitation also in immunoelectrophoresis. The site of the precipitation arch corresponded to the electrophoretic location of the ribosomal fraction [24].

The ribosome-latex agglutination which gives a positive result not only with immune sera but also with sera from patients with certain diseases (e.g. liver disease, SLE) probably demonstrates the presence of RNA antibodies [23, 28]. Thus in our ribosome-latex agglutination experiments we must have

measured the reaction of RNA and of its specific antibody. The positive reaction obtained by horse serum immunized with bacterial ribosomes also supports this assumption. According to BARBU and PANIJEL [1], the antibodies to RNA should be held responsible for the cross reaction between bacterial and mammalian ribosomes. On the other hand, the protein components probably also interfere with the precipitation reaction.

Heat-labile factors proved to be of importance in the precipitation reaction and the complement factors still present in the immune sera might also interfere with the reaction and the action of further factors cannot be excluded.

As to the serum fraction or, more precisely, the immunoglobulin responsible for the precipitation, the fact that precipitation had been accomplished also in an almost Mg^{++}-free medium, excluded the possibility of some non-specific aggregation. The presence of IgG in the precipitate could be demonstrated in the serum of rats immunized with the washed precipitate of the ribosome-anti-ribosome precipitin. However, without controlling the presence of antibodies to heavy chains, one cannot declare that activity is found in the IgG fraction only [2, 24]. In all probability, the IgM fraction also has a share in the reaction [3, 5, 6]. The histologically observed lesions might have accounted for the auto-antibody nature of the produced antibodies. DODD [5, 6] published data on the anti-RNA auto-antibodies in the 19S fraction. It is questionable whether anti-ribosome activity present in the normal serum is due to normal auto-antibodies [19, 20].

Only sporadic data are available concerning the effect of anti-ribosome serum on the cell-free amino acid incorporation system. Using a high quantity of whole serum, MEYER-BERTENRATH [15] succeeded in appreciably reducing ^{3}H-phenylalanine incorporation by rat liver ribosomes. Normal serum failed to give a reaction in this system. This suggests the relative resistance to RNase of the system, since the RNase content of the whole serum *in se* also reduces amino acid incorporation. PANIJEL [21] determined two contrasting effects by ^{14}C-phenylalanine incorporation. Dispersed and washed ribosome-antibody precipitates showed an appreciably reduced level of biosynthesis. The soluble antigen-antibody complex seemed to be more active than the ribosomes themselves. Applying a different antigen-antibody ratio, and in highly diluted IgG solution, we failed to demonstrate an increase in biosynthesis. Soluble antigen-antibody complexes inhibited the functional activity of ribosomes in our experiments and TANENBAUM [29] also observed inhibition for various RNA-reactive antibodies.

The IgG fractions used also had some RNase activity, of the same degree both in normal and in immune IgG fractions. This fact has confirmed our statement in that the inhibitory effect of the immune IgG fraction on the function of ribosomes, at least the action exceeding the effect of the normal IgG fraction, may be attributed to the presence of specific antibodies.

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GENETIC MAPPING OF RHIZOBIOPHAGE 16—3

By

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Summary. The partial genetic map of the Rhizobium phage 16—3 was constructed by complementation and two or three point crosses. Temperature sensitive *(ts)*, thermo inducible *(ti)*, plaque morphological *(N2; C)*, host range *(h)* and altered phage antigen *(Ant⁻)* mutants were isolated and used. The mapped genes were found in the sequence of *N2-C-ts2-ts6-ts4-h-ts5--Ant.*

The bacteriophage 16—3 of *Rh. meliloti* was isolated and characterized as temperate by Szende and Ördögh [1], its nucleic acid was studied by Sik [2]. In order to study this phage genetically, some basic requirements of the bacterium and phage growth had to be known and the system for phage crosses had to be worked out.

Mutants were isolated with altered plaque morphology and antigen character as well as temperature sensitive [3, 4], thermo inducible [5, 6, 7] and host range [8] ones.

By crossing these and counting the recombinants under different selective conditions it was possible to construct the linear genetic map of this phage.

Materials and methods

Abbreviations. m.o.i. = multiplicity of infection; E.O.P. = efficiency of plating; P.F.U. = = plaque forming unit; K = velocity constant of antiphage serum [9]; Rh. 41 = *Rhizobium meliloti* 41; his = histidine; Str^r = streptomycin resistant; ph^r = phage resistant; mi = minute plaques; s = small plaques.

Bacteriophages. 16—3 wild type phage, forms turbid plaques 3—5 mm in diameter at 25—37°C. Its lysogenisation frequency is about 30% [1]. N2 — nitrous acid induced plaque morphology mutant of 16—3, forming sharp, dark centred turbid plaques. K — clear plaque mutant with a lysogenisation frequency of about 10^{-5}.

Bacteria. Rhizobium meliloti 41 (Rh. 41) — wild type bacterium [1]. It requires biotin for growth. A-1 — histidine requiring mutant of Rh. 41, induced by nitrosoguanidine. G-1 — streptomycin resistant and phage resistant spontaneous derivative of A-1 (Rh. 41 *his⁻*, Str^r, ph^r). This was used as host for host range mutants.

Media. MRP. NaCl, 1 g; NH_4Cl, 3 g; glucose, 2 g; KH_2PO_4, 0.7 g; $Mg^{\cdot\cdot}$, 10^{-3} *M*; $Ca^{\cdot\cdot}$, 10^{-3} *M*; 0.2 *M* tris (hydroxymethyl)-aminomethane, 125 ml; 0.1 *n* HCl, 200 ml in one litre. pH: 7.2. BRP—MRP supplemented with biotin 10^{-5} g/litre; YRP—MRP supplemented with yeast extract 1 g/litre; YTB — tryptone, 10 g; yeast extract, 1 g; NaCl, 5 g; $Ca^{\cdot\cdot}$, 10^{-3} *M*; $Mg^{\cdot\cdot}$, 10^{-3} *M* in one litre. pH was adjusted with NaOH to 7.

Mutagenesis. Nitrous acid treatment [10]. 0.5 ml of 16—3 or N2 phage (titre, 10^8/ml); 0.5 ml of 0.2 *M* acetate buffer (pH 4.5) and 0.5 ml $NaNO_2$ solution (1.5; 0.75; 0.375 *M*) were mixed at 30°C and after 20 minutes the mixture was diluted into MRP medium and plated. The survival phage fractions were 10^{-4}; 10^{-3} and 10^{-2}, respectively

Nitrosoguanidine treatment. The lysogenic form of Rh. 41 was induced by mitomycin C (5 μg/ml at 30°C [11]) in YRP medium containing 5 μg/ml nitrosoguanidine. After 4 hours the lytic yield was plated.

Temperature sensitive mutants. The optimum temperature for both bacterium and phage was 30°C, but they still grew at 37°C. The nitrosoguanidine or nitrous acid treated phage population was plated at 32°C, and after 14 hours of incubation the minute plaques were picked out and tested at 25°C and 36°C, respectively. Those unable to grow at 36°C were qualified as "*ts*" (Table I).

Table I

Some characteristics of the ts mutants

Mutant	Mutagen	Origin	Frequency of ts^+ revertants at 36°C	Plaque size	Average burst size
ts2	NTG	16—3	$< 10^{-8}$	normal	40
ts3	N	N2	$< 10^{-7}$	normal	20—25
ts4	N	N2	$< 10^{-7}$	normal	20—25
ts5	N	N2	$< 10^{-8}$	small	10
ts6	N	N2	$< 10^{-6}$	normal	20—25

NTG = nitrosoguanidine
N = nitrous acid

Heat inducible mutants. Mutagen treated phages at 32°C or 36°C and clear plaques were picked and tested at 25°C and 36°C. Those giving clear plaques at 36°C but turbid at 25°C were characterized as heat inducible mutants.

Isolation of host range mutants. From the histidine requiring and streptomycin resistant bacterium (*A-1 Str^r*) 40 phage resistant mutants were isolated for different clear plaque phages. Host range mutants of the K phage (Kh) were isolated from 6 of these resistant bacteria, with a frequency of about 10^{-8} (Table II).

Table II

Characteristics of Kh

Phage	m.o.i.	Adsorption %		Plaque size		E.O.P.	
		on Rh. 41	on G—1	on Rh. 41	on G—1	on Rh. 41	on G—1
Kh	17—0.5	95	20	mi	varying	1	0.2—0.4
K	15—0.4	95	0	normal	—	1	—

One-step growth of the phage. The recipient bacteria A-1 (5×10^8/ml) were infected with a multiplicity of 0.01—0.1 in BRP medium. After 20—60 minutes adsorption, the free phages were removed by centrifugation or antiphage serum (K-1) and infective centres were diluted 10^4 times into YTB medium. Samples of 0.1 ml were plated for plaque forming units [12]. In the case of wild type recipient bacterium (Rh. 41) the cells were infected in YTB or YRP medium and adsorption time was 15—20 minutes at 30°C. The latent period was 90 minutes at 30°C, the average burst size of phage was 40—70.

Isolation of lysogens containing ti prophages. 10^7—10^8 phages were spotted onto a plate inoculated with Rh. 41 bacterium and incubated at 25°C. After the appearance of the lytic spot, bacteria were picked from the centre of the spot and suspended in BRP containing phage antiserum [K = 8]. A few hours later the bacteria were plated for single colonies. These were tested for phage producing ability at 25°C, 30°C, 32°C, 36°C, and for immunity.

Antiphage serum was prepared from rabbits injected with phage 16—3, according to the method described [9].

Results

Ca⁺⁺ requirement of phage multiplication. The role of Ca^{++} was studied under the conditions of single-step growth. To avoid lysogenisation, the thermo-inducible, clear plaque double mutant phage *ti4C*$_1$ was used in this experiment. No phage production could be detected without Ca^{++} as shown in Fig. 1. Multiplication started after the addition of 10^{-3} *M* Ca^{++}. The same effect was found using the A-1 mutant as host. As expected, the histidine, required by this host bacterium, was indispensable for the growth of phages.

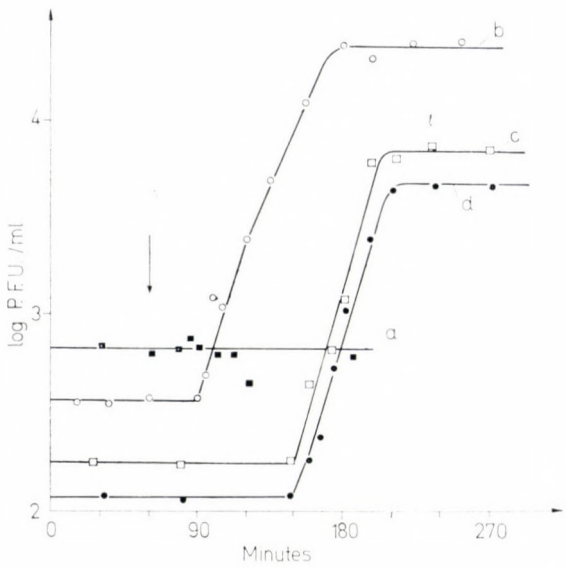

Fig. 1. One step growth of phage 16—3. Recipient bacteria. a = Rh. 41 or A-1, without Ca^{++}; b = Rh. 41, normal one step growth; c = Rh. 41, after 60 minutes Ca^{++} was added; d = A-1 (Rh. 41 *his*⁻), after 60 minutes diluted into complete medium

Thermo-induction of lysogenic bacteria containing ti prophages. From the isolated lysogens carrying *ti* phages, three were chosen to test inducibility. The bacteria were grown to a cell number of 2×10^8/ml in YTB or YRP medium without Ca^{++}. After induction at 36°C for 10 or 20 minutes the culture was cooled to 30°C for the multiplication of phages. As shown in Fig. 2, the burst started 90 minutes after the induction. Average burst size was 40 for the *ti3* and *ti4* phages, and 22 for the *ti5* phage. The increased temperature had no effect on the lysogen carrying wild type phage. Ca^{++} was not necessary for the replication and maturation of heat induced phages.

Comparing the *ti* mutants at different temperatures, differences were found in thermosensitivity of their repressor. As shown in Table III, the plaques formed by mutant *ti4* were already clear at 32°C.

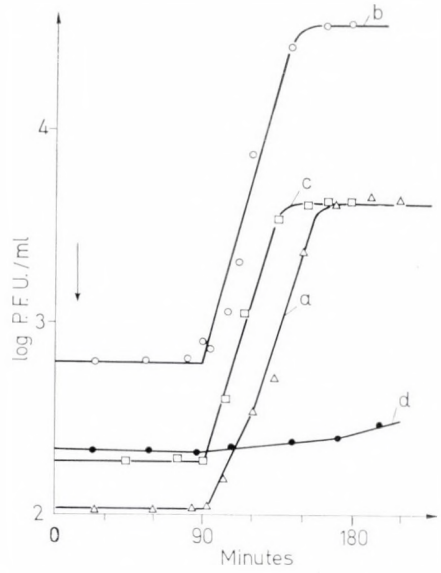

Fig. 2. Thermo-induction of lysogenic bacteria. a = Rh. 41 *(ti3)*; b = Rh. 41 *(ti4)*; c = Rh. 41 *(ti5)*; d = Rh. 41 *(C+)*; ↓ = cooled to 30°C

Table III

Plaque form of ti mutants at different temperatures

Mutant	Plaques at			
	25°C	30°C	32°C	36°C
ti3	t	t	t	C
ti4	t	t	C	C
ti5	t	t	t	C

t = turbid, lysogenisation
C = clear, no lysogenisation

Complementation of ts, ti and clear plaque mutants. Complementation was carried out by spotting pairs of mutants onto a plate with a lawn of Rh. 41 as host [13, 14, 3]. The *ts* mutants were tested with each other at 36°C, the clear plaque and *ti* mutants with the phage K. The complementation groups are shown in Table IV.

Phage crosses. The phage stocks were mixed and m. o. i. 5 was applied with each mutant. The host was A-1. Adsorption took place in BRP. The unadsorbed phages were removed by two centrifugations. The infected cells were diluted 10^2—10^3 times into YTB medium at 30°C. 2.5—3 single growth cycles were allowed, then $CHCl_3$ was added and the progeny analysed.

Table IV

Complementation groups

Group				
1	2	3	4	5
ts2	*ts3*	*ts5*	*ts6*	*ti3*
	ts4			*ti4*
				ti5
				K and all the tested clear mutants

(a) Mapping of ts points. Plating was made at 30°C and at 36°C. The frequency of recombinants was calculated as

$$R = \frac{2 \times \text{titre at } 36°C}{\text{titre at } 30°C}$$

(b) Mapping of the host range (h) character. The Kh mutant phage was crossed to the two distal *ts* sites. The *ts2C* and *ts5C* mutant phages were partners in these crosses. The number of turbid plaques on the mixed indicator at 36°C corresponded to the h^+ ts^+ recombinants. The percentage of recombinants was calculated as twice the number of h^+ ts^+ phages divided by the number of plaque forming units at 30°C.

It is also possible to differentiate *h* and h^+ phages on Rh. 41 host because "h" mutants are forming minute *(mi)* plaques (see Table V).

(c) Mapping of the clear (C) character. The clear mutant site was crossed to the known end points of the chromosomal segment determined with our *ts* mutants. The crosses were made with the markers in both the cis and the trans positions. In the case of cis the clear plaques at 36°C are corresponding to the recombinants ts^+C, whereas in trans crossings to the turbid plaques ts^+C^+.

We can also distinguish recombinants at 30°C based on the size and turbidity of plaques since *ts5* is forming small *(s)* plaques. The recombination frequency between *C* and *h* could be determined by plaque size. The hC^+ phages form minute, whereas h^+C recombinants normal plaques on 41 or mixed *(41 + G—1)* indicator (Table V).

(d) The position of serum character (Ant). One of our mutants was serologically different from the wild type 16—3 or other mutants. The antiphage serum from rabbits injected with phage 16—3 gave a value of K = 300 with Ant^+ mutants and K = 40 with the Ant^- one. The recombinants can be classified using a serum concentration, when 30-minute treatment resulted in a titre of 10^{-5} survival with Ant^+ phages, but only in 10^{-1} with the Ant^- mutants.

Table V

Results of the phage crosses

Mapping of mutant sites		Crossing		Observed recombinants	Frequency of recombinants % R
ts points			*ts2* +		
	1	*ts2*×*ts5*		*ts*⁺	5.5
			+ *ts5*		
	2	*ts2*×*ts4*		*ts*⁺	4.7
	3	*ts2*×*ts6*		*ts*⁺	3.3
	4	*ts5*×*ts4*		*ts*⁺	1.15
	5	*ts5*×*ts6*		*ts*⁺	1.7
	6	*ts4*×*ts6*		*ts*⁺	0.7
h gene					
ts2 — *h*	7	*Cts2h*⁺×*Cts2*⁺*h*	*C ts2* +	*ts*⁺*h*⁺	5.4
			C + *h*		
3 point	8	*C*⁺*ts2h*⁺×*Cts2*⁺*h*		*ts*⁺*h*⁺	
		mi⁺ *mi*		*mi*⁺	
ts5 — *h*	9	*Cts5h*⁺×*Cts5*⁺*h*		*ts*⁺*h*⁺ at 36°	0.3
				s⁺ *h*⁺ at 25°	
3 point	10	*C*⁺*ts5h*⁺×*Cts5*⁺*h*		*ts*⁺*h*⁺ at 36°	
		mi⁺ *mi*		*s*⁺ *h*⁺ at 25°	
				mi⁺	
C gene					
ts2 — *C*					
cis	11	*ts2C*×*ts2*⁺*C*⁺	*C ts2*	*ts*⁺*C*	18
trans	12	*ts2C*⁺×*ts2*⁺*C*	+ +	*ts*⁺*C*⁺	
3 point	8	*ts2C*⁺*h*⁺×*ts2*⁺*Ch*		*ts*⁺*C*⁺	
ts5 — *C*					
cis	13	*ts5C*×*ts5*⁺*C*⁺		*ts*⁺*C* at 36°	21.5
				s⁺ *C* at 25°	
			C +	*s* *C*⁺ at 25°	
trans	14	*ts5C*⁺×*ts5*⁺*C*		*ts*⁺*C*⁺ at 36°	
				s⁺ *C*⁺ at 25°	
				s *C* at 25°	
3 point	10	*ts5C*⁺*h*⁺×*ts5*⁺*Ch*	+ *ts5(s)*	*ts*⁺*C*⁺ at 36°	
				s⁺ *C*⁺ at 25°	
C — *h*					
	15	*Ch*×*C*⁺*h*⁺	*C h*(mi)	*C*⁺*mi*	21.5
		Cmi×*C*⁺*mi*⁺		*C mi*⁺	
			+ +		
3 point	8	*C*⁺*ts2h*⁺×*Cts2*⁺*h*		*C*⁺*mi*	
		mi⁺ *mi*		*C mi*⁺	
3 point	10	*C*⁺*ts5h*⁺×*Cts5*⁺*h*		*C*⁺*mi*	
		mi⁺ *mi*		*C mi*⁺	

Mapping of mutant sites		Crossing		Obrerved recombinants	Frequency of recombinants % R
N2 point					
N2 — C	16				
cis		*N2C* × *N2⁺C⁺*	*N2 C* / + +	*N2C⁺*	
			N2 + / + *C*		1
trans	17	*N2C⁺* × *N2⁺C*		*N2⁺C⁺*	

Phage mutant genotypes by genetic symbols: Kh = *Cts2⁺h* (cross 7,8), *Cts5⁺h* (cross 9, 10), *Ch* or *Cmi* (cross 15); K = *ts2⁺C* (cross 11), *ts5⁺C* (cross 14), *N2⁺C* (cross 17). Frequency data represent average from 3—4 crosses except for 1 and 4 where they represent average from 10 crosses.

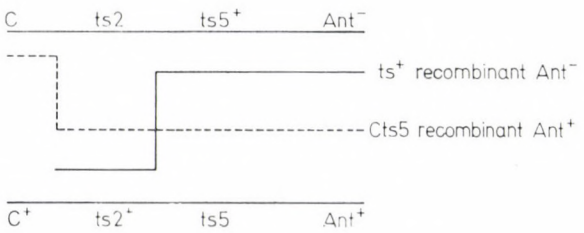

Fig. 3. Mapping of *Ant* character

The *ts2 Ant⁻* was crossed with *ts5 Ant⁺*, *ts6 Ant⁺* and *ts4 Ant⁺* phages and *ts⁺* recombinants were propagated. The *ts⁺* yield as population was tested for antigen *(Ant)* character. These recombinants were serologically altered *(Ant⁻)* phages. This was confirmed by checking single *ts⁺* recombinant plaques. All *ts⁺* recombinants were showing altered antigen character *(Ant⁻)*. From this it was concluded that the *Ant* region has to be situated to the right of point *ts2* in our map (Fig. 3).

From the crossing of *ts2C Ant⁻* × *ts5C⁺ Ant⁺* shown in Fig. 3, it was possible to isolate at 36°C the *ts⁺* recombinants. These were all showing altered antigen character *(Ant⁻)*. At 30°C we isolated the small clear recombinants. In view of their small size, these were *ts5* and *Ant⁺*. To check the genotype, these recombinants were back-crossed to *ts2* and *ts5* mutants; this has confirmed that the real genotype was *C ts2⁺ ts5 Ant⁺*. Thus, the *Ant* region is also situated to the right of point *ts5*.

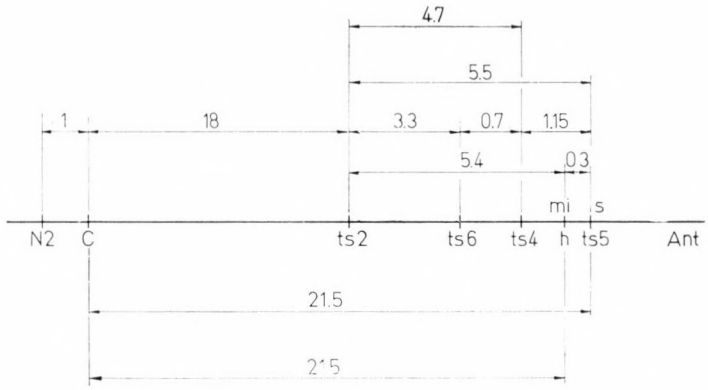

Fig. 4. Order of mutant points on 16—3 chromosome

(e) Mapping of N2. The cross was made in both cis and trans positions. *N2* was mapped to *C* gene only. There were detectable *N2* recombinants in cis and 16—3 ones in trans (Table V).

Discussion

The first part of our experiments was concerned with the conditions necessary for the crosses. The conditions of one-step growth, adsorption, and the period of phage maturation were studied.

Similarly as for the infection of other well-described phages in this case, too, Ca^{++} was found necessary. The lack of Ca^{++} did not influence the adsorption of phages and its presence was not necessary in thermo-induction. Since phage production could not be detected when infection was carried out in the absence of Ca^{++}, it was concluded that Ca^{++} was involved in the injection of phage DNA, probably in the contraction of the tail sheath [15].

From the frequency of recombinants summarized in Table V, we have constructed the linear map of the chromosome of Rhizobium phage 16—3.

The possibility to detect ts^+ recombinants allowed the mapping of *ts* sites. After carrying out all the possible crossings between pairs of these mutants we were able to construct the unambiguous order *ts2-ts6-ts4-ts5* which covers a region of map length 5.5 with strict additivity (crosses 1—6 in Table V).

In the mapping of our other mutants, the extreme loci of the *ts* region *(ts2, ts5)* were used as relative points. In this way the *ts* parents could be eliminated from the progeny at higher temperatures. The crosses with mutants in the *C* gene resulted in a higher frequency of recombination with *ts5* than with *ts2* (crosses 11—14, 8, 10) whereas the *h* locus was found close to the *ts5* point (crosses 7—10 in Table V). The order was confirmed by the high frequency of

recombinants in crosses between *C* and *h*, making use of the distinct minute character of plaques of *h* mutants (crosses 8, 10, 15 in Table V).

The mutant displaying a changed antigenic character *(Ant⁻)* was unusual. The possibility of contamination could be eliminated. In the crosses of phages with different protein capsule the chance of phenotypic mixing [16, 12] has to be taken into account. The progeny of the crossing between *ts2 Ant⁻* and *ts2⁺ Ant⁺* was treated with antiphage serum. Only 15% of phages surviving serum treatment at 30°C could be detected at 36°C.

The serum inactivation of *h* and *Ant⁻* mutants was almost the same. Both were less sensitive than the wild type phage. Their specificity could be distinguished by different hosts. All the resistant bacteria for the wild type phage were equally resistant to the *Ant⁻* mutants and vice versa. The *Ant⁻* could also represent an *h* mutant certainly different from the isolated *h*, but no adequate host was found among the 40 resistant bacteria tested.

The results of two and three point crosses with the *Ant* gene added, gave the genetic map shown in Fig. 4.

The evaluated three point crosses fitted well into the constructed map. Thus $C^+ts2h^+ \times Cts2^+h$ three point cross (cross 8, Table V) resulted in a map distance of 17 between *C* and *ts2*, 5.4 between *ts2* and *h*, whereas one of 20 between *C* and *h (mi)*. The frequency of double recombinants between *C* and *h* was 2.2% (1.1% $C^+ts^+h^+$ was detected). From the crossing of $C^+ts5h^+ \times$ $\times Cts5^+h$ (cross 10 in Table V) the map distance was 22.5 between *C* and *h*, and 0.3 between *h* and *ts5*.

In other three point crosses *ts* mutants and loci in *C* gene were combined. Among the ts^+ recombinants of very close *ts* mutants the frequency of double recombinants was higher than expected. In the case of $ts5C^+ \times ts4C$ crossing, 50% of the ts^+ recombinants were clear. This was probably due to the high negative interference but crosses between the extreme *ts* points resulted in an even higher frequency (80% when $ts2C \times ts5C^+$ was performed) and this cannot be explained at the moment.

The *N2* plaque morphology mutant was only crossed to *C* locus and will have to be related to other points of the map.

The heat inducible mutants and other clear mutants gave no complementation with the mapped *C* mutants. Therefore the mapped *C* cistron is thought to be the regulator gene of the prophage. The *h* mutants contain altered sites in one of the genes of tail fibres and *Ant⁻* mutants may have also altered genes of morphogenesis. In analogy with other phages [4, 17, 18], the left arm of the map contains the non-vegetative functions *(C)* and the right side controls the morphogenetic steps of phage 16—3 *(h, Ant)*.

Our conditional lethal mutants are under functional specification and further details of the genetic map of Rhizobium phage are continuously studied with further new mutants.

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J. G. WEISZFEILER

DIE BIOLOGIE UND VARIABILITÄT DES TUBERKELBAKTERIUMS UND DIE ATYPISCHEN MYCOBAKTERIEN

In deutscher Sprache · 311 Seiten · 81 Abbildungen · 62 Tafeln · 17 × 25 cm Ganzleinen

Der Verfasser berichtet über seine experimentellen Untersuchungen, welche die Hauptprobleme der Biologie der Tuberkelbakterien und der atypischen Mycobakterien umfassen. Besondersinteressant ist das Material über die Reaktivierung von Mycobakterien mit geschädigter Lebensfähigkeit, über erbliche Änderung der Arzneimittelresistenz, der Virulenz und der vom Verfasser als komplexe plurifikatorielle Mutation gekennzeichnete gleichzeitige Wandlung mehrerer Eigenschaften sowie die Analyse der Gesetzmäßigkeiten dieser Erscheinungen auf Grund des Darwinismus. Die Untersuchungen über die nichtsäurefesten und filtrierbaren Formen der Tuberkelbakterien, der Antigenstruktur im Zusammenhang mit dem Problem der atypischen Mycobakterien und der Klassifikation haben ebenfalls zu zahlreichen neuen Beobachtungen geführt. Die aus Affen herausgezüchteten neuen, fakultativ pathogenen Mycobakterien werden eingehend beschrieben, und das Problem der möglichen Herkunft von atypischen Mycobakterien aus Tuberkelbakterien wird beleuchtet. Ein besonderer Abschnitt von E. Vandra verfaßt, ist den Mycobacteriophagen gewidmet.

AKADÉMIAI KIADÓ
VERLAG DER UNGARISCHEN AKADEMIE
DER WISSENSCHAFTEN, BUDAPEST

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Acta microbiol. Acad. Sci. hung. 17, 195—198, 1970

ANAEROBIC TRANSFORMATION OF STEROIDS BY MYCOBACTERIUM PHLEI

By

K. ALBRECHT, E. TÖMÖRKÉNY and A. SZABÓ

Research Institute for Pharmaceutical Chemistry (Director: L. VARGHA), *Budapest*

(Received December 23, 1969)

Summary. Under anaerobic conditions *Mycobacterium phlei* transformed androst-4-ene-3,17-dione, 17β-hydroxyandrost-4-en-3-one and its 17β-acylates, as well as 3β-hydroxyandrost-5-en-17-one and 17α-methyl-17β-hydroxyandrost-4-en-3-one to 5α- and 5β-saturated compounds. From the reduction of the 3-oxo and occasionally of the 17-oxo groups, saturated hydroxy derivatives were obtained. Steroid ester formation also occurred under anaerobic conditions.

Since the investigations into steroid transformation by MAMOLI *et al.* [1], a number of authors studied the microbial reduction of steroids. Observations concerning saturation of the molecule and keto-reduction were made mainly under aerobic conditions [2]. The present paper deals with the transformation of androstenes by *Mycobacterium phlei*, under anaerobic conditions.

Materials and methods

Growth, microbial transformation and extraction. The *Mycobacterium phlei* strain used in the experiments was cultivated under aerobic conditions [3]. The culture was centrifuged and the cells were either resuspended in sterile distilled water to make a normal cell suspension with 0.5% dry material content or dried in vacuo in the presence of phosphoric anhydride, powdered and stored in a glass-stoppered bottle, and used in 0.5% suspension in distilled water. One hundred ml amounts of the cell suspension were distributed each into 500 ml Erlenmeyer flasks. In the centre of the bottom of each flask was soldered a glass tube, 12 cm long, 1.5 cm in diameter and open at the top. Into this tube were poured 8 ml of an aqueous solution containing 10% pyrogallol and 16% sodium hydroxide. Subsequently an oxygen-free nitrogen flow was passed through the culture suspension at a rate of 200 ml/min for 40 minutes. Before stopping the nitrogen flow, 20 mg of one of the substrates androst-4-ene-3,17-dione (I), 17β-hydroxyandrost-4-en-3-one (II), the latter's 17β-acetate (IIa) or 17β-propionate (IIb), 3β-hydroxyandrost-5-en-17-one (III) or 17α-methyl-17β-hydroxyandrost-4-en-3-one (IV), dissolved in 1 ml acetone, was added to each flask, then the flask was closed airtight. Absence of oxygen was checked with MARSHALL's indicator [4]. Incubation of flasks and conditions of extraction were as described previously [3], except that no distinction was made between experiments for analytical and preparative purposes and therefore the cell extract was used throughout.

Analytical methods. In every case, transformation was followed by thin-layer chromatography on silica gel, using in succession 95:5, 90:10, 80:20 and 70:30 mixtures of benzene and ethyl acetate as running solvent (system A). Ester derivatives were separated by a single running in a 95:5 mixture of benzene and ethyl acetate (system B). The chromatograms were detected with a 1:1 mixture of sulphuric acid and ethanol. Quantitative estimation of Δ4-3-oxo and Δ1,4-3-oxo derivatives was carried out by means of paper chromatography [5].

Determination of 5α-androstane-3,17-dione (V), and 5β-androstane-3,17-dione (VI) (5α to 5β ratio). The dry residue of the extract obtained by transformation of 20 mg substrate

was dissolved in 2 ml glacial acetic acid and oxidized with a mixture of 0.4 ml of 2% aqueous CrO_3 and 1.6 ml of glacial acetic acid at room temperature for 2 hours. Subsequently the solution was diluted with water, extracted with dichloroethane, washed and evaporated. 5α-androstane-3,17-dione and 5 β-androstane-3,17-dione were determined separately in the above extract by the TLC method, running in succession in 95:5 and 90:10 mixtures of benzene and ethyl acetate. The dried chromatogram was sprayed with 2,4-dinitrophenylhydrazine reagent (saturated solution of 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid, filtered at 20 °C) and the coloured spots of compounds V and VI were eluted with dichloroethane. The eluate was washed with 3 *N* aqueous sodium hydroxide solution and measured photometrically at 410 mμ. With steroids of known amount, the loss was about 20% and this was the case with the 5 α and 5 β steroids as well.

Isolation and identification of the transformation products. After extraction and evaporation the metabolites were isolated by repeated chromatography on silica gel column. Elution was made with benzene, containing increasing amounts of ethyl acetate. The product was compared with authentic substances in respect of melting point, mixed melting point, R_f value and IR spectrum.

Results and discussion

Using a normal cell suspension the following compounds were obtained from androst-4-ene-3,17-dione in 24 hours during which the substrate disappeared completely: 5α-androstane-3,17-dione (V), 5β-androstane-3,17-dione (VI), 3β-hydroxy-5α-androstan-17-one (VII), 3α-hydroxy-5β-androstan-17-one (VIII), 5β-androstane-3α, 17β-diol (IX), 3β-hydroxy-5β-androstan-17-one (X), 5α-androstane-3β, 17β-diol (XI) and 17β-hydroxy-5β-androstan-3-one (XII). In some fractions eluted with benzene, at least seven compounds more apolar than V could be detected in the TLC system B. Transformation of these fractions with *M. phlei* under aerobic conditions in the presence of 8-hydroxyquinoline resulted in the formation of androsta-1,4-diene-3,17-dione [6]. Other aliquots of the fractions showed, after hydrolysis with potassium carbonate, compounds VII, VIII, IX and X when chromatographed in system A. These two results indicated that the substances more apolar than compound V were the esters of the reduced derivatives.

The ratio of the saturated 5α and 5β derivatives was ~ 0.9. Among them, the 3,17-diones (V and VI) were predominant in the early stage, whereas the reduced derivatives in the later stage. On shorter transformation 17β-hydroxyandrost-4-en-3-one (II) was also demonstrable in traces.

Transformation of 17β-hydroxyandrost-4-en-3-one (II), its 17β-acetate or 17β-propionate and of 3β-hydroxyandrost-5-en-17-one (III) with normal cell suspension in 24 hours yielded the same results as when androst-4-ene-3,17-dione (I) had been the initial substrate. Distinct formation of I from the above substrates was in fact noted at earlier points of time.

Using the dried cell suspension, formation of all foregoing derivatives took place from substrates I, II and III, but the ratio of 5α to 5β saturated compounds was 0.1—0.2.

17α-methyl-17β-hydroxyandrost-4-en-3-one (IV) was transformed into 17α-methyl derivatives analogous to those of the former substrates, but transformation to 5α saturated compounds was less distinct with the normal

cell suspension and only traces of them were demonstrable with the dried cell suspension.

As it was shown Δ4-3-oxo steroids were transformed anaerobically into both 5α and 5β saturated compounds. The finding that with the dried cell

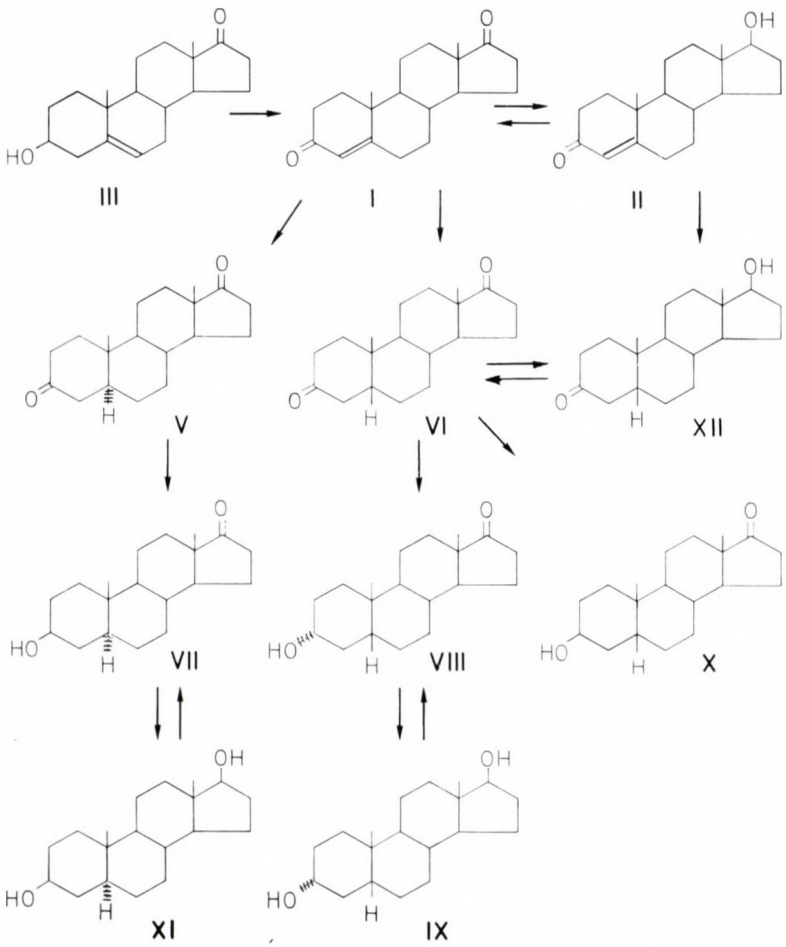

Fig. 1. Anaerobic transformation of compounds I, II and III

suspension the rate of 5α saturation decreased considerably suggested that saturation was actually related to the activities of two different enzymes, at least at the co-factor level. This seems to verify the simultaneous presence of Δ4-5α and Δ4-5β reductases in *Mycobacterium phlei*, as in higher organisms, too [7].*

* In preliminary experiments performed under less anaerobic conditions, increase of 5α compounds at the cost of 5β reduced derivatives was noted while small amounts of androst-4-ene-3,17-dione (I) and, occasionally, of androsta-1,4-diene-3,17-dione appeared. Thus 5β-androstane-3,17-dione (IV) could be transformed to 5α reduced derivatives through I.

Reduction of the 3-oxo group resulted chiefly in equatorial hydroxy-derivatives, but in the 5β-series axial epimeres were also demonstrated (X and XII). We failed to demonstrate 3-hydroxy-Δ4 intermediate, the saturation of Δ4 double bond had apparently preceded the 3-oxoreduction.

The considerable amount of androst-4-ene-3,17-dione (I) formed in the early transformation phase of 3β-hydroxyandrost-5-en-17-one (III) suggests that in accordance with the finding of SCHUBERT *et al.*, saturation of the Δ5-3β-hydroxy compounds took place through the Δ4-3-oxo derivatives [8].

Both 5α- and 5β-androstanes could be esterified with *Mycobacterium phlei*. The formation of steroid esters by microorganisms was studied by SCHUBERT *et al.* [9], but its occurrence under anaerobic conditions was noted first in the present study, with the applied *M. phlei* strain. The esterifying acids have not been identified, but the ester formed was found to be enzymatically hydrolysable during aerobic transformation.

Reduction of compounds I, II and III by *Mycobacterium phlei* under anaerobic conditions is shown in Fig. 1, with indication of the temporal sequence and eventual reversibility of the reduction processes.

Acknowledgement. We are indebted to Mrs. M. KOVÁCS for analyses and to Dr. G. TÓTH for taking the IR spectra.

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TRANSFORMATION OF 4,5-EPOXY STEROIDS WITH MYCOBACTERIUM PHLEI

II. TRANSFORMATION UNDER ANAEROBIC CONDITIONS

By

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(Received December 23, 1969)

Summary. Under anaerobic conditions, 3-oxo-(3-hydroxy)-4,5-epoxy steroids could be transformed to 5α-H and 5β-H derivatives through the Δ4-3-oxo intermediate. Elimination of the oxido group supposedly took place through the 3-hydroxy-4,5-epoxy, and, in succession, the 3,5-dihydroxy derivatives.

It was shown previously that 3-oxo-(3-hydroxy)-4,5-epoxy steroids were transformed by *Mycobacterium phlei* under aerobic conditions, through androst-4-ene-3,17-dione to androsta-1,4-diene-3,17-dione, which accumulated in the presence of 8-hydroxyquinoline. Elimination of the oxido group could not be explained by the formation of a 4,5-dihydroxy compound as described in the literature and the process was accompanied also by reductive transformations [1]. Supposing that the elimination took place through a monohydroxy derivative and with the fact in mind that elimination by microorganisms of the C_4-hydroxyl-group has not yet been observed, the presence of 3-oxo-5-hydroxy and 3,5-dihydroxy intermediates had to be postulated. The formation of these could be a reductive process, the biochemical analogue of the cleavage of epoxy steroids with *e.g.* lithium aluminium hydride [2, 3]. To substantiate this theory, 3-oxo-(3-hydroxy)-4,5-epoxy steroids were transformed with different cell suspensions of *Mycobacterium phlei* under anaerobic conditions.

Materials and methods

Melting point, IR and NMR spectra were determined as described previously [1].

Preparation of 4,5-epoxy steroid substrates. The 3-oxo-4,5-epoxy compounds were obtained from the appropriate Δ4-3-oxo compounds in alkaline medium, using hydrogen peroxide: 4β,5β-epoxyandrostane-3,17-dione (I) [4]; 4α, 5α-epoxyandrostane-3,17-dione (II) [5]; 17α-methyl-4β,5β-epoxy-17β-hydroxyandrostan-3-one (III) and 17α-methyl-4α,5α-epoxy-17β-hydroxyandrostan-3-one (IV) [6]. The compounds 4β, 5β-epoxyandrostane-3α, 17β-diol (V), 17α-methyl-4β, 5β-epoxyandrostane-3α,17β-diol (VI) and 17α-methyl-4α, 5α-epoxyandrostane-3β,17β-diol (VII) isolated also as transformation products, were prepared from the appropriate 3-oxo compounds by means of sodium borohydride [2, 7].

Growth, microbial transformation, extraction. Growth of the *Mycobacterium phlei* strain [9] used in the experiments and the conditions of transformation and extraction were the same as described previously [9].

Analytical methods. Transformation was followed by thin layer chromatography (system A) [9]. The Δ4-3-oxo derivatives obtained on transformation of I and II (Figs 1—3) were determined by paper chromatography [10]. In the early stage of transformation the

epoxy substrates and metabolites were determined quantitatively by forming a sulphuric acid chromogen [1]. In the later stage, owing to the presence of 8—10 metabolites the epoxy derivatives were analysed semi-quantitatively on the basis of the colour intensity after development with sulphuric acid, by comparison to known amounts of the compound estimated on the thin layer chromatogram. The total quantity of 5α-H and 5β-H saturated derivatives as well as their ratio were determined as described previously [9]. If there was also an epoxy compound present, the above semi-quantitative determination was performed owing to the uncertainty of separation.

Isolation and identification of transformation products. The metabolites were isolated as described previously [1]. Table I shows the appropriate conditions of transformation of different substrates, for the best isolating effect.

Table I

Optimal conditions of transformation for isolating the metabolites

Substrate	I		II		III		IV	
Cell suspension	A	B	A	B	A	B	A	B
Metabolites	Time of transformation in hours							
V	24							
VI					3—4	6—8		
VII								6—8
VIII	6—7		1.5—2					
IX					5—6		5—6	
XV	3—4	4—5						
XVI				6—8				

A: Transformation with normal cell suspension; B: Transformation with dried cell suspension: V = 4β,5β-epoxyandrostane-3α,17β-diol; VI = 17α-methyl-4β,5β-epoxy-androstane-3α,17β-diol; VII = 17α-methyl-4α,5α-epoxyandrostane-3β,17β-diol; VIII = androst-4-ene-3,17-dione; IX = 17α-methyl-17β-hydroxyandrost-4-en-3-one; XV = 4β,5β-epoxy-3α-hydroxyandrostan-17-one; XVI = 4α,5α-epoxy-3β-hydroxyandrostan-17-one.

Identification of all metabolites except compound XVI was made by comparison with the earlier prepared [1, 2] or other authentic substances, with regard to melting point, mixed melting point, IR, UV and NMR spectra, as well as R_f.

The 4α,5α-epoxy-3β-hydroxyandrostan-17-one (XVI) had, on repeated recrystallization from acetone–petrol ether: Mp 175—179 °C; $[\alpha]_D$: +124° (c = 1, acetone). Analysis calculated for $C_{19}H_{28}O_3$ (304.2):C 74.95, H 9.27%. Found C 74.84, H 9.33%. IR spectrum: $\nu\overline{CO}$: 1730 cm^{-1}; νOH: 3330 cm^{-1}. NMR spectrum: C_4—H:7.08 ppm (singulette) in $CDCl_3$.

17β-hydroxyandrost-4-en-3-one (X), which was formed in a small quantity, was identified by the R_f value and UV spectrum, whereas the saturated derivatives of Δ4-3-oxo steroids (5β-androstane-3,17-dione (XI), 5α-androstane-3,17-dione (XII), 17α-methyl-17β-hydroxy-5β-androstan-3-one (XIII), 17α-methyl-17β-hydroxy-5α-androstan-3-one (XIV) as well as their further reduced derivatives [9]) were identified by thin layer chromatography [9].

Results and discussion

On the transformation of 3-oxo-4,5-epoxy compounds with a normal cell suspension no substrate, and starting from 3-hydroxy-4,5-epoxy steroids, no 3-oxo-4,5-epoxy analogue could be detected. Depending on the substrate applied and on the type of cell suspension a 3-hydroxy-4,5-epoxy compound,

subsequently a Δ4-3-oxo compound and its 5α-H or 5β-H saturated derivatives had formed in the same proportion as *e.g.* on the anaerobic transformation of androst-4-ene-3,17-dione or 17α-methyl-17β-hydroxyandrost-4-en-3-one [9]. This proved that the Δ4-3-oxo compounds are intermediates also under anaerobic condition. On transformation of I and V, reduction of the 17-oxo group and dehydrogenation of the 17β-hydroxyl group took place like in other anaerobic transformations [9].

Transformations performed with the dried cell suspension differed from the above in the decreased elimination rate of the oxido group; *e.g.* XVI could be isolated only under such conditions. The ratio of 5α-H and 5β-H derivatives was as described previously [9].

Transformation of I and II with normal cell suspension as well as transformation of II with a dried cell suspension were followed quantitatively (Figs 1—3). Changes in substrate and metabolite concentration were plotted against time and per cent molarity of the substrate. The quantities of 5α-H and 5 β-H derivatives were expressed in terms of the sum of XI and XII after oxidation of the reaction mixture with chromic acid [9].

Considering again the reduction of the 3-oxo groups of epoxyketones, in every case 3-pseudo-equatorial hydroxy derivatives were isolated [1, 2] which, when derived from the 17α-methyl-17β-hydroxy derivatives (III, IV, VI and VII) were directly identifiable with the chemically prepared compound [2]. The 17α-methyl-17β-hydroxy derivatives behaved under aerobic conditions in the same manner as did the corresponding 17β-hydroxy or

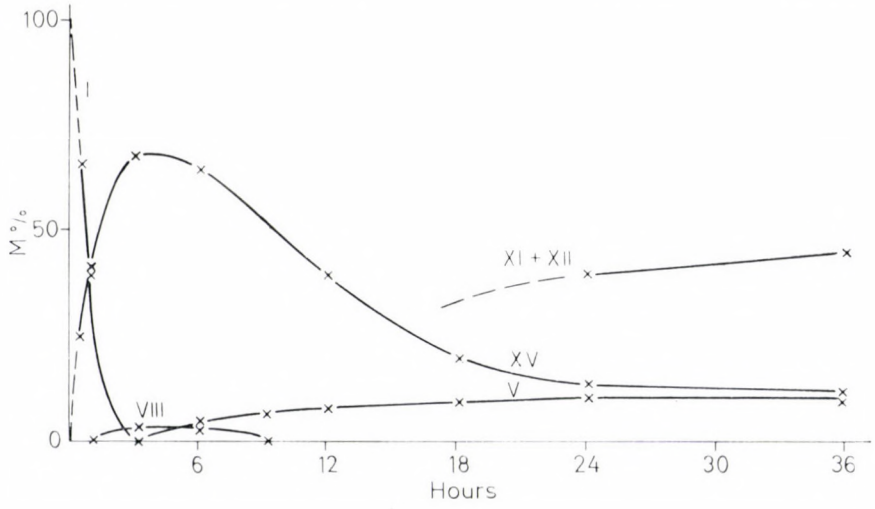

Fig. 1. Transformation of 4β,5β-epoxyandrostane-3,17-dione (I) with normal cell suspension. I = 4β,5β-epoxyandrostane-3,17-dione; V = 4β,5 β-epoxyandrostane-3α,17β-diol; VIII = androst-4-ene-3,17-dione; XI + XII = 5 β-androstane-3,17-dione + 5α-androstane-3,17-dione; XV = 4β,5β-epoxy-3α-hydroxyandrostane-17-one

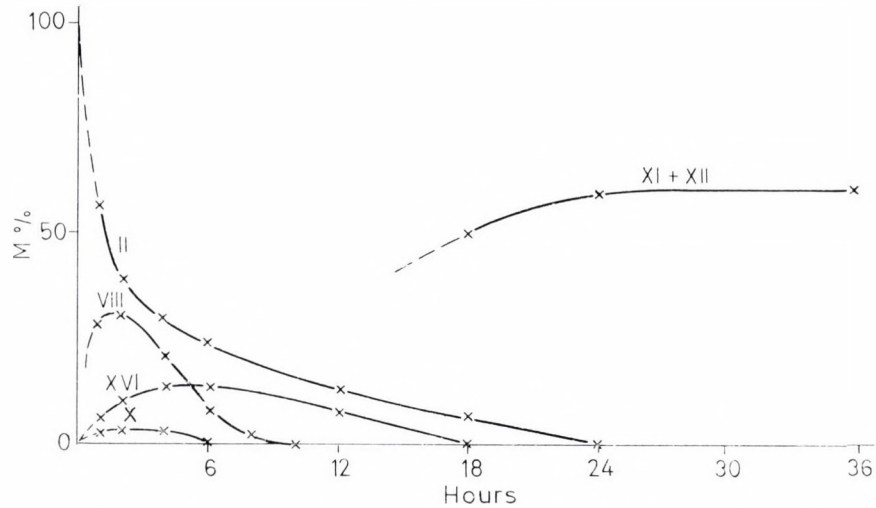

Fig. 2. Transformation of 4α,5α-epoxyandrostane-3,17-dione (II) with normal cell suspension. II = 4α,5α-epoxyandrostane-3,17-dione; VIII = androst-4-ene-3,17-dione; X = 17β-hydroxyandrost-4-en-3-one; XI + XII = 5β-androstane-3,17-dione + 5α-androstane-3,17-dione; XVI = 4α,5α-epoxy-3β-hydroxyandrostan-17-one

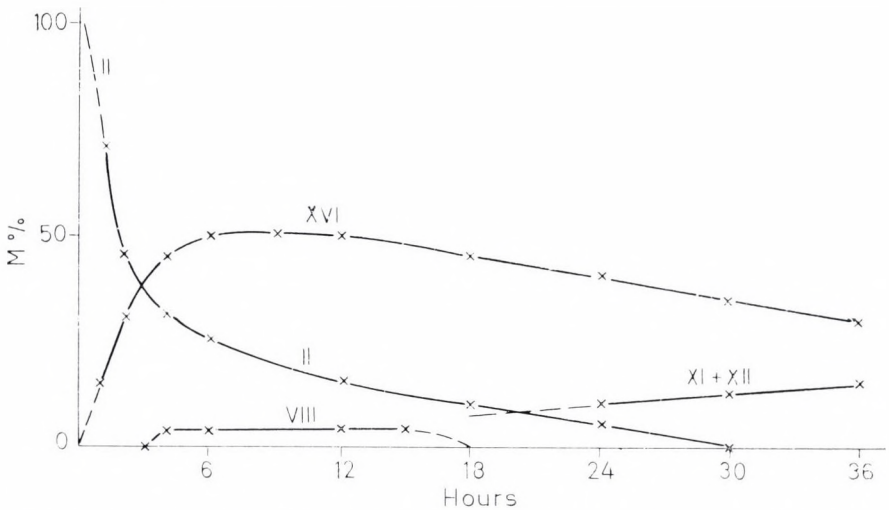

Fig. 3. Transformation of 4α,5α-epoxyandrostane-3,17-dione (II) with dried cell suspension. II = 4α,5α-epoxyandrostane-3,17-dione; VIII = androst-4-ene-3,17-dione; XI + XII = = 5β-androstane-3,17-dione + 5α-androstane-3,17-dione; XVI = 4α,5α-epoxy-3β-hydroxyandrostan-17-one

17-oxo analogues, *i.e.* they could be transformed into 17α-methyl-17β-hydroxyandrosta-1,4-dien-3-one [1, 11] through a Δ4-3-oxo derivative. RINGOLD *et al.* have demonstrated that an electron-attractive substituent (halogen) at C_2, C_4 or C_6 of the steroid molecule promotes the enzymic reduction of the 3-oxo group [12]. It seems likely that in the present case and

also in others [13, 14, 15], C_3 was rendered suitable for hydride transfer and accordingly for reduction of the oxo group by the electron-attraction of the oxido group, under aerobic and anaerobic conditions alike.

Direct evidence is lacking as to the role of the 3-keto or the 3-hydroxy compound in the elimination of the oxido group. The fact that on transformation of 3-hydroxy-4,5-epoxy compounds, no 3-oxo-epoxy compound had formed, indicated that it was the 3-hydroxy derivative which underwent further transformation. The same conclusion is arrived at if Figs 2 and 3 are compared; with nearly identical rates of consumption of the substrate, XVI accumulates if the dried cell suspension is used, while VIII and in succession its saturated derivatives are formed at the former's cost if a normal culture is used. The probable sequence of these processes is shown in Fig. 4, using 17β-hydroxy and 17-oxo derivatives as examples.

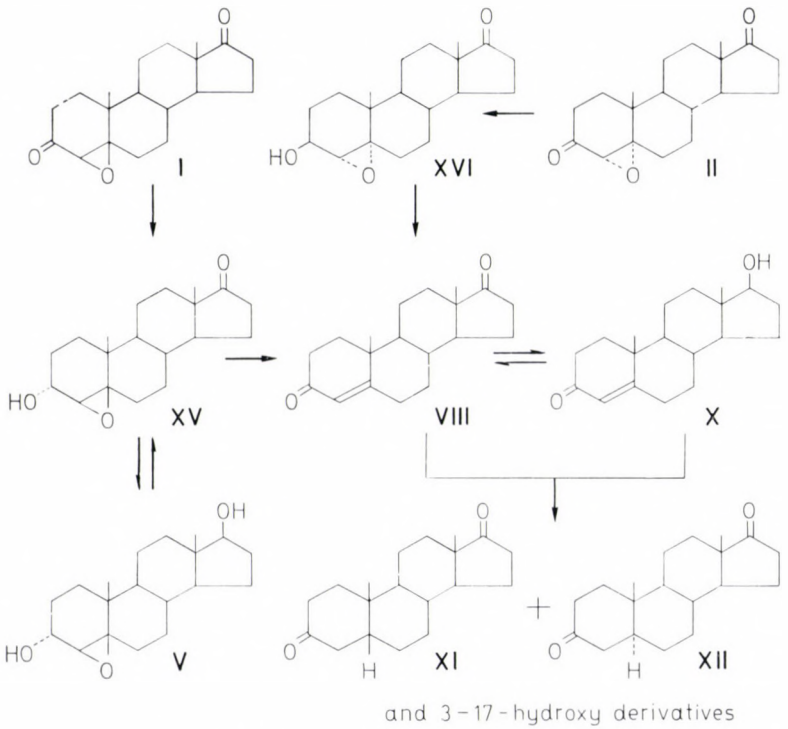

Fig. 4. Tentative sequence of transformational steps

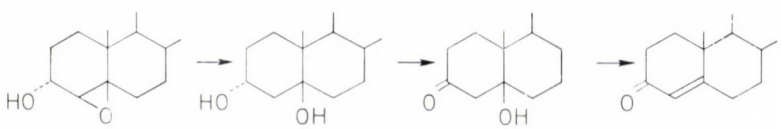

Fig. 5. Tentative sequence of transformational steps

The intermediate between epoxy and Δ4-3-oxo compounds was not demonstrable under anaerobic conditions either. In preliminary examinations, neither the expected hydrolytic cleavage products nor their dehydrated derivative (3,4,5-trihydroxy, 3-oxo-4,5-dihydroxy, Δ4-3-oxo-4-hydroxy) transformed in the expected direction. But the products of oxido substrates obtained on cleavage with lithium aluminium hydride (3,5-dioles [2]) could be transformed at a high rate to 5α-H and 5β-H derivatives [16] through the Δ4-3-oxo intermediate. All these findings underline the possibility of a reductive cleavage of the oxido group not yet noted with microorganisms, as shown *e.g.* in Fig. 5.

It seems unlikely that the 3-oxo-(3-hydroxy)-4,5-epoxy compounds would have a role in steroid metabolism in vivo. 16α,17α-epoxypregn-4-ene-3,20-dione, too, seems to be an artefact; its metabolism was explained by KADIS [17] by an analogous mechanism in mammals [17]. In contrast, OSUCH and CHEN [18] stated that in the biosynthesis of bufadienolides, the 14β,15β-oxido compound could be an intermediate in the formation of the 14β-hydroxy derivative which means that a similar process takes place also in nature.

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APPLICATION OF GEL ADSORPTION TO CHARACTERIZE STRAINS OF THE FAMILY ENTEROBACTERIACEAE

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(Received December 29, 1969)

Summary. Cell suspensions of strains belonging to the family Enterobacteriaceae were found to be bound to the surface of $Al(OH)_3$ gel with different intensity. As adsorption was inhibited by phosphate ions, an attempt was made to characterize the degree of adsorption by the molarity of the phosphate buffer. Regarding as strain characteristic the numerical value of the molarity of that buffer concentration at which 50% of the cells were adsorbed to the gel surface (AC_{50}), bacterial strains belonging to identical serological and phage types could be distinguished from each other.

On the basis of the AC_{50} values, 25 *E. coli* O-type strains showed the following distribution. Three exhibited very weak adsorption properties even in the absence of phosphate ions. Seven strains displayed an AC_{50} of 0.0035, while four strains fell in the 0.0025—0.005, seven in the 0.005—0.01, three in the 0.01—0.02 and one in the 0.04—0.08 phosphate buffer *M* intervals. Strains isolated from patients showed a similar distribution as O-type strains. The majority of the cells of the pathogenic *E. coli* strains exhibited very weak adsorption properties even in the absence of phosphate ions. A reasonable difference of the AC_{50} values was found among *Shigella flexneri* strains. *Shigella flexneri* and *sonnei* strains showing identical serological and phage type could be differentiated on the basis of their different AC_{50} values. The Salmonella strains, except *S. typhi*, were characterized by protracted adsorption curves. The AC_{50} values for two *S. typhi murium* strains were different.

Pathogenic *E. coli dispepsiae* cells, owing to their weak adsorption character, could be regained from a suspension prepared from normal *E. coli* bacteria adsorbing stronger to the gel, in spite of the greater number of the latter.

The relation between antigenic and adsorption character is discussed.

Reports suggesting dissimilar chromatographic properties of virus species and also similar intratypic differences between virus strains were first published during the past decade [1—3]. In the meantime, chromatographic and gel adsorption methods have increasingly been applied in virology, as they allow a ready demonstration of intra-typic differences of the various enterovirus strains [4—8].

The present studies were undertaken to adapt the gel adsorption method for the strain characterization of enteral bacteria. The working principle of the method was that cell suspensions of various bacterial strains were found to adsorb to the surface of $Al(OH)_3$ gel with different intensity. As phosphate ions inhibited the adsorption, its degree could be expressed in terms of the inhibitory concentration of phosphate ions.

Materials and methods

Bacterial growth medium: Broth.

Buffer solutions. TRIS buffer, 0.05 *M*, pH 7.5; phosphate buffer, 0.66 *M*, pH 7.5.

Serial dilutions were prepared, using as diluent TRIS-buffer in the range below 0.1 *M*, whereas bisdistilled water in the range above 0.1 *M*.

Adsorbent. $Al(OH)_3$ gel $C\gamma$ (Behring Werke) was used throughout. The $Al(OH)_3$ content of the preparation corresponded to 1 g Al_2O_3/100 ml distilled water. For experimental purposes, this stock solution was diluted 1:4 in distilled water.

Bacterial strains. Escherichia coli, Shigella and *Salmonella* strains, prototypes and isolates from patients, were used.

Preparation of bacterial cell suspension for gel adsorption studies. The bacteria were grown in 100 ml Erlenmeyer flasks fitted with a communicating tube for density reading. Culturing was made in 20 ml medium per flask, in a water bath at 37°C. During incubation, the flasks were shaken at a frequency of 120/min. Cultivation was continued until turbidity had reached a degree of 1.6, as read in a Magnephot II, type 2213 apparatus. Subsequently, the cultures were centrifuged and the cell sediment was suspended in 20 ml TRIS-buffer.

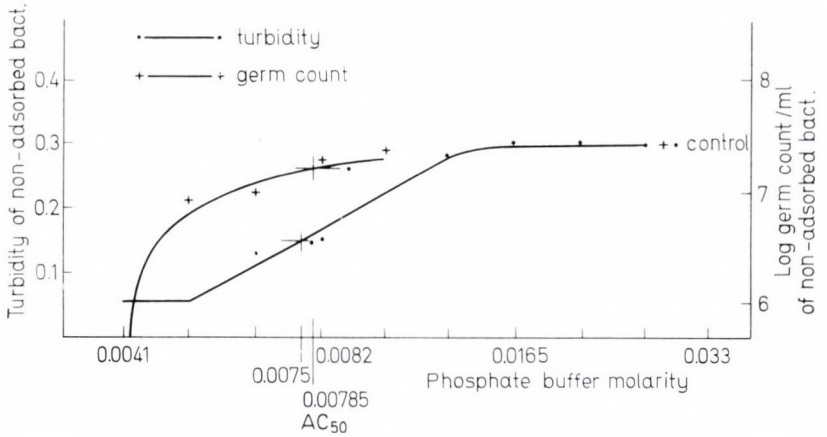

Fig. 1. Adsorption of *E. coli* (strain 30018) to $Al(OH)_3$ gel

Gel adsorption test. In 9 of 10 test tubes (100×15 mm) each was placed 1 ml $Al(OH)_3$ gel and in the 10th tube was placed 1 ml distilled water for control. Two ml TRIS-buffer was then added to the first tube and subsequently 2 ml phosphate buffer of rising molarity, graded at 0.1 log, was added to each tube in succession. The molarity interval was selected with regard to the strength of binding of the bacterial cells to the gel, as assessed in preliminary experiments. In every case, the molar concentration of the buffer added to tubes Nos 9 and 10 was 0.32 *M*. Then 1 ml of the bacterial suspension to be examined was added to each tube, thoroughly mixed, and allowed to stand at room temperature for 20 minutes. After adsorption, the material was centrifuged at 1000 r.p.m. for 2 minutes and the proportion of non-adsorbed bacterial cells was assessed by estimating the density of the supernatant. At a concentration of 0.32 *M*, the phosphate buffer completely inhibited adsorption of all the strains studied up to now, thus it was expected that this suspension and the control suspension not containing adsorbent will be identical in density. Plotting the adsorption curve, the abscissa value corresponding to the intersection point of 50% adsorption represented the buffer molarity characteristic of the examined strain. Reading this value, care was taken to consider the twofold dilution of the buffer effected by the addition of $Al(OH)_3$ gel and of the bacterial suspension. The characteristic molarity was termed AC_{50}.

The proportion of non-adsorbed bacterial cells can be determined also by germ counting, using the pour-plate method. Both methods of determination yielded identical AC_{50} values. The adsorption curve for the *E. coli* strain No. 30018, shown as example, is presented in Fig. 1.

Results

First of all it had to be clarified whether the gel adsorption behaviour was a stable property of the population of a given bacterial strain. For this purpose, average AC_{50} values were determined for selected strains in parallel measurements and the standard deviation was calculated. From the same

strains, several passage materials were prepared under identical conditions and examined for AC_{50} values and standard deviation. Results are summarized in Table I.

As can be seen from Table I, the average AC_{50} values for the two groups were in good correlation and the standard deviations did not differ notably.

O-prototype strains belonging to the *E. coli* serotypes most frequent in Hungary were examined for adsorption properties on $Al(OH)_3$ gel. The exam-

Table I

AC_{50} values for E. coli and Shigella flexneri strains in different passages

Bacterium	Designation of strain	Number of parallel examinations	AC_{50}	
			Log $\bar{x}$	Standard deviation
Escherichia coli	30018	8	−2.131	±0.0197
	30018	9(1—9 passage)	−2.152	±0.0190
	30023	8(1—8 passage)	−2.933	±0.0066
	38	8	−3.251	±0.096
Shigella flexneri	38	8(1—8 passage)	−3.397	±0.092
	1286	6	−1.633	±0.0615
	1286	6(1—6 passage)	−1.783	±0.0762

ined strains were classified by AC_{50} values into molarity intervals of $\log_2$ in rising sequence. Results are shown in Table II.

The majority of the strains was found to belong to groups 2, 3 and 4. Cells of these strains were characterized by a weak binding to the gel surface; a phosphate buffer concentration of the order of *M*/1000 was sufficient to inhibit their adsorption which always took place if no phosphate ions were present.

Distribution of the adsorption properties was similar among the prototype strains and isolates.

The adsorption characteristics of pathogenic enteral bacteria are summarized in Table III.

The gel adsorption capacity of the greater part of enteral bacteria was very weak. This applied chiefly to the pathogenic *E. coli* group in which every strain except O124 the majority of cells were found in the supernatant, even if no phosphate ions were present. The per cent values in Table III show the proportion of non-adsorbed bacterial cells, as assessed by turbidimetry.

Shigella flexneri strains too displayed low AC_{50} values, though 2 of the examined 8 strains had a very distinct adsorption capacity. The dissimilar adsorption properties enabled the differentiation of strains of identical serotype or phage type, as *e.g.* of two type 3a strains and of type 6 strains.

Table II

Distribution by adsorptivity to $Al(OH)_3$ gel of the most frequent E. coli O-type strains

Bacterium	Phosphate buffer nil	Phosphate buffer molarity intervals						Total
		≤0.0025	0.0025–0.005	0.005–0.01	0.01–0.02	0.02–0.04	0.04–0.08	
	1	2	3	4	5	6	7	
O-type strains	4:3L:5 5:4L:4 25:—:—	8:8L:4 10:5L:4 9:9L:12 18:76B:14 19b.:7 20:17L:— 101:—:—	2:1L:4 21:20L:— 22:13L:1 65:—:—	1:1L:7 7:1L:— 16:1L:— 25:19L:12 15:14L:4 24:.:. 3:2a,bL:2	6:2acL:1 17:16L:18 23:18L:15		20:—:—	
No.	3	7	4	7	3	0	1	25
%	12	28	16	28	12	0	4	100
Strains isolated from patients								
No.	2	22	15	12	4	3	2	60
%	3.33	36.7	25.00	20.00	6.65	5.00	3.33	100

Table III

Adsorption characteristics of pathogenic enteric bacteria

Bacterial strain	Serotype	Phage type	No. of strains examined	Adsorption to $Al(OH)_3$ gel	
				Without phosphate buffer, per cent	AC_{50} $\overline{X}$
	026 K 60	—	10	60—80	—
	055 K 59	—	10	58—75	—
E. coli	086 K 61	—	7	70—85	—
pathogenic	0111 K 58	—	4	65—80	—
	0125 K 70	—	3	70—85	—
	0124 K 72	—	2	—	0.0026
	1 b	109	1	80	—
	2 a A	109	1	10	0.00048
Shigella flexneri	3 a	62	2	—	<0.00030
				—	0.00055
	3 a	30	1	20	0.00046
	3 a	68	1	20	0.00065
	6	121	2	—	0.023
				—	0.06
		65	2	—	0.0012
Shigella sonnei				—	0.00041
		6	1	—	0.0008
		32	1	—	0.0015
Salmonella typhi	9,12, Vi: d	D	1	—	0.0065
		A	1	—	0.006
S. enteritidis	1,9,12: g, m	—	1	20	0.005
S. typhi-murium	1,4,5,12: i	—	2	20	0.002
		—		10	0.013
S. derby	1,4,5,12: f, g	—	1	20	0.006
S. abony	1,4,5,12: b	—	1	40	<0.0003
S. paratyphi-B	1,4,5,12: b	—	1	10	0.001
S. infantis	6,7: r	—	1	30	0.001
S. meleagridis	3,10: e, h	—	1	—	0.003

The *Shigella sonnei* strains had a more distinct adsorption capacity. Two strains of the phage type 65 had dissimilar adsorption properties.

With the majority of salmonella strains, part of the cells failed to adsorb to the gel even if no phosphate buffer was present, though their AC_{50} values

were relatively high. The adsorption properties of two *S. typhi-murium* strains differed considerably.

Gel adsorption allowed to distinguish between bacterial cells with different adsorption properties even in mixtures. As pathogenic *E. coli* and salmonella cells had a very weak adsorptivity as compared to non-enteropathogenic *E. coli* bacteria, gel adsorption appeared to offer a suitable basis for the elaboration of a routine diagnostic method for their isolation from faecal samples.

To obtain more information, a model experiment was carried out as follows. Cell suspensions prepared from non-enteropathogenic *E. coli* and pathogenic *E. coli* (O55) strains in saline were mixed so that the non-enteropathogenic cells were present in greater number. The suspension was added to a mixture of 1 ml $Al(OH)_3$ gel + 2 ml TRIS buffer and allowed to adsorb for 20 minutes. Then the material was centrifuged at 1000 r.p.m. In the supernatant, cells were counted by the pour-plate method to assess the ratio of pathogenic and non-pathogenic *E. coli* cells. Results are shown in Table IV.

Table IV

Separation by gel adsorption of pathogenic and non-enteropathogenic E. coli bacteria

Components of mixed bacteria	Germ count/ml	Distribution of components Nos 1 and 2 between 100 colonies on agar plate	
		before adsorption	after adsorption
1. *E. coli* 30018	2.7×10^6	No. of colonies ≤ 100	35
2. *E. coli* O55	5.3×10^4	No. of colonies 0:13.5%*	65
		No. of colonies $\geq$ 1:86.5%	

* Values calculated according to Poisson distribution.

If the original cell suspension (see Table IV) was so diluted that an inoculum of unit volume gave rise to about 100 colonies on agar plate, colony outgrowth took place according to Poisson distribution. Thus, no outgrowth of pathogenic *E. coli* colonies was expected on 13.5% of the agar plates, while growth of one or more colonies on 86.5% of the plates. After gel adsorption, the average proportion of pathogenic *E. coli* growth was 65% per plate.

Discussion

Under the given conditions of experiment, the adsorption property of bacterial cells appeared to be a stable feature characteristic of the strain. The AC_{50} value for bacterial strains passaged under identical conditions remained within the limits of the standard deviation originally assessed (Table I). The

AC_{50} value for a Shigella strain which had undergone one human passage by a laboratory accident, also remained unchanged.

The AC_{50} value, nevertheless, represents only a single point of the adsorption curve and as such may not always be sufficient to characterize the adsorption property of the strain.

When examining intratypic differences, the course of the adsorption curve should also be taken into consideration.

As can be seen from the adsorption curve of the *E. coli* strain 30018 (Fig. 1), the difference between the molarities of the phosphate buffer concentrations not inhibiting and inhibiting adsorption was about 0.4 log, thus the AC_{50} value fell into the steep part of the curve and as such characterized well the adsorption property of the strain.

The adsorption curves of the examined *E. coli* O prototype strains as well as of the non-enteropathogenic *E. coli* strains isolated from patients had all steep slopes.

The adsorption curves of the pathogenic enteral bacteria were variable. The weakest adsorption capacity was found with pathogenic *E. coli* strains. Type O124 strains differed from the latter group also by their greater adsorptivity.

Shigella flexneri strains, too, varied in adsorption property. The weak adsorptivity of the type 1b strain resembled that of the pathogenic *E. coli* strains, whereas two strains of serotype 6, phage type 121, were in the highest adsorptivity range.

The dissimilarity of the slopes of adsorption curves is indicated by the adsorption data of strains serotype 2aA (phage type 109) and serotype 3a (phage type 62) in that the former strain's AC_{50} value was higher though 10% of the cells had not bound to the gel even in the absence of phosphate ions, whereas the cells of the other strain, the AC_{50} value for which indicated a weak binding, had fully adsorbed also in the absence of phosphate ions.

Strains of the *Salmonella* group, except *Salmonella typhi* yielded curves indicating a protracted adsorption. This was clearly shown by the numerical data of Table III showing that the proportion of unbound cells was great also without phosphate ions, even if the AC_{50} was relatively high. Steep and protracted curves seem, therefore, to suggest a homogeneous and heterogeneous population, respectively, of the strains in question.

There was no parallelism between serological type and adsorptivity of the strains examined. Though the *E. coli* material did not comprise strains of identical serotype but different origin and, therefore, intratypic differences could not be pursued, the data of Table II clearly indicate that none of the antigenic components (O, K, H) was in itself responsible for the adsorption property. For example, one strain each of type O25 was found in groups 1 and 4, both with and without phosphate buffer, and O20 strains were found

in both the 2nd and 7th adsorption groups, which were fairly distant. Several such examples might be cited also in respect of the antigenic components K and H, as *e.g.* the K antigen designated 1L as well as the H antigen designated 4 were demonstrated in strains of dissimilar adsorptivity. The dissimilar nature of antigenic and adsorption properties was still more conspicuous with the shigella strains, as many of them differed in adsorptivity though their serological and phage types were identical. Thus, though the available data are not too numerous, there is reason to conclude that the study of adsorption properties may be helpful in demonstrating intratypic differences of various enteral bacterial strains.

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EFFECT OF FACTOR C ON GLUCOSE REPRESSION OF INDUCED β-GALACTOSIDASE SYNTHESIS

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(Received February 27, 1970)

Summary. Factor C, a glycoproteid-type macromolecule-containing agent regulating cytodifferentiation in *Streptomyces griseus*, increases the amount of 5%-cold-TCA-insoluble ^{14}C-uracil fraction incorporated in the presence of actinomycin D in both *Str. griseus* and *Escherichia coli* and raises the Tm point of DNA.

Studies on glucose repression of induced β-galactosidase synthesis in *E. coli* showed that at various glucose concentrations factor C decreased glucose-induced transient and catabolite repression.

In view of data on the mechanism of glucose repression it has been assumed that factor C acts primarily on DNA.

Factor C was isolated in this laboratory from shaken cultures of *Streptomyces griseus* strain 45H [1]. When added to the culture of *Str. griseus* strain 52—1, the agent caused an important cytomorphological change in that culture, which had been unable to reach the reproductive stage in normal submerged fermentation, began to differentiate for conidium production [2]. As to chemical structure, it has been concluded that the active substance in extracts containing factor C is a glycoproteid-type macromolecule (I. BÉKÉSI, personal communication).

The mode of action of factor C was examined by SZABÓ, BÉKÉSI and VITÁLIS [3]. It was shown that, in the presence of actinomycin D, factor C increased the amount of incorporated ^{14}C-uracil fraction not soluble in 5% cold TCA in *Str. griseus* and also in *E. coli* and *Bacillus subtilis*. Factor C altered the denaturation profile of DNA, it raised the Tm point. From these it was concluded that factor C acted primarily on DNA.

In the present work we have attempted to determine more exactly the site of action of factor C. As described in [3] the substance was effective in *E. coli;* this organism was chosen, accordingly, for studying the effect of factor C at the level of gene regulation. The influence of factor C on glucose repression of the well-known *lac* operon [4] was investigated.

Materials and methods

E. coli K-12 wild-type strain was stored on agar slant in the refrigerator. The culture was transferred into Oxoid nutrient broth and shaken at 37°C for 8 hours. The liquid culture was inoculated into the following medium: NH_4Cl, 2.0 g; Na_2HPO_4, 6.0 g; KH_2PO_4, 3.0 g; NaCl, 3.0 g; $MgCl_2$ 0.04 g; Na_2SO_4, 0.116 g; sodium succinate, 10.0 g; acid casein hydrolysate,

1.0 g per litre; pH 7.4. The culture was aerated overnight by blowing air through the medium, then centrifuged and washed in a medium containing the above ingredients except sodium succinate and casein hydrolysate. Finally the cells were resuspended in the complete medium and optical density was set to approximately 0.300 in Spektromom 360 (MOM, Budapest) spectrophotometer at 570 mμ.

The suspension was induced by adding methyl-β-D-thiogalactopyranoside (TMG, Koch-Light) at 1 mg/10 ml concentration (approximately 5×10^{-4} *M*), then transferred immediately into a water bath at 37°C and shaking was started (0 minute).

Aliquots of the culture were transferred at intervals indicated in Figs 1, 2, 3, 4 into Erlenmeyer flasks containing the substance(s) tested at the required concentration, and shaking was continued. At intervals shown in Figs 1, 2, 3, 4, 1.0 ml samples were taken and pipetted into aliquots of 0.1 ml solution of cetyl-trimethyl-ammonium bromide (Fluka, 1.0 mg/ml) and chloramphenicol (0.5 mg/ml) kept in flasks immersed in ice bath. After rapid and brisk mixing the samples were incubated in ice till the end of the experiment.

For the determination of β-galactosidase activity the method described in [5] was slightly modified. To the chilled samples, 1.5 ml o-nitrophenyl-β-D-galactopyranoside (ONPG, Koch-Light) solution (1 mg/ml) was added and the mixture was placed in a water bath at 37°C. The reaction was terminated by adding 1.0 ml 0.1 *M* Na_2CO_3 after 10 minutes. Then the mixture was again chilled in ice, centrifuged and the supernatant's absorbance was estimated in the Spektromom 360 spectrophotometer at 420 mμ wave length. Enzyme activity was calculated on the basis of 1U = 1 μM o-nitrophenol released per hour per dry weight of bacteria. The enzyme activity estimated under constant conditions was proportional to the amount of enzyme produced.

Glucose (in 0.08, 0.2, 20 and 40% solutions) was pipetted in the flasks to give the required final concentrations. Factor C was added as dry substance to give 200 μg/ml final concentration. When factor C was measured to the culture before the addition of glucose, the latter was added with a micropipette, when the sequence of additions was reversed then the culture already containing glucose was pipetted to the flask into which factor C had been added.

The factor C used in these experiments was prepared from 69-hour submerged cultures of *Str. griseus* strain 45H by salting out with $(NH_4)_2SO_4$ and passing the liquid through Dowex 50 column. The purified and freeze-dried product was effective in morphological experiments [2]. According to electrophoretic experiments, the preparation consisted of several different compounds (I. BÉKÉSI, personal communication).

Results

Before describing the results, it seems advisable to summarize the data applied in these experiments as a model for the study of the mode of action of factor C.

Today two kinds of repression are distinguished: transient and catabolite repression [6—8].

If glucose is added to an induced *E. coli* culture grown on weak carbon source (glycerol or succinate), the rate of its β-galactosidase synthesis rapidly decreases to nil. After a certain interval, enzyme synthesis reappears even when glucose is still present in the medium [9]. This phenomenon is termed transient repression.

If one culture is grown in weak carbon source, and another on glucose, the rate of β-galactosidase synthesis is much less in the latter than in the former. This type of repression is known as catabolite repression. After the release of the transient repression the process changes into catabolite repression.

Transient repression is produced by glucose (or any adequate carbohydrate) at the level of transcription [10—13].

The mechanism of catabolite repression has not been elucidated; in the opinion of some authors this also takes place at transcription level [11]; other investigators assume that it occurs at the translation level [7, 8].

If factor C was added to the induced culture alone, there was no difference in β-galactosidase activity as compared to the culture not supplemented with factor C; it was only after 90 minutes that a minimal, practically not

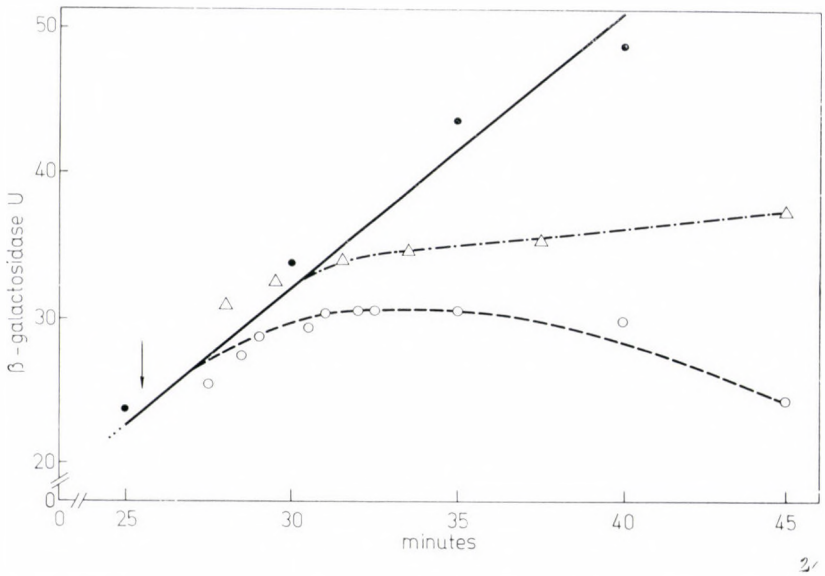

Fig. 1. E. coli K-12 cells were induced with TMG (1 mg/10 ml, approximately 5×10^{-4} *M*) at 0 minute, then shaken at 37° (●——●). Glucose (2×10^{-2} *M*) (○– – –○) or glucose (2×10^{-2} *M*) + factor C (200 μg/ml) (△–.–.–△) were added (arrow) then shaking was continued. Samples of 1.0 ml were taken at intervals and examined for enzyme activity

evaluable effect was noted. This means that factor C *per se* does not influence the induction of β-galactosidase and the enzyme synthesis [14]. If factor C was added 1—2 minutes after the addition of glucose, it failed to influence the repressing effect of the latter; the enzyme activity curve was similar to that obtained with glucose alone.

The present results are summarized in Figs 1—4.

Fig. 1 shows experiments with 2×10^{-2} *M* glucose. With glucose alone the rise in enzyme activity decreased and after 3—4 minutes enzyme synthesis stopped. After reaching the maximum activity, the curve shows a decreasing tendency, probably owing to a deterioration of the enzyme. (There are no data in the literature that glucose would directly decrease enzyme activity [10] and we also failed to demonstrate such an effect. In contrast, a slight degree of increase has been described.) When the same concentration of glucose was supplemented with factor C, the decrease in enzyme synthesis occurred

about 1.5 minutes later and no perfect termination was observed: enzyme synthesis continued, although at a much lower level than in the control culture.

Similar results were obtained at lower glucose concentrations. While in the above experiment the examined period of time was insufficient for demonstrating the release of transient repression, in the following experiments it was evident that transient repression had been released after a certain interval and enzyme synthesis started again.

If glucose was added at 10^{-3} *M* final concentration (Fig. 2), the result was similar as in Fig. 1. The rate of enzyme synthesis decreased to nil. The

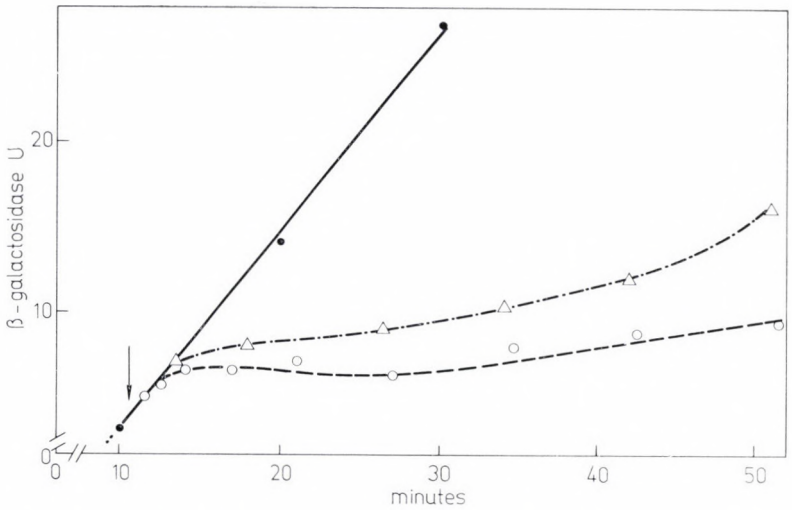

Fig. 2. Conditions as in Fig. 1, except glucose concentration ($10^{-3}M$)

repression lasted only for 14—15 minutes, then enzyme synthesis reappeared, though at a much lower rate than in the control. This finding may be explained by a catabolite repression following transient repression. In the presence of factor C the decrease in the rate of enzyme synthesis was delayed; the activity curve showed a slight rising tendency and at the end of the experiment the rate of enzyme synthesis was definitely higher as compared to the culture containing glucose alone.

In the experiments shown in Fig. 3, 10^{-4} *M* glucose was used. Transient repression was observed not only with glucose but also with glucose + factor C. In the presence of the latter, repression lasted a shorter time; it began later and was released earlier than with glucose alone. After the release the rate of enzyme synthesis increased gradually and by the end of the experiment it had considerably exceeded the control value. In the presence of glucose alone, transient repression was followed by catabolite repression; the rate of enzyme synthesis was lower than in the control culture.

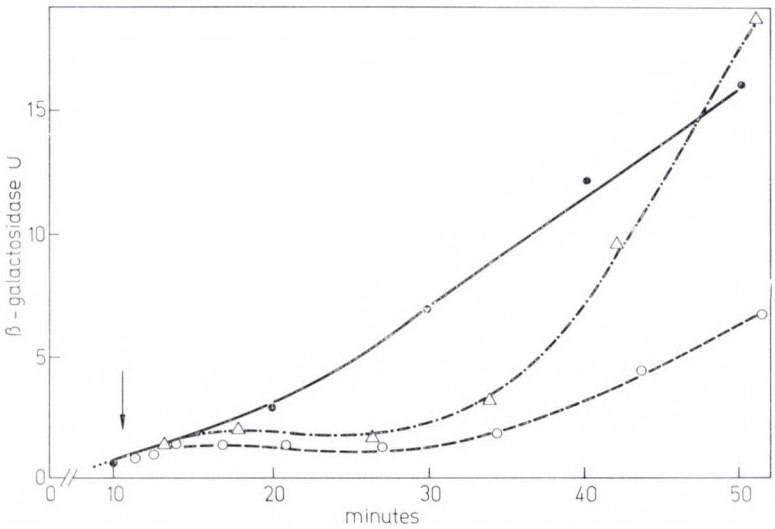

Fig. 3. Conditions as in Fig. 1, except glucose concentration (10^{-4} *M*)

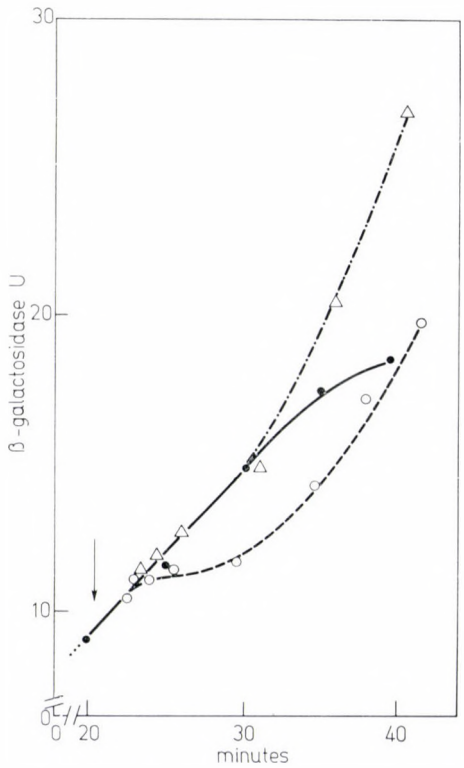

Fig. 4. Conditions as in Fig. 1, except glucose concentration (4×10^{-5} *M*)

Fig. 4 presents results obtained at 4×10^{-5} *M* glucose concentration. The small amount of glucose was also able to evoke transient repression, though this was shorter than that observed at higher glucose concentrations. After the release of transient repression the rate of enzyme synthesis increased gradually and at the end of the experiment it was higher than the control value, *i.e.* catabolic repression did not occur (even a stimulating effect was evident). At the same glucose concentration, factor C eliminated transient repression. The rate of enzyme synthesis was identical with that of the period of transient repression; subsequently β-galactosidase production rate increased and was considerably higher than in the control and in the glucose-containing culture.

If the culture was supplemented with factor C before the addition of glucose, the results were similar to the above findings.

Discussion

On the basis of previous findings [1] in the present experiments we have attempted to perform a detailed analysis of the effect of factor C.

It has been demonstrated that factor C, if added together with or before glucose, is able to influence the transient repression brought about by glucose. The highest concentration of glucose tested (2×10^{-2} *M*) caused a total repression of enzyme synthesis not released during the experiment. The increase in enzyme activity was delayed also in the presence of glucose + factor C, but enzyme synthesis continued at a rate markedly lower than in the control. At 10^{-3} *M* glucose, an essentially similar effect of factor C was demonstrated, but in this experiment the release of transient repression took place during the time of observation. Transient repression in the presence of factor C was observed only at 10^{-4} *M* glucose. In all experiments the repression, in other words the onset and the decrease of the rate of enzyme synthesis was delayed in the presence of factor C as compared to cultures supplemented only with glucose. In experiments where the release of transient repression occurred within the examined period of time, in the presence of factor C the release appeared earlier. At the lowest glucose concentration tested (4×10^{-5} *M*), development of transient repression was entirely prevented by factor C. In the presence of glucose + factor C the rate of enzyme synthesis was identical with that in the control in the period of transient repression.

The assumption that factor C stabilizes the already synthesized messenger RNA [1] and is thus responsible for the slight β-galactosidase synthesis taking place despite the inhibition of messenger RNA synthesis by glucose, cannot be denied on the basis of experiments 1, 2 and 4. However, experiment 3 and the fact that factor C *per se* failed to influence the inductive synthesis

of β-galactosidase in *E. coli* [14] contradict that assumption. Messenger stabilization, *i.e.* the possibility that on one messenger RNA molecule more enzyme molecules can be synthesized than in the control, ought to result in a higher enzyme level.

The course of the enzyme synthesis curve in the presence of glucose + factor C indicates rather that as an effect of factor C the development of repressor—operator complex is delayed in transient repression.

At lower glucose concentrations, transient repression was released during the course of the experiments. In experiments 2 and 3 the rate of enzyme synthesis was lower than in the control, even after release. This finding may be explained by a transition of transient to catabolite repression. In the presence of factor C, the release occurred earlier and the curve rose steeper: catabolite repression was slighter in degree. Thus it may be concluded that factor C generally inhibitis the development of glucose repression: it decreases both transient and catabolite repression.

In Fig. 4, where at 4×10^{-5} *M* glucose, factor C completely eliminated transient repression, the rate of β-galactosidase synthesis reappearing after an interval is higher in the presence of glucose alone than in the control, that is, no catabolite repression occurs. At the same time in the presence of factor C the rate of synthesis is even higher. No data are available in the literature as to this effect of glucose. This may be due to the fact that glucose repression is usually studied with cells grown on glycerol, a poorly metabolized carbon source and at higher glucose concentrations.

On the basis of the effects of factor C (delaying the development and promoting the release of transient repression and decreasing the degree of catabolite repression) its mode of action may be explained by an inhibition of catabolite-corepressor and repressor interaction and by an influence on repressor-operator binding. Accordingly, in view of earlier findings [3, 14] DNA is the most probable site of action of factor C.

If new theories concerning the development of transient repression and assumptions as to the role of cyclic 3',5'AMP [15, 16] are considered, there is a remarkable similarity between the effect of factor C and cyclic 3',5'AMP. This suggests that our product, which has not so far been obtained in electrophoretically pure form, may be contaminated with cyclic 3',5'AMP, a substance seemingly ubiquitous in microorganisms and in higher animals [17]. This assumption, however, may be refuted on the basis that while cyclic 3',5'AMP is thermostable, our factor C preparation had lost its effect after heating and also that the newest fraction kindly purified by Dr. J. Erdei by gel filtration through Sephadex G 75 column was more effective than the fraction passed through Dowex 50X2 ion exchange resin.

The similarity of effects is indicative of a similarity of the mode of action. According to Perlman *et al.* [18, 19] cyclic 3',5'AMP acting at the

promotor site of *lac* operon [20] is responsible not for induction but for the rate of transcription and is a molecule present in the cells at physiologically determined concentration. As an effect of glucose, first the decrease of 3',5'AMP level appears and then follows the deceleration of the transcription of gene *z*. It may be assumed that factor C exerts its effect at the same site, either directly or indirectly, by maintaining the actually effective cyclic 3',5'AMP level.

Preparation of an electrophoretically pure fraction needed for elucidating whether or not the above-discussed effect was due to a complex activity of ingredients of the product, is in progress.

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ELECTRON MICROSCOPIC STUDIES IN EXPERIMENTAL KERATOCONJUNCTIVITIS LISTERIOSA

I. PENETRATION OF LISTERIA MONOCYTOGENES INTO CORNEAL EPITHELIAL CELLS

By

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Summary. Electron microscopic studies have been carried out in guinea pigs on the penetration into corneal epithelial cells of *Listeria monocytogenes*. Epithelial cells were invaded usually by one or two Listeria each. Before the advancing organism, the cell membrane became invaginated. As the organism advanced further into the cytoplasm, the invagination developed into a membrane-bound vacuole containing the agent. Subsequently, the membrane disintegrated and the organism remained surrounded by a characteristic granular material. When the organism released in this manner again approached the cell membrane it protruded at that site. Depending on the absence or presence of oedema in the intercellular space, this part of the host cell together with the agent was either pushed into the neighbouring cell which later incorporated the organism, or it was demarcated into the intercellular space in case of a considerable intercellular oedema.

In later stages, 36—48 hours after infection, the number of cellular organelles increased in the cells containing the bacterium and in the neighbouring cells.

It has been concluded that in connection with keratoconjunctivitis listeriosa the term "facultative intracellular parasitism", which has so far been applied only to the interaction of host and parasite in relation to macrophage-type cells should be extended to the epithelial cells of the cornea.

SUTER [1] in 1956 applied the term facultative intracellular parasites to certain pathogenic bacteria. The most important organisms belonging to this group are *Mycobacterium tuberculosis*, *Francisella tularensis*, *Salmonella* and *Brucella*. These bacteria are not destroyed by macrophages at the beginning of the infective process but are able to multiply in the cytoplasm of the macrophage. After a given time varying with different organisms, a state of cellular immunity develops [2—6].

In earlier studies on the facultative intracellular parasitic properties of *Listeria monocytogenes* the organism was introduced intravenously or intraperitoneally into experimental animals. Under these circumstances the interaction of host and parasite appeared mainly in relation to macrophage-type cells. Under natural conditions the causative agent gains entrance to the body through an epithelial barrier between the body and the environment. The keratoconjunctivitis listeriosa model in guinea pigs [7, 8] described by ANTON [9] in 1934, offers an adequate method for investigating the development on infection when the agent enters the body through the epithelial barrier.

We have performed an electron microscopic study to establish the mode of entrance of the organism into the epithelial cell, the ultrastructural changes developing in the host cell, the fate of the organism released from the host cell, and the spread of infection from one cell to another.

Materials and methods

Culture preparation. L. monocytogenes serotype 1 strain "Bodajk" was isolated by Dr. G. SZEMERÉDI (National Institute of Veterinary Hygiene, Budapest) from the brain of a sheep dying from listeriosis. Before conjunctival infection of guinea pigs, the organism was cultured on blood agar at 37°C for 18 hours.

Technique of infection. Guinea pigs of both sexes of our own breed, weighing 250—300 g were used. Groups of 3 animals were infected by means of a platinum loop into the conjunctival sac of both eyes with 10^9 live cells. The animals were sacrificed by cervical dislocation 1, 3, 6, 9, 12, 24, 36, 48 and 72 hours after inoculation. Five animals were used as controls.

Electron microscopic technique. The method of KAYE [10] was modified as follows. Two per cent OsO_4 solution (pH 7.2) containing 0.045 g per ml of sucrose was stored at 4°C and used for fixation. After sacrificing the animals, a small amount of the solution was injected into the anterior chamber of the eye so that the cornea became slightly protruded and opalescent. After rapid enucleation the whole eyeball was fixed for 40 minutes at 4°C, cut into pieces of 0.5×3 mm and fixed for further 50 minutes at 4°C. The preparation was then washed in Soerensen, phosphate buffer, dehydrated in alcohol series contrasted with 1% uranyl acetate dissolved in 70% alcohol for 60 minutes and embedded in Durcupan. Thin and ultrathin sections were cut with a glass knife in the Reichert ultramicrotome. Post-contrasting was done with uranyl acetate and REYNOLDS' lead citrate [11]. Thin sections were stained with 1% toluidine blue. Ultrathin sections were examined with an SEM 2—3 electron microscope operating at 80 kV.

Results

The electron microscopic structure of the normal guinea pig cornea (Fig. 1) has been dealt with in a previous study [9].

Light microscopic examination indicated that as soon as 3 hours after the inoculation of 10^9 *Listeria*, a few cells of the outer layer of the corneal epithelium already contained the organism [7]. For electron microscopic examination of initial changes, sections prepared 9 hours after infection were the most suitable as by that time the number of cells containing the agent had increased sufficiently for an easy detection on the screen.

At this stage, corresponding to the site where the organism has come near to the cellular surface the plasma membrane of the host cell which has been undulate before became smooth and invaginated in front of the advancing *Listeria* (Fig. 2 *Aa*, Fig. 3). Then the invaginated part was separated and appeared in the cytoplasm as a vacuole containing the microorganism (Fig. 2 *Ab*). Later the wall of the vacuole was partly or totally destroyed and the cytoplasm showed a characteristic change by becoming more electron dense and granulate (Fig. 2 *Ac*, Fig. 4). This change in the cytoplasm was so characteristic that when it was observed in one of the serially cut sections with-

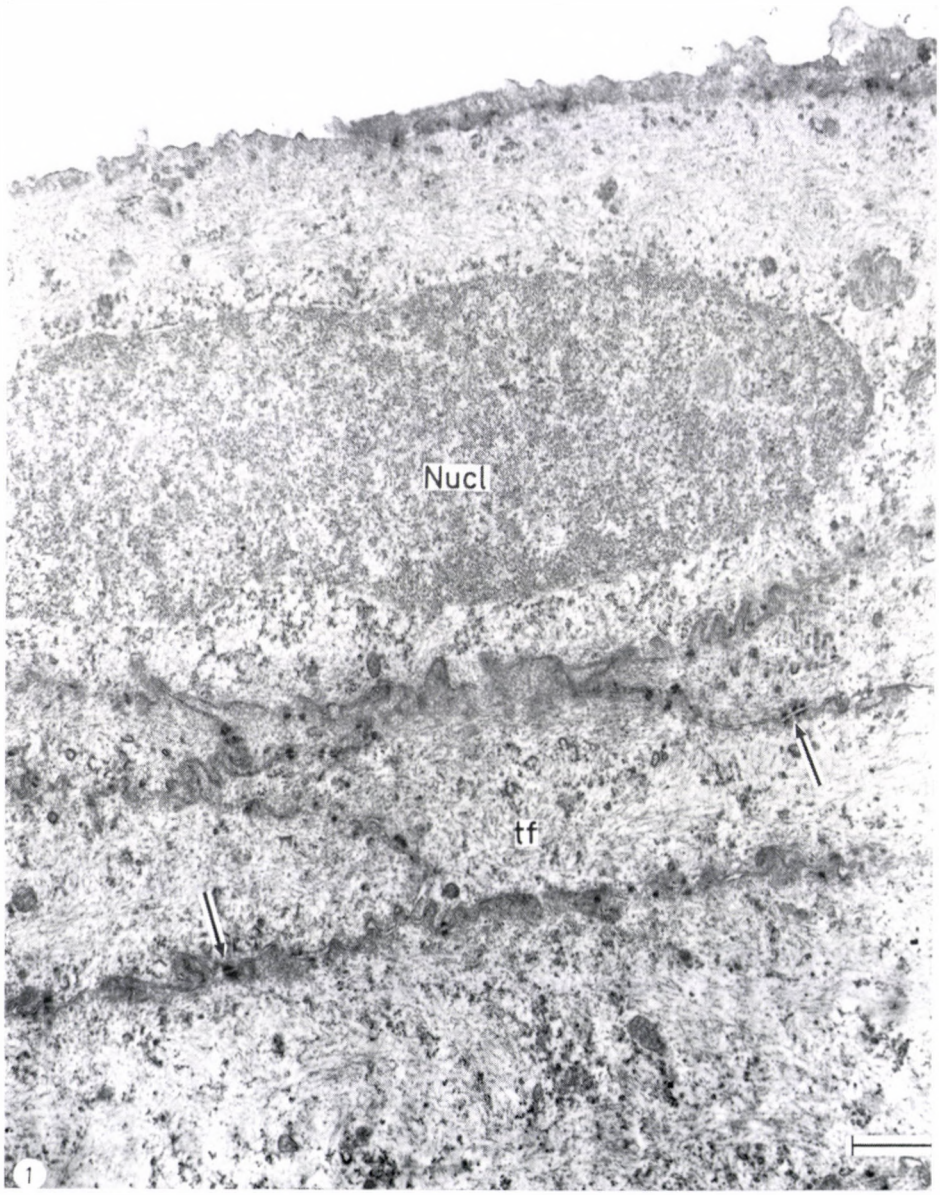

Fig. 1. Corneal epithelium in control animals. Cells of the stratified squamous epithelium are in close contact with one another. The cytoplasmic membrane is undulate or dentate. Numerous desmosomes (arrow). Tonofilaments situated at various sites in the cytoplasm. The cytoplasm is poor in organelles

out the microorganism, it was known for certain that *Listeria* will be found in the sections of deeper parts (Fig. 5).

In subsequent stages when, probably due to cytoplasmic flow, the agent again advanced near the plasma membrane, the membrane of the host cell protruded into the neighbouring cell, pushing forward the plasma membrane of the latter (Fig. 2 *Ad*, Fig. 6). Where the two parallel membranes had formed a cavity around the agent, no desmosomes were visible (Fig. 2 *Ae*, Fig. 6). The cavity containing the organism intruded more and more deeply into the neighbouring cell and finally separated so that both the organism

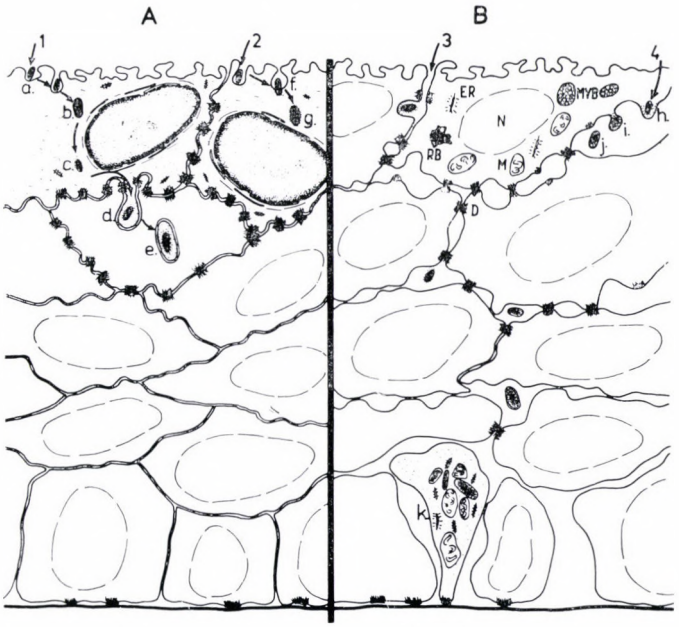

Fig. 2. A. Early stage of *Listeria* infection. The epithelial cells are in close contact with one another. *Aa.* The agent reaches the cell surface. The plasma membrane becomes smooth and invaginates before the agent; *Ab.* The invaginated part of the plasma membrane is separated and appears in the cytoplasm as a vacuole containing the agent; *Ac.* The wall of the vacuole disintegrates and the cytoplasm becomes more granular and more electron dense around the organism; *Ad.* The plasma membrane of the host cell when the organism approaches it protrudes into the neighbouring cell and pushes its membrane. At this site desmosomes are absent; *Ae.* The cavity containing the organism separates and the wall of the vacuole developing in this manner is constituted by a double membrane originating from two different adjacent plasma membranes; *Af.* At the site where the agent advances from the direction of the surface, the invaginating cell membrane disintegrates. Around the agent the cytoplasm shows a special change; *Ag. Listeria* surrounded by characteristically changed cytoplasm of the host cell. *B.* Corneal epithelium 36 hours after infection. Intercellular oedema in every layer of the epithelium. Contact between epithelial cells persists only at the site of the desmosomes (D) or has been lost (k). Increased numbers of mitochondria; they are swollen and their cristae are fragmented. Numerous multivesicular bodies (MVB). The ribosomes are detached from the rough-surfaced endoplasmic reticulum (ER). Rest bodies (RB) appear; *Bh.* The plasma membrane protrudes before the advancing organism into the intercellular space; *Bi.* The cavity formed in this manner separates; *Bj.* Vacuole containing the agent in the intercellular space

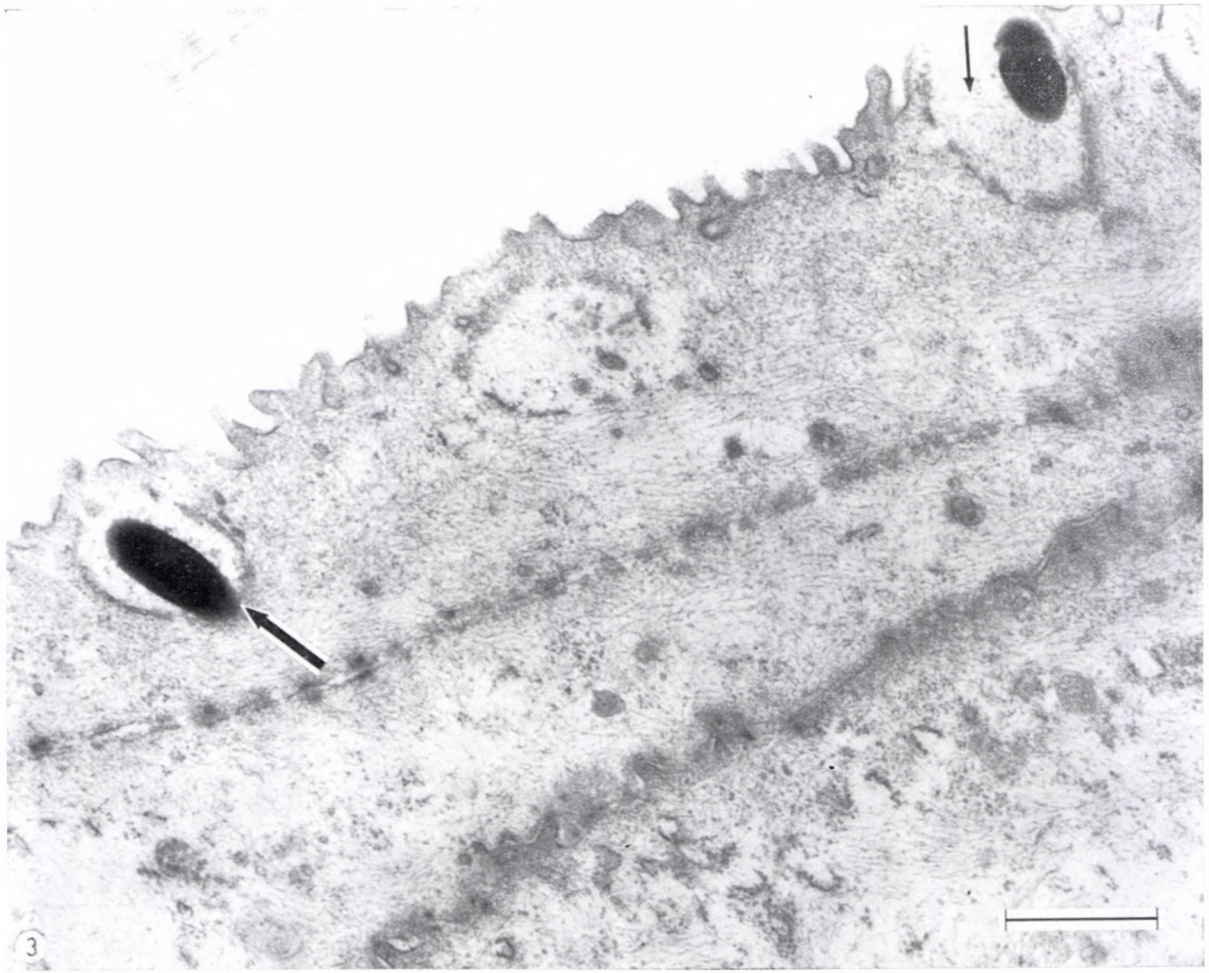

Fig. 3. Nine hours after infection. Two *Listeria* organisms entering an epithelial cell in the surface layer. Before one of the organisms (thin arrow) the plasma membrane is invaginated (see Fig. 2 *Aa*). In front of the other *Listeria* (thick arrow) the cytoplasmic membrane is destroyed at a circumscribed area and the cytoplasm shows characteristic changes (see Fig. 2 *Af*)

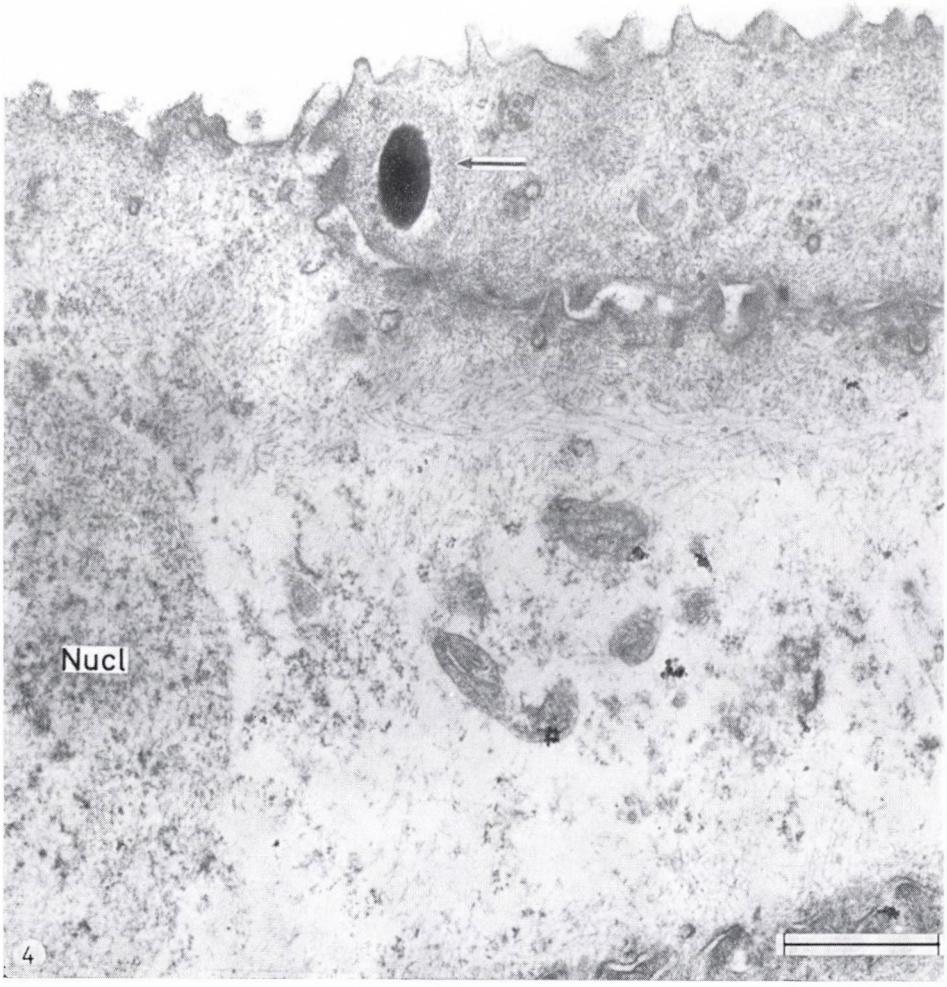

Fig. 4. Nine hours after infection. The *Listeria* within the cell is not surrounded with phagosome membrane. The cytoplasm has became more electron dense (arrow) (see Fig. 2 *Ag*)

and the cytoplasmic part surrounding it became incorporated by the second cell. In this the agent is enveloped by a double membrane; unlike on its entrance into the first cell where it had been surrounded by a single plasma membrane (Fig. 2 *Ae*, Fig. 5).

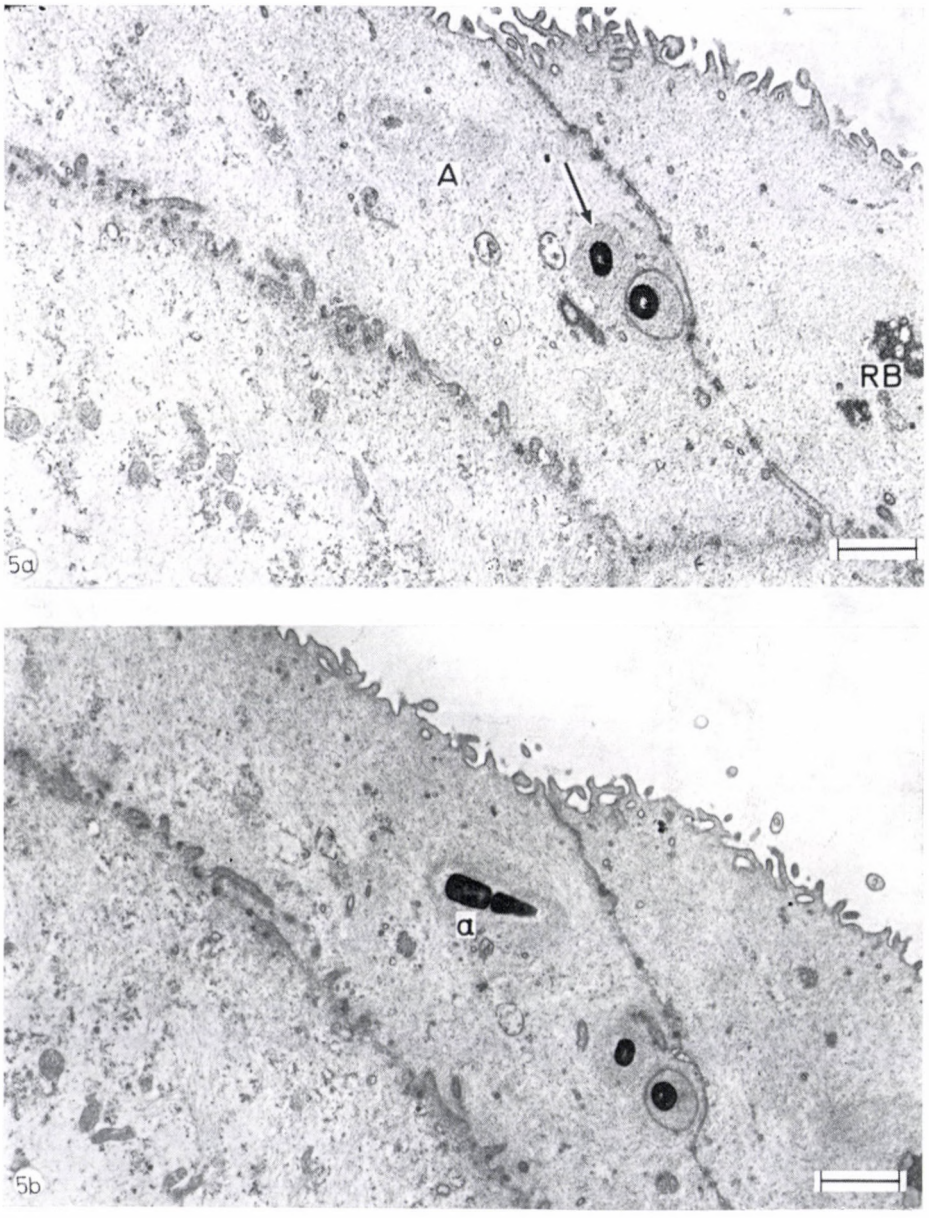

Fig. 5. Nine hours after infection. The same area cut at different levels (*a* and *b*). Characteristic density of cytoplasm (A) at site corresponding to the position of the more deeply situated dividing *Listeria* (*a*). Beside the plasma membrane an intracellular agent is surrounded by characteristic denser cytoplasm and membrane fragments (arrow); another agent is surrounded by double unite membrane. In the neighbouring cell a rest body (RB) is visible

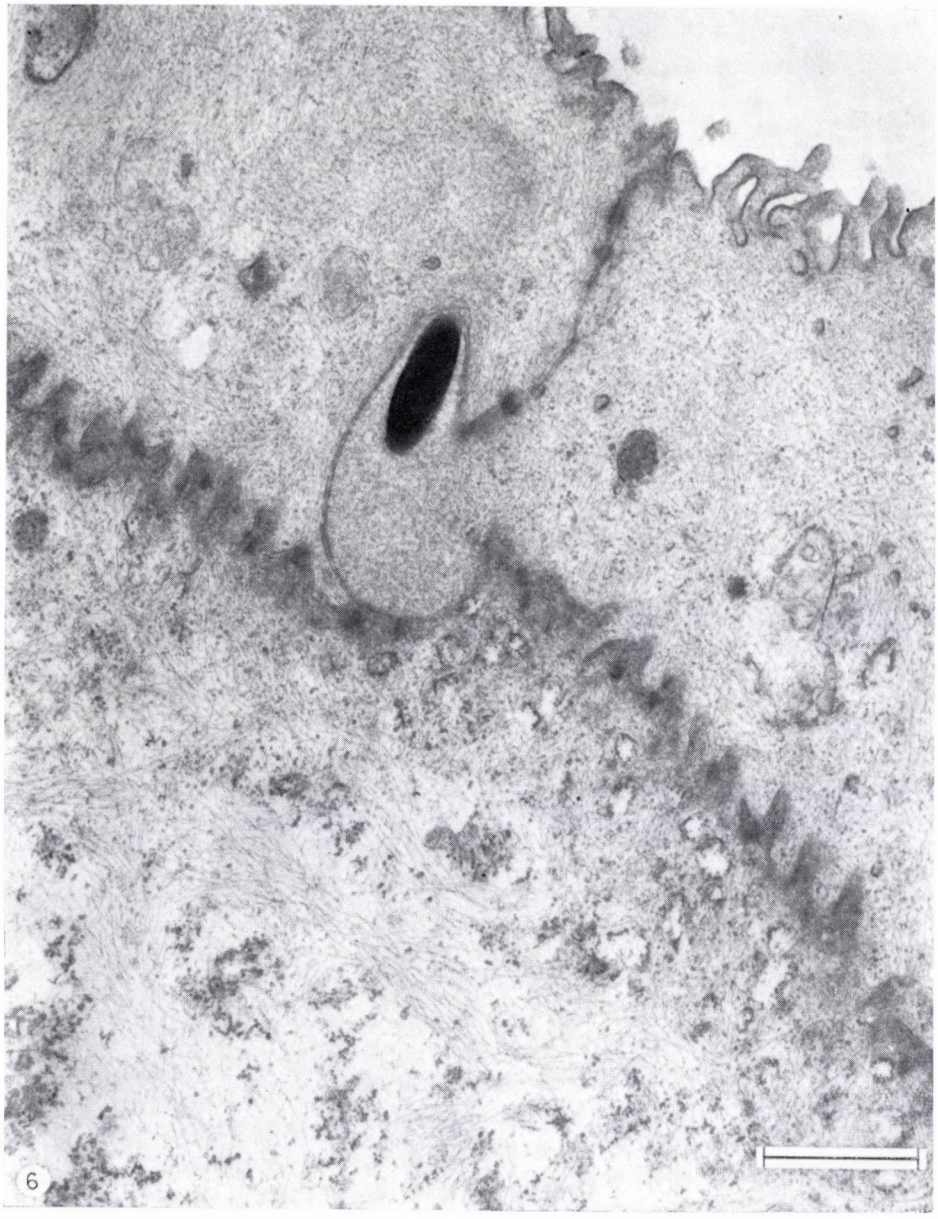

Fig. 6. Nine hours after infection. *Listeria* entering the neighbouring cell. Around the advancing organism plasma membranes form a cavity. At this site of the plasma membrane no desmosomes are visible. Increased density of cytoplasm around the organism (see Fig. 2 *Ad*)

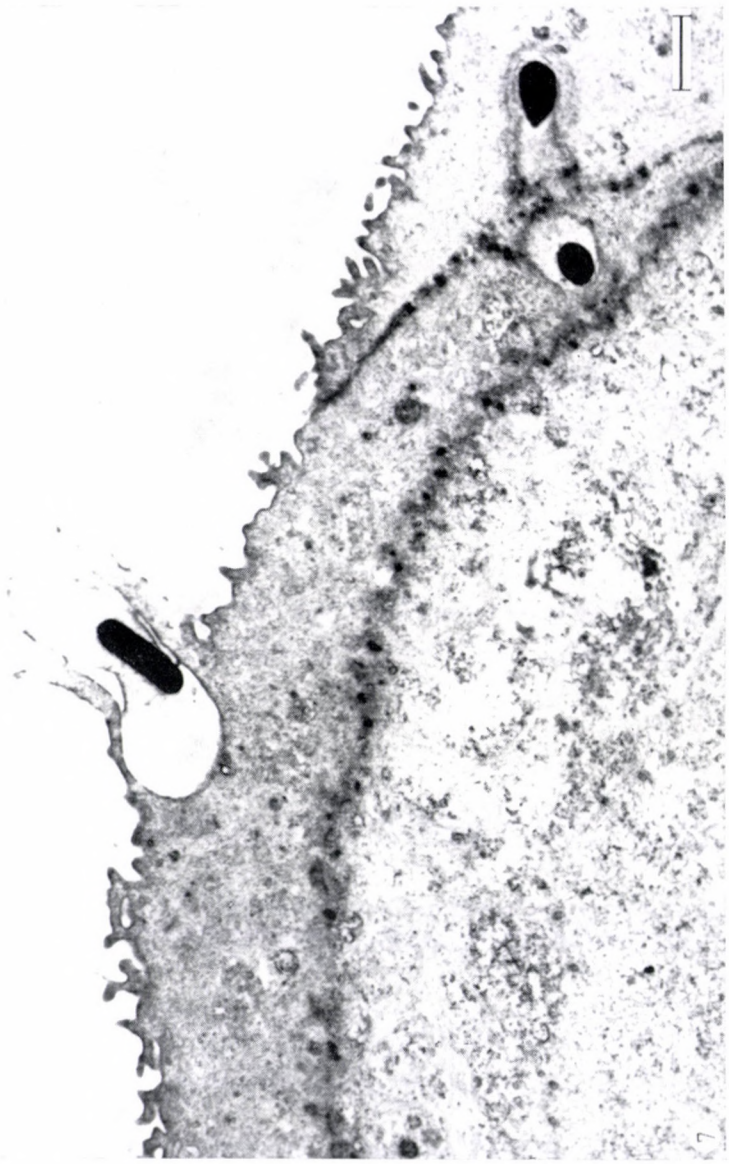

Fig. 7. Nine hours after infection. Listeria entering (?) or leaving (?) a superficial epithelial cell

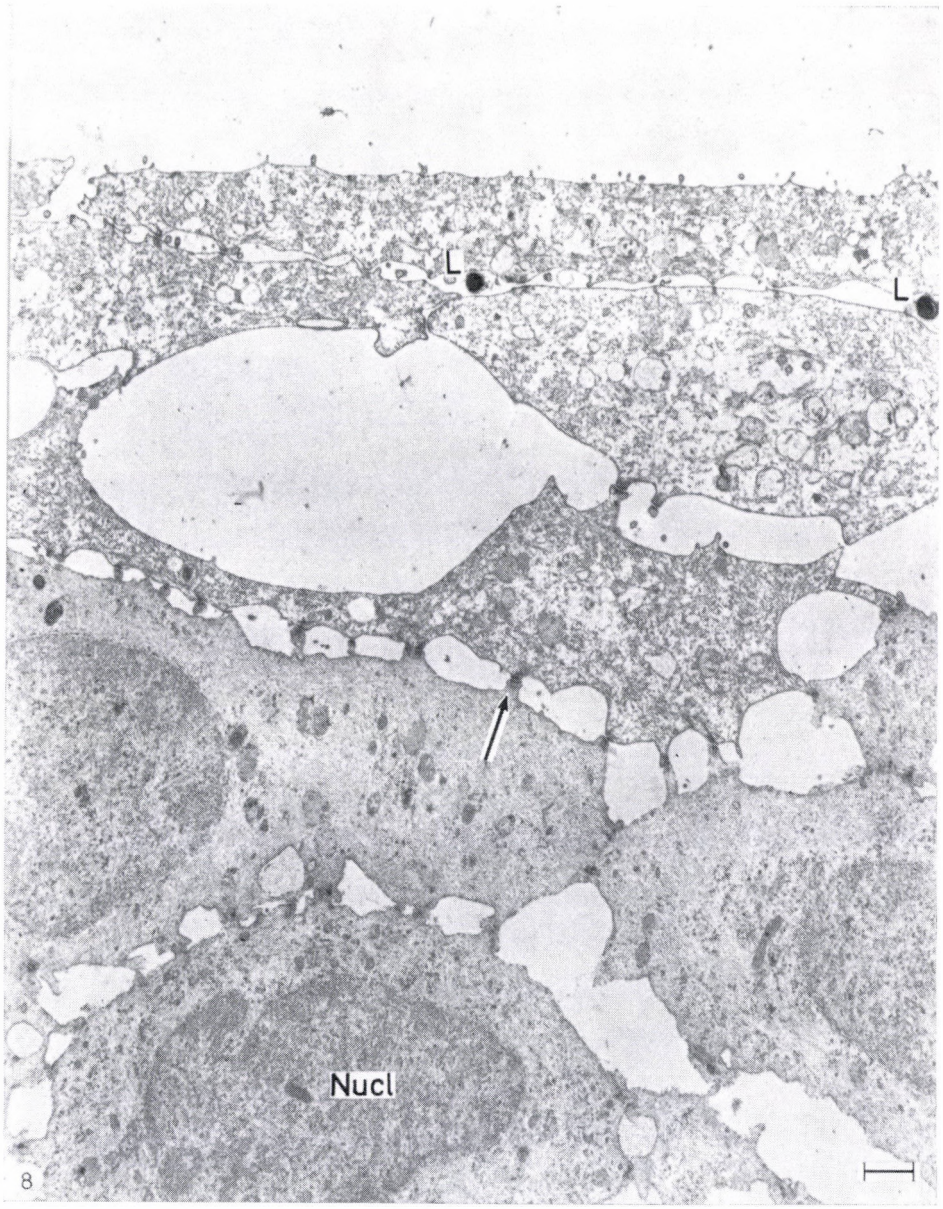

Fig. 8. Thirty-six hours after infection. Severe ultrastructural damage in the three external cell-row layers of the cornea. More deeply situated cells retained their ultrastructure well. Widely dilated intercellular space. The connection between neighbouring cells remained unbroken, the longest at the site of the desmosomes (arrow). Listeria organisms are present in the intercellular space (L)

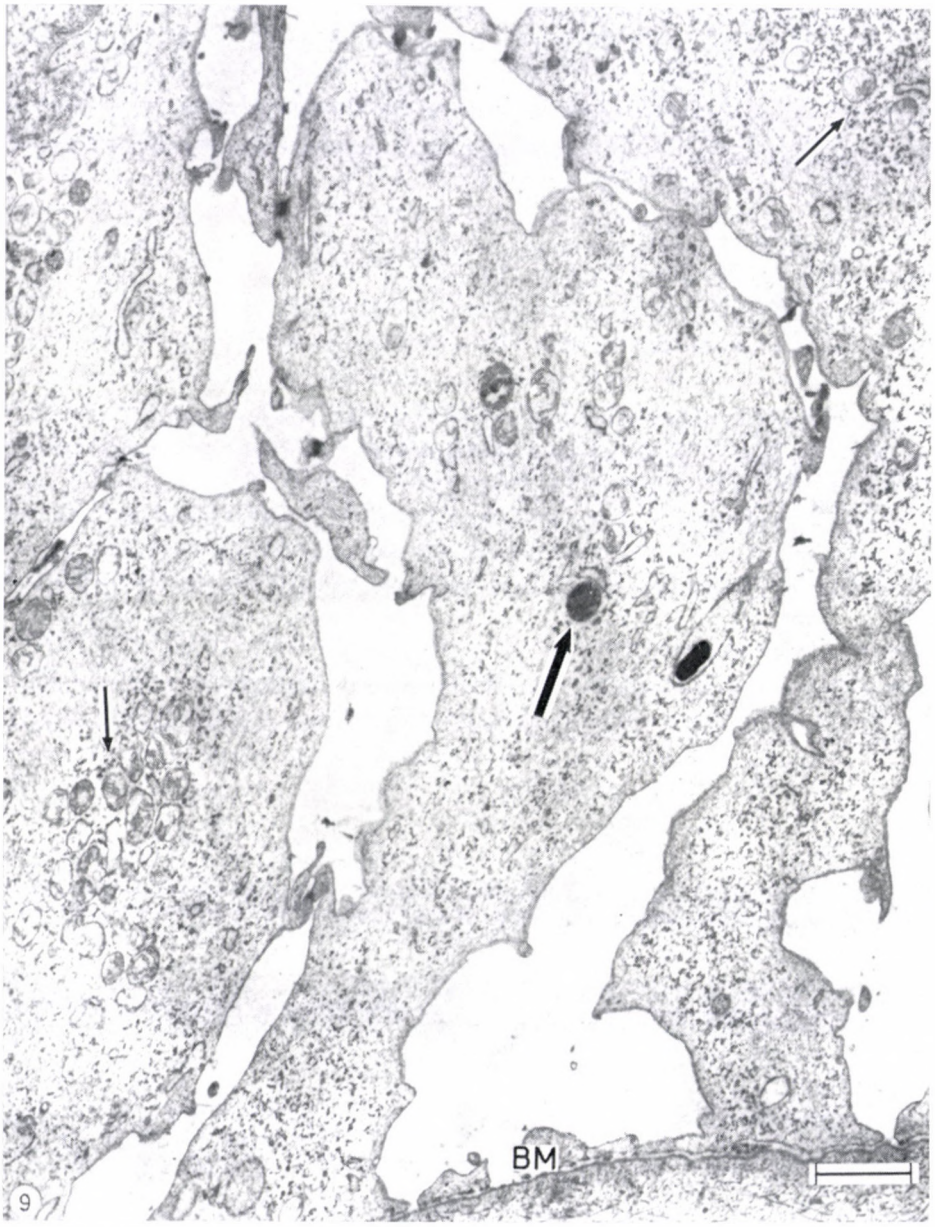

Fig. 9. Forty-eight hours after infection. Basal layer of the corneal epithelium. The cells are separated by intercellular oedema. The contact between the cells is broken in a large area, even at the site of the desmosomes. Increased number of mitochondria (arrow). In the cell shown in the middle of the section the agent is surrounded by double unite membrane, beside which a lysosome-like formation is present (thick arrow). The basal membrane (BM) is retained

A different manner of entrance of *Listeria* into the cell was also observed. Sometimes the plasma membrane invaginating before the advancing organism was destroyed at a site and thus the *Listeria* and the cytoplasm came into direct contact (Fig. 2 *Af*, Fig. 3). Around the microorganism gradually advancing towards deeper areas of the cell, the above-mentioned characteristic changes of the cytoplasm were visible (Fig. 2 *Ag*).

Occasionally the direction of the movement of the agent could not be determined exactly and it was assumed that the cell might cast out the phagosome together with the organism (Fig. 7).

Nine hours after the infection there was still a close contact between the epithelial cells of the cornea. This contact was ensured by the undulate or dentate course of the cell membrane and the junctional complex described by FARQUHAR and PALADE [12]. In subsequent stages of the process, however, the initial cellular oedema (Fig. 4) changed into a severe cellular dystrophy and at the same time an intercellular oedema has became evident in every layer of the epithelium. The contact between the epithelial cells remained longest at the site of the desmosomes; later even this contact ceased at several sites (Fig. 8). In this manner a new way has opened for the spread of the infective process and the organism can reach the cells situated more deeply either by advancing from the direction of the surface or by wandering through the dilated intercellular space after having been released from the epithelial cell.

While the normal corneal epithelium contains a small number of mitochondria as well as rough and smooth endoplasmic reticula (Fig. 1), 38—48 hours after the infection the number of mitochondria increased considerably (Fig. 1) and various dystrophic conditions (fragmentation of christae, swelling of mitochondria) were observed. The number of rough and smooth-surfaced endoplasmic reticula increased, and the ribosomes separated from the rough-surfaced endoplasmic reticulum (Fig. 9). At this stage many multivesicular bodies appeared.

Listeria cells usually retained their ultrastructure in later stages of the infection, dividing cells still were present and only a small number of osmiophilic rest bodies could be detected.

Discussion

On the basis of light microscopic studies [7, 8] it would appear that in the case of keratoconjunctivitis listeriosa the epithelial barrier of the cornea and conjunctive exert not only a protective function as they do in other infections, but also promote the spreading of the pathological process. Corneal epithelial cells ensure favourable conditions for the multiplication of the causative agent and at the same time protect them against the phagocyting

activity of polymorphonuclear leucocytes. Our electron microscopic studies have confirmed that living epithelial cells are entered by the agent which is able to multiply in it. It seems justified to assume that the host cell is viable at the time of the organism's entrance since its ultrastructure is more or less retained and it shows a vital reaction against *Listeria* production of phagosomes, changes in cytoplasmic structure, etc. (as shown in Figs 3, 4, 5, 6, 7 and 9). The appearance of dividing bacteria indicates the viability of the agent (Fig. 5). In addition to *Listeria*, other, mainly facultative intracellular pathogens may also enter the cells of the epithelial barrier [8, 13—23].

The process of intrusion of *Listeria* into the cytoplasm of epithelial cells may be regarded as a special form of phagocytosis, or, using a different nomenclature, of endocytosis. The phenomenon is similar to that observed in keratoconjunctivitis shigellosa [8, 20, 21] and differs of course from what can be seen in endocytosis observed in the case of microphages and RES cells. An important point of the difference is that this form of endocytosis is a specific one because the epithelial cells are phagocyting only *Listeria* [7, 8], human pathogenic *Shigella* [8, 20, 21] and certain *E. coli* strains [14, 22]. Several authors have supposed the phagocytic activity of intestinal epithelial cells [24, 27]. The extraneous coat covering the surface of the corneal cells may act in selective endocytosis similarly to the complex acid mucopolysaccharide material covering the cell surface lining the gastrointestinal tract.

To study the intracellular parasitism upon the penetration of *Listeria* into the host cell cytoplasm, our observations have been compared with other data on the ultrastructural changes in macrophages phagocyting this organism.

North and Mackeness [28, 29] injected *Listeria* intraperitoneally into immunized and control mice and examined the peritoneal macrophages 10 minutes after the infection. Armstrong and Sword [30] also injected *Listeria* intraperitoneally and examined the macrophage-type cells in the spleen of mice at 72 hours. In every case of the 10 minute experiment and in most cases of the 72 hour experiment the agent was found in the phagosome within the cytoplasm. In both experiments it was evident that the content of cytoplasmic vesicles (probably lysosomes) had entered the phagosome containing the organism.

In the amorphous electron-dense material surrounding the organism, less electron dense areas were observed after 72 hours, they spread into the cytoplasm surrounding the phagosome at those circumscribed sites where the phagocytic vacuolar membrane had broken. The phenomenon was regarded as a toxic, degenerative process.

The disintegration of the phagocytic vacuolar membrane is a process leading to a basic alteration of the relationship between the organisms and the host. Some parallelism seems to exist between the ability of certain bacteria for intracellular parasitism and the disintegration of the phagocytic

vacuolar membrane surrounding the infective agent. For example, STALEY *et al.* [27] found that around cells of non-enteropathogenic *E. coli* strains unable for intracellular parasitism, disintegration of the phagosomic membrane can never be observed. ROTH and WILLIAMS [31] injected virulent and avirulent *B. anthracis* intravenously into mice and observed that in the reticuloendothelial cells of the spleen the virulent bacillus, in contrast to the avirulent one, was never surrounded by phagosomic membrane. ARMSTRONG and SWORD [30] assumed that the haemolysin released from *Listeria*, which according to JENKINS *et al.* [32] also had a lecithinase activity, played a part in the disintegration of the phagosomic membrane. In our opinion, for the disintegration of the phagosome the haemolysin is not primarily or not alone responsible, since this phenomenon occurs even more definitely in keratoconjunctivitis shigellosa, too.

In contrast to the macrophages, the corneal epithelial cells contained very few lysosome-like formations. Consequently, it may be assumed that only the first stage of phagocytosis, the ingestion of the agent, has taken place. In the host cell there are no larger amounts of enzymes harmful for the infective agent so that it is able to multiply freely in the cytoplasm of epithelial cells. We failed to observe the grave injury around the bacteria described by ARMSTRONG and SWORD [30]. This finding is perhaps in analogy with the observation of DICAPUA *et al.* [33] who, after implanting a diffusion chamber with *Listeria* into the peritoneal cavity of mice, recorded no toxic effect. The bacterium-free filtrate of the content of the chamber when injected intracerebrally to mice also failed to exert any appreciable toxicity.

On the basis of the above data and our findings it would appear that the activity of lysosomal enzymes is needed for the release by *Listeria* of substances injurious to the cytoplasm of the living host cell. The damage observed in the superficial cell layer of the cornea at later stages of the infection (Fig. 8) may not be due to a direct effect of the agent present intracellularly in the epithelium, but rather to toxic substances released as an effect of the *Listeria*-phagocyting activity of polymorphonuclear leucocytes. In this stage, considerable leucocytic infiltration occurs in various areas of the eye [7].

The special electron dense granular material appearing in the cytoplasm of corneal epithelial cells around the causative agent develops probably from the ground plasma and its microfilaments and disintegrated membrane materials. This formation, localized to a small area of the cell, does not seem to affect the viability of the host cell. An analogous cytoplasmic reaction has been observed in intestinal epithelial cells [27, 34].

In view of the spreading of the infective process, an important phenomenon shown in electron microscopic examinations [23] is the agent's passage from one host cell to another without passing through the intercellular space (Fig. 6). In later stages of the inflammatory process, when there is a definite

intracellular oedema, the terminal barriers and desmosomes are damaged (Figs 8 and 9) and the spreading of the organism occurs by paracellular passage. This process opens the way for the passage of different ions, water soluble molecules and drugs [35].

Parallel with the progression of the infection, the number of organelles, mainly mitochondria and smooth-surfaced endoplasmic reticulum, increases in cells containing the organism and in the neighbouring cells. This phenomenon is an important indicator of the interaction of the organism and host cell resulting in an increase of cellular metabolism, but is not a specific response to the presence of the microorganism, since it may also occur in intoxications due to parasitic infection [36].

It may be concluded that the living corneal epithelium is a suitable medium for the multiplication of *Listeria* and, therefore, the term intracellular parasitism of bacteria which has so far been applied only to the interaction of host and parasite in relation to macrophages, should be extended in relation of the epithelial cells of the cornea.

Acknowledgement. We are indebted to Dr. E. MÉRŐ, Section of Bacteriology of our Institute for the *Listeria* culture, and to Mr. L. KISS, Mrs. I. PÁLL and Mr. L. SZABÓ for skilled technical assistance.

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EFFECT OF SORBIC ACID ON THE GROWTH OF SOME SPECIES OF YEAST*

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Summary. The effect of sorbic acid (SA) on growth kinetic parameters in aerobic and anaerobic cultures of *Candida beverwijkii, C. claussenii, C. krusei, C. pseudotropicalis, C. utilis, Procandida albicans, Pc. tropicalis* and *Saccharomyces carlsbergensis* has been studied. SA exerted different effects on growth rate constant, lag phase and final density depending on the concentration of the agent, mode of incubation, presence of reduced glutathione (GSH) and species of the examined organism. As a main effect of SA the lag phase became prolonged. The substance caused an approximately identical inhibition of growth rate and development of final density. The effect of SA was more definite under anaerobic conditions. This was due partly to the fact that during aerobic cultivation of certain species the concentration of SA decreased and thus the inhibitory effect of the substance became weaker and weaker. As in the presence of GSH the decrease of SA was absent or less definite, it has been concluded that GSH indirectly increased the effect of SA.

In a previous work [9] it has been pointed out that although sorbic acid (SA) is a widely applied preservative used especially in food industry for the inhibition of the growth of yeasts and molds, only few authors have studied the kinetics of its inhibitory effect. Our growth kinetic studies on *Procandida albicans* model have shown that the effect of SA depends on the aeration of the culture and is to some extent influenced by the ingredients of the medium. We have also demonstrated that under aerobic conditions the agent is detoxicated by *Pc. albicans*. Detoxication of SA is prevented by reduced glutathione (GSH) and thus the growth inhibitory effect becomes more definite and prolonged.

Since many data indicate that the fungistatic effect of SA depends on the species examined [26], on the basis of the above-mentioned model experiments we investigated the action of SA on some further yeast species and devoted special attention beside the effect on growth kinetics to the possibility of detoxication of the agent and the influence of GSH in this respect.

Materials and methods

The methods were as described in our previous work [9] but only one kind of medium (Sabouraud glucose broth) was used. Sorbic acid was added to the medium as potassium sorbate (PS) produced by Farbwerke Hoechst A.G. Anaerobic (more correctly, semianaerobic

* Part of this investigation has been reported by T. DEÁK [8].

standing tube) cultures were incubated with 0.3 and 0.6 mg/ml, aerobic shaken T-tube cultures with 0.5 and 1.0 mg/ml concentrations of the agent. When GSH was added, its molar concentration was always 10 times less than that of PS. The concentration of PS in the cultures was determined at 0, 16, 24, 48 and 72 hours by direct photometry at 254 mμ by preparing suitable dilutions of 0.1—0.2 ml samples. Distillation was omitted, since in preliminary experiments distilled and direct samples yielded identical results.

Evaluation was made on the basis of the growth rate constant (k), lag phase (L) and final density (D). Initial optical density was set to 0.050 for all species. In comparing various species, differences in final density due to variations in the initial cell count were corrected by calculating the multiplication index (M) [21].

In order to make direct comparisons with the results of our previous study [9], *Pc. albicans* (OKI 85/1957) was re-examined together with the following cultures: *Candida beverwijkii* (OKI CLXXV/1967), *C. claussenii* (OKI 460 sect/1961), *C. krusei* (OKI 287/1964), *Pc. tropicalis* (OKI 302/1964), *Saccharomyces carlsbergensis* (OKI II/1966). Maintenance and cultivation of the strains were performed as described previously [9, 21].

All results represent mean values obtained in two parallel experiments. Analysis of variance was used for statistical evaluation [11, 35].

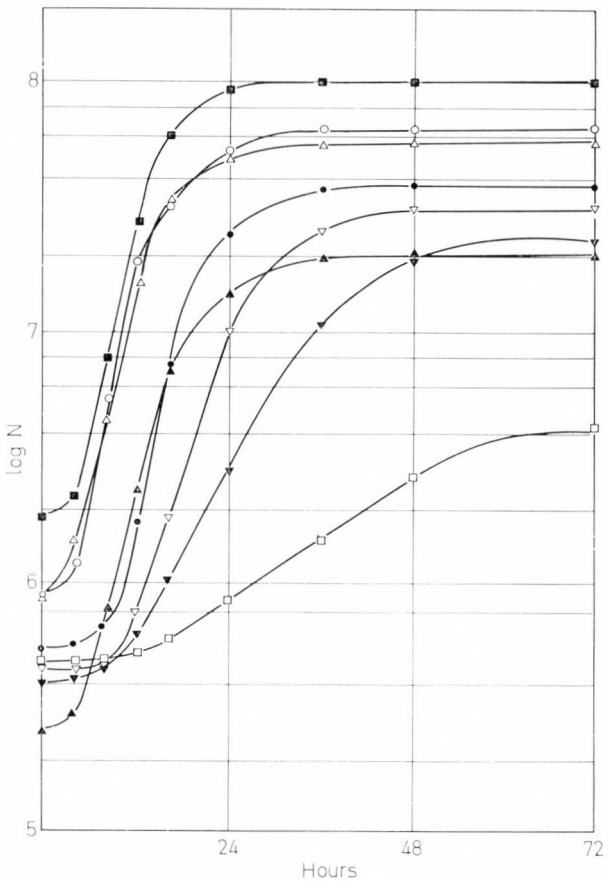

Fig. 1. Control growth curves for the examined yeast species. Aerobic cultures in Sabouraud glucose broth. ▽ = *C. beverwijkii*; ● = *C. claussenii*; ○ = *C. krusei*; □ = *C. pseudotropicalis*; ■ = *C. utilis*; △ = *Pc. albicans*; ▲ = *Pc. tropicalis*; ▼ = *S. carlsbergensis*

Results

For characterizing and comparing the growth properties of the examined species, the control growth curves are presented first (Figs 1 and 2). Differences in growth among the various organisms were evident. With the exception of *C. pseudotropicalis* and *C. carlsbergensis* all yeasts grew more rapidly and reached higher final densities than in anaerobic cultures. The differences in growth among the various species were more definite in aerobic cultures. It is evident that a shorter lag phase and a higher growth rate resulted in a higher final density and vice versa (Fig. 3). However, there was not always such an interrelationship between the growth parameters (see curves for *C. krusei* and *Pc. albicans* in Fig. 2). The influence of these two factors of final density differed also with the conditions of incubation; the interrelationship between k and M was closer in anaerobic than in aerobic cultures (Fig. 3).

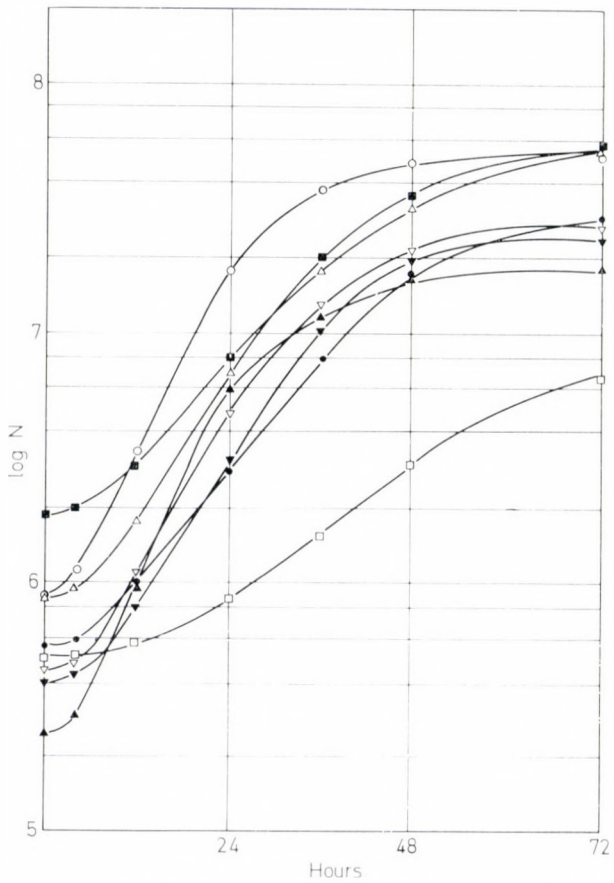

Fig. 2. Control growth curves for the examined yeast species. Anaerobic cultures in Sabouraud glucose broth. ▽ = *C. beverwijkii*; ● = *C. claussenii*; ○ = *C. krusei*; □ = *C. pseudotropicalis*; ■ = *C. utilis*, △ = *Pc. albicans*; ▲ = *Pc. tropicalis*; ▼ = *C. carlsbergensis*

Regarding all parameters, under the present experimental conditions the best growth was exhibited by *C. krusei*.

C. pseudotropicalis differed from all cultures presented in Figs 1 and 2 in showing a very weak growth. As compared to the control, growth of this

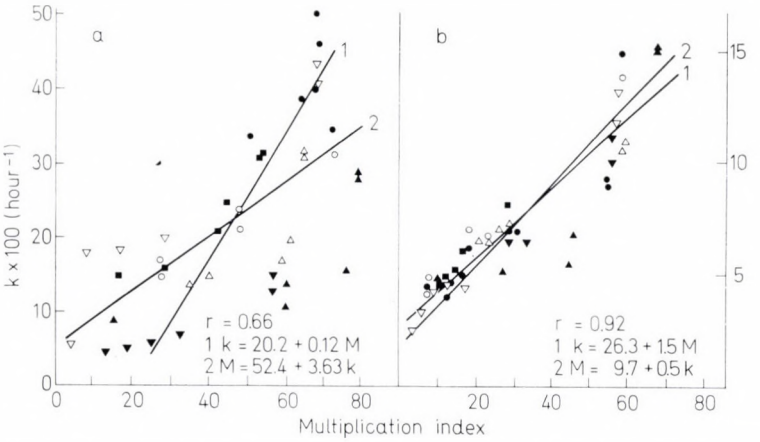

Fig. 3. Interrelationship of growth rate constant and multiplication index. Sabouraud glucose broth cultures. a = aerobic, b = anaerobic incubation. ▽ = *C. beverwijkii*; ● = *S. claussenii*; ○ = *C. krusei*; □ = *C. pseudotropicalis*; ■ = *C. utilis*; △ = *Pc. albicans*; ▲ = *Pc. tropicalis*; ▼ = *S. carlsbergensis*

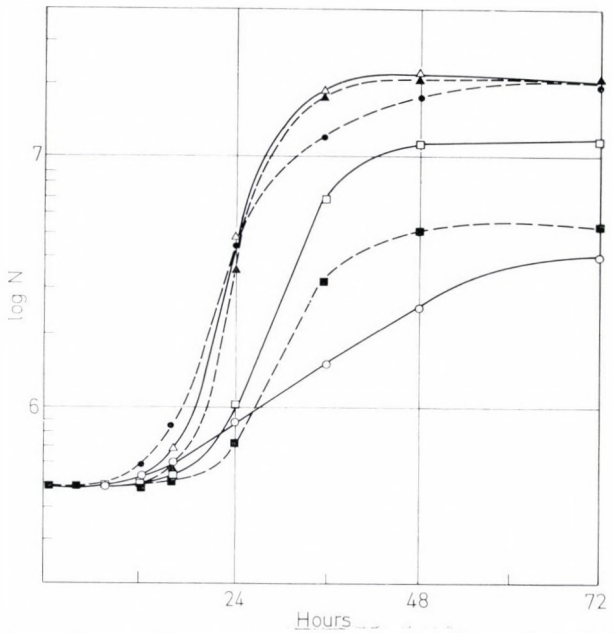

Fig. 4. Effect of potassium sorbate on growth curves for *C. pseudotropicalis* in the presence and in the absence of reduced glutathione. Aerobic cultures in Sabouraud glucose broth. Circles, control; triangles, 0.5 mg PS/ml; squares, 1.0 mg PS/ml. Continuous line: without GSH, dotted line: with GSH. GSH:PS molar ratio 1:10

species was always better, though different in degree, in the presence of SA, GSH or SA + GSH, as it is seen in aerobic growth curves in Fig. 4. Similar results were obtained under anaerobic conditions. This unexpected finding was explained by morphogenetic changes observed at the microscopic checking of the cultures. While in the control culture this species grew in pseudomycelian form, in the presence of the examined substances, however, it always exhibited a budding-type growth. This morphological change highly influenced the shape of growth curves obtained on turbidimetric estimation. Consequently, the results for growth inhibition experiments could not be evaluated and in the following their numerical data are omitted.

Values for the growth kinetic parameters, as determined from the growth curves, are summarized in Tables I—VI. These data and average values of the given parameter for 7 different species as well as results obtained in cultures with added substances and expressed as percentage of the control (inhibition % for k and M, prolongation % for L) demonstrate well the effect of various treatments, though do not allow a complex evaluation of the individual effects and the interactions of the examined factors: PS, GSH, mode of incubation (Inc) and sensitivity of species (Spec). These were estimated by analysis of variance.

Effect of PS on growth rate constant. On the basis of data in Tables I and II, Table VII shows the analysis of the effect of the examined factors on the alteration of the growth rate constant. It is evident that every factor exerts a significant effect by itself, that is, influences the k value. Double

Table I

Alteration of growth rate constant under the effect of potassium sorbate in the presence and absence of reduced glutathione in aerobic cultures

Species	$k \times 100$ (hr^{-1})					
	1	2	3	4	5	6
C. beverwijkii	43.0	40.0	19.8	18.0	17.6	5.4
C. claussenii	45.5	50.0	40.0	38.7	33.2	29.6
C. krusei	31.0	34.0	23.6	21.0	16.7	14.6
C. utilis	31.0	30.7	24.2	20.2	15.8	14.6
Pc. albicans	30.2	31.4	18.7	16.9	14.8	13.6
Pc. tropicalis	28.0	28.0	15.4	13.4	10.6	8.4
S. carlsbergensis	12.6	14.5	6.9	5.9	5.0	4.7
Mean	31.6	32.7	21.2	19.2	16.2	13.0
Effect in per cent inhibition	0.0	—4.0	33.0	39.0	49.0	59.0

1 = control, 2 = GSH 0.2 mg/ml, 3 = PS 0.5 mg/ml, 4 = PS 0.5 mg/ml + GSH 0.1 mg/ml, 5 = PS 1.0 mg/ml, 6 = PS 1.0 mg/ml + GSH 0.2 mg/ml.

Table II

Alteration of growth rate constant under the effect of potassium sorbate in the presence and absence of reduced glutathione in anaerobic cultures

Species	$k \times 100$ (hr^{-1})					
	1	2	3	4	5	6
C. beverwijkii	11.8	13.2	4.2	4.5	3.3	2.5
C. claussenii	9.0	8.8	6.8	4.1	6.2	4.5
C. krusei	14.0	15.0	7.0	6.6	4.8	4.3
C. utilis	8.1	7.0	6.0	5.2	4.9	4.5
Pc. albicans	11.1	10.7	7.0	7.0	6.5	6.5
Pc. tropicalis	15.0	15.2	6.9	5.5	5.2	4.7
C. carlsbergensis	11.2	10.4	6.4	6.4	4.5	5.0
Mean	11.5	11.5	6.3	5.6	5.1	4.6
Effect in per cent inhibition	0.0	0.0	45.0	51.0	56.0	60.0

1 = control, 2 = GSH 0.12 mg/ml, 3 = PS 0.3 mg/ml, 4 = PS 0.3 mg/ml + GSH 0.06 mg/ml, 5 = PS 0.6 mg/ml, 6 = PS 0.6 mg/ml + GSH 0.12 mg/ml.

Table III

Alteration of lag phase under the effect of potassium sorbate in the presence and absence of reduced glutathione in aerobic cultures

Species	L (hr)					
	1	2	3	4	5	6
C. beverwijkii	10.2	10.2	17.3	22.4	23.5	24.4
C. claussenii	9.2	6.5	14.7	13.8	17.5	18.4
C. krusei	3.2	2.6	4.5	4.8	5.4	7.3
C. utilis	3.5	3.5	6.6	8.0	9.8	10.5
Pc. albicans	2.9	2.6	4.6	6.4	9.0	10.2
Pc. tropicalis	3.8	3.4	10.2	11.0	13.7	24.7
S. carlsbergensis	8.4	5.9	15.0	16.8	20.0	28.5
Mean	5.9	5.0	10.4	11.9	14.1	17.7
Effect in per cent prolongation	0.0	−15.0	76.0	102.0	139.0	200.0

1—6 = as in Table I.

and triple interactions between the factors are mostly also significant, that is, their effect on growth rate is not independent from one another. From the analysis of interactions the following were concluded.

Table IV

Alteration of lag phase under the effect of potassium sorbate in the presence and absence of reduced glutathione in anaerobic cultures

Species	*L* (hr)					
	1	2	3	4	5	6
C. beverwijkii	4.5	5.5	15.0	17.5	22.0	26.0
C. claussenii	5.0	5.5	14.5	17.5	25.0	31.0
C. krusei	2.0	3.0	6.5	6.5	10.0	10.0
C. utilis	6.0	7.0	15.0	18.0	25.5	27.0
Pc. albicans	5.0	5.0	12.0	14.0	20.0	22.0
Pc. tropicalis	3.0	3.5	9.5	9.0	18.5	27.0
S. carlsbergensis	6.0	5.0	8.5	8.5	14.5	14.5
Mean	4.5	4.9	11.6	13.0	19.4	22.5
Effect in per cent prolongation	0.0	2.0	158.0	189.0	330.0	400.0

1—6 = as in Table II.

Table V

Alteration of multiplication index under the effect of potassium sorbate in the presence and absence of reduced glutathione in aerobic cultures

Species	*M* (ratio of final and initial density)					
	1	2	3	4	5	6
C. beverwijkii	69	69	29	18	9	5
C. claussenii	69	69	64	64	51	27
C. krusei	73	73	48	48	28	28
C. utilis	54	54	45	43	29	17
Pc. albicans	65	65	61	59	40	35
Pc. tropicalis	80	80	76	60	60	16
S. carlsbergensis	57	57	33	25	19	14
Mean	67	67	51	45	34	20
Effect in per cent inhibition	0	0	24	33	48	70

1—6 = as in Table I.

Table VI

Alteration of multiplication index under the effect of potassium sorbate in the presence and absence of reduced glutathione in anaerobic cultures

Species	*M* (ratio of final and initial density)					
	1	2	3	4	5	6
C. beverwijkii	58	58	10	12	6	4
C. claussenii	55	55	31	13	18	8
C. krusei	59	59	19	23	8	8
C. utilis	28	28	17	15	12	11
Pc. albicans	60	59	29	26	23	21
Pc. tropicalis	68	68	46	44	27	10
S. carlsbergensis	57	57	34	29	18	16
Mean	55	55	27	23	16	11
Effect in per cent inhibition	0	0	51	58	71	80

1—6 = as in Table II.

Table VII

Analysis of variance of growth rate constant

Variance	Sum of squares	Degrees of freedom	Mean squares	F
Inc	4,680.4	1	4,680.4	3,900.3***
Spec	1,487.8	6	248.0	206.6***
GSH	16.6	1	16.6	13.8**
PS	2,133.5	2	1,066.8	889.0***
Inc × Spec	1,708.0	6	248.7	207.2***
Inc × GSH	7.1	1	7.1	5.9*
Inc × PS	428.2	2	214.1	178.4***
Spec × GSH	13.9	6	2.3	1.9
Spec × PS	254.0	12	21.2	17.6***
GSH × PS	20.4	2	10.2	8.5**
Inc × Spec × GSH	30.5	6	5.1	4.3*
Inc × Spec × PS	106.6	12	8.9	7.4***
Inc × GSH × PS	15.1	2	7.5	6.3*
Spec × GSH × PS	26.2	12	2.2	1.8
Residual (error)	14.8	12	1.2	
Total	10,943.1	83		

*P = 5%, **P = 1%, ***P = 0.1%.

1. Alteration of the mode of incubation from aerobic to anaerobic decreases the k value; the degree of this decrease strongly depends on the species (Table VIII). Under anaerobic conditions all species show an approximately identical growth rate (Fig. 5).

Table VIII

Alteration of growth rate constant as a function of the interaction of mode of incubation and species

Species	Mode of incubation	
	Aerobic	Anaerobic
C. beverwijkii	24.0	6.7
C. claussenii	39.7	6.7
C. krusei	23.7	8.7
C. utilis	22.8	6.0
Pc. albicans	21.0	8.3
Pc. tropicalis	17.2	8.8
S. carlsbergensis	8.5	7.2

The data represent mean values of $k \times 100$ (hr^{-1}). Confidence interval (I) for the difference of mean values (d): $I_{P1\%} = d \pm 1.93$.

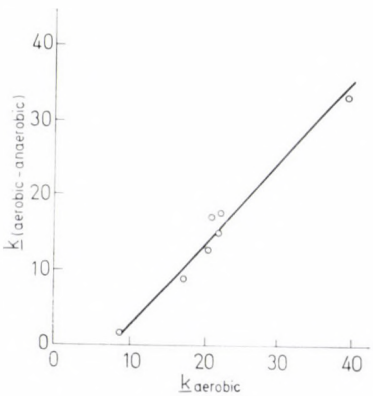

Fig. 5. Alteration of growth rate constant as a function of the mode of incubation; k values in hr^{-1}

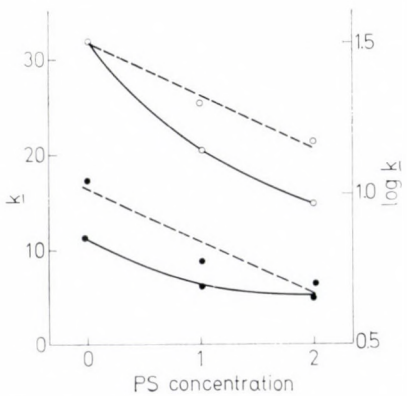

Fig. 6. Alteration of growth rate constant as an effect of incubation mode and potassium sorbate. ○ = aerobic, ● = anaerobic incubation. Continuous line: k, dotted line: log k. PS concentration in aerobic incubation, 0.5 and 1.0 mg/ml, in anaerobic incubation 0.3 and 0.6 mg/ml. The abscissa indicates only that the experiment was performed at a concentration of zero (0), at a certain level (1) or at twice the latter (2)

2. As compared to aerobic incubation, under anaerobic conditions the k value decreases to 1/3 at all concentrations of PS. The increase of the effect at increasing PS concentrations is more marked under aerobic than under anaerobic conditions (Fig. 6).

3. In aerobic incubation, GSH *per se* does not influence the k value significantly. Combined with PS, however, they considerably decreased the growth rate constant. Under anaerobic conditions the effect of PS+GSH does not differ significantly from the effect of PS (Table IX).

Table IX

Alteration of growth rate constant as a function of the triple interaction of mode of incubation, potassium sorbate and reduced glutathione

	PS					
	Aerobic			Anaerobic		
	0*	0.5	1.0	0	0.3	0.6
O	31.7	21.5	16.4	11.4	6.3	5.1
GSH	32.7	19.1	13.1	11.4	5.7	4.9

$I_{P_1\%} = d \pm 1.6$.
The data represent mean values of $k \times 100$ (hr^{-1}).
*Potassium sorbate concentration in mg/ml; GSH:PS = 1:10 mole; the higher GSH concentration in the control.

Effect of PS on lag phase. On the basis of the data in Tables III and IV the analysis of variance of the effect of factors tested is presented in Table X. All factors exerted statistically significant effects when analysed separately. The effect shown with GSH was due probably to an interaction of GSH+PS, since GSH failed to exhibit any significant effect in double or triple combination with other factors. Of the triple interactions, only Inc×Spec×PS was significant. Conclusions of the analysis were as follows.

1. When conditions are changed from aerobic to anaerobic, the L value increases in the majority of organisms. Under the effect of PS the lag phase is prolonged in all species about twofold at lower and threefold at higher concentrations (Fig. 7).

2. Under anaerobic conditions PS acts more markedly and the effect of increased concentrations is also more definite (Fig. 8).

3. GSH exerted a significant effect only in the presence of PS; the L value increased when PS was supplemented with GSH and the effect increased at higher concentrations of either PS or GSH (Table XI).

Table X

Analysis of variance of lag phase

Variance	Sum of squares	Degrees of freedom	Mean squares	F
Inc	81.9	1	81.9	40.9***
Spec	948.4	6	158.1	79.1***
GSH	50.2	1	50.2	25.1***
PS	2,524.3	2	1,262.2	631.1***
Inc×Spec	474.5	6	79.1	39.6***
Inc×GSH	1.1	1	1.1	0.6
Inc×PS	124.4	2	62.2	31.1***
Spec×GSH	20.9	6	3.5	1.9
Spec×PS	244.6	12	20.4	10.2***
GSH×PS	41.8	2	20.9	10.5**
Inc×Spec×GSH	14.3	6	2.4	1.2
Inc×Spec×PS	151.4	12	12.6	6.3**
Inc×GSH×PS	1.7	2	0.8	0.4
Spec×GSH×PS	52.3	12	4.4	2.3
Residual (error)	24.2	12	2.0	
Total	4,756.0	83		

**P = 1%, ***P = 0.1%.

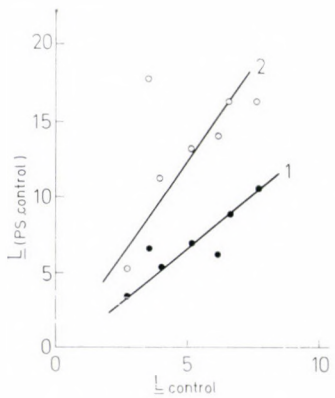

Fig. 7. Prolongation of lag phase as a function of potassium sorbate concentration. PS levels (1 and 2) as in Fig. 6

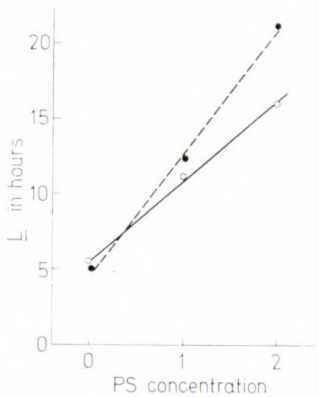

Fig. 8. Alteration of lag phase under the effect of incubation mode and potassium sorbate. Continuous line: aerobic incubation, dotted line: anaerobic incubation. PS levels (0, 1, 2) as in Fig. 6

Table XI

Alteration of lag phase as a function of the interaction between potassium sorbate und reduced glutathione

	PS		
	0	1	2
O	5.2	11.2	16.9
GSH	5.1	12.6	20.2

$I_{P1\%} = d \pm 0.52$.

PS concentrations as in Fig. 6. GSH levels corresponding to 0, 1 and 2 PS levels: 0.2, 0.1 and 0.2.

Effect of PS on multiplication index. Variance analysis of the multiplication index (Table XII on the basis of Table V and VI) gave generally the same results as that of the two other parameters. Among double interactions in combination Inc×Spec, the character of alteration was similar as with *K* value. According to the analysis of the combination Inc×PS, potassium

Table XII

Analysis of variance of multiplication index

Variance	Sum of squares	Degrees of freedom	Mean squares	F
Inc	5,376.0	1	5,376.0	480.0***
Spec	5,428.8	6	904.8	80.0***
GSH	505.2	1	505.2	45.1***
PS	23,235.0	2	11,617.5	1,037.3***
Inc×Spec	1,740.0	6	290.0	25.9***
Inc×GSH	58.3	1	58.3	5.2*
Inc×PS	494.9	2	246.5	22.0***
Spec×GSH	407.8	6	68.0	6.1**
Spec×PS	2,205.5	12	183.8	16.4***
GSH×PS	291.7	2	145.9	13.0***
Inc×Spec×GSH	100.4	6	16.7	1.5
Inc×Spec×PS	700.6	12	58.4	5.2**
Inc×GSH×PS	74.7	2	37.4	3.3
Spec×GSH×PS	448.8	12	37.4	3.3*
Residual (error)	134.1	12	11.8	
Total	41,199.8	83		

*P = 5%, **P = 1%, ***P = 0.1%.

sorbate at low concentrations was less effective under aerobic than under anaerobic conditions; at higher concentrations the increase in effectiveness was more definite in aerobic incubation (Fig. 9).

The effect of the various combinations of the mode of incubation, PS and GSH may be analysed as triple interactions (Table XIII). It is seen that, similarly as on parameters k and L, GSH acts only in the presence of PS and its effect increases when the concentration of either PS or GSH is increased

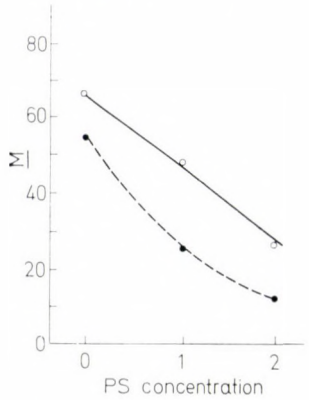

Fig. 9. Alteration of multiplication index under the effect of incubation mode and potassium sorbate. *M* value: ratio of final and initial density. Continuous line: aerobic, dotted line: anaerobic incubation. PS levels (0, 1, 2) as in Fig. 6

under both aerobic and anaerobic incubations. Under aerobic conditions GSH + PS decreased the *M* value more markedly than PS *per se*; in anaerobic cultures the difference was not significant.

Effect of the decrease of PS concentration. As in our previous experiments [9] PS concentration decreased during aerobic incubation of *Pc. albicans*. In aerobic cultures the same result was obtained for *C. claussenii*, *C. pseudotropicalis*, *C. utilis* and *Pc. tropicalis* but not for *C. beverwijkii*,

Table XIII

Alteration of multiplication index (M) as a function of the triple interaction of mode of incubation, potassium sorbate and reduced glutathione

	PS					
	Aerobic			Anaerobic		
	0*	0.5	1.0	0	0.3	0.6
O	66.5	51.5	33.7	55.0	28.0	11.0
GSH*	66.5	45.3	20.3	55.0	23.3	11.2

$I_{P1\%} = d \pm 5.48$.
M = ratio of final and initial density; *PS and GSH concentrations as in Table IX.

C. krusei and *S. carlsbergensis* (Table XIV). Under anaerobic conditions none except two organisms were able to decrease PS concentration. With *C. claussenii* and *Pc. tropicalis* there was a slight decrease (17 and 10%, respectively) but only in the absence of GSH and at low initial PS concentrations (0.3 mg/ml). In aerobic conditions and at higher PS concentrations in the presence of GSH the decrease of PS was always considerably less; at lower PS concentrations the effect of GSH appeared only as a slight delay in PS decrease.

Accordingly, the yeast species examined could be classified into two groups on the basis of their ability to decrease PS concentration in aerobic

Table XIV

Decrease of potassium sorbate concentration in aerobic cultures of some yeasts in the presence and absence of reduced glutathione

Species	Time of incubation, hours	Decrease of PS concentration, %			
		1	2	3	4
C. claussenii	16	0	0	6	6
	24	34	34	8	6
	48	90	96	10	3
	72	100	100	43	9
C. pseudotropicalis	16	0	0	0	0
	24	10	15	0	4
	48	70	67	10	5
	72	100	100	24	11
C. utilis	16	0	6	4	10
	24	16	20	14	8
	48	44	44	36	8
	72	100	96	36	12
Pc. albicans	16	8	12	2	8
	24	28	16	0	8
	48	48	50	8	15
	72	100	100	40	22
Pc. tropicalis	16	14	10	5	2
	24	44	24	11	5
	48	46	32	12	2
	72	100	92	48	14

1 = PS initial concentration, 0.5 mg/ml, 2 = PS initial concentration, 0.5 mg/ml + GSH 0.1 mg/ml, 3 = PS initial concentration, 1.0 mg/ml, 4 = PS initial concentration, 1.0 mg/ml + GSH 0.2 mg/ml.

cultures. This difference was expected to be reflected in the effect on the growth kinetic parameters of PS or of its combination with other factors. The above analyses failed to indicate this difference clearly as the 7 species were evaluated separately. In Table XV a new kind of variance analysis is presented: the species were divided into the above two groups and two concentration levels for each of PS and GSH were united.

Table XV

Analysis of variance of multiplication index. Analysis according to 2^4 factorial experiment

Variance	Sum of squares	F
Inc	812.25	361.00***
Spec	144.00	64.00*
GSH	49.00	21.77*
PS	4,422.25	1,965.44***
Inc × Spec	121.00	53.77*
Inc × GSH	9.00	4.00
Inc × PS	42.25	18.77*
Spec × GSH	12.25	5.44
Spec × PS	256.00	113.77**
GSH × PS	49.00	21.77*
Inc × Spec × PS	25.00	11.11
Inc × GSH × PS	9.00	4.00
Spec × GSH × PS	12.25	5.44
Inc × Spec × GSH*	2.25 } 4.5	
Residual (error)	2.25 }	
Total	5,967.75	

*Inc × Spec × GSH united with the residual (degrees of freedom, 2); degrees of freedom in all other variances, 1 (total 15), that is, the mean of squares is equal with the sum of squares (2.25 in the residual).

*P = 5%, **P = 1%, ***P = 0.1%.

The analysis indicated that the effects of PS and mode of incubation were highly significant even separately, while the significant effect of the species factor and GSH was probably due to a double interaction. The analysis of the latter (Table XVI) showed that the *M* values decreased significantly upon the effect of both PS and anaerobiosis (Table XVI A) and that GSH caused a significant alteration only in the presence of PS (Table XVI B), as it was expected from the former analysis. The new analysis, however, showed also that while the effect of PS was significant in both species groups, the

difference between the groups appeared only in the presence of PS (Table XVI C). Similarly, although a change in the mode of incubation caused significant alterations in both groups, the difference between the two groups appeared only under aerobic conditions (Table XVI D). This finding indicates that the basis of the significant difference between the groups really consisted of the decrease of PS in aerobic cultures and that differentiation of the species into the two groups was justified. It is also evident that in yeasts causing the decrease of PS in aerobic cultures significantly higher M values may be obtained, in other words the degree of growth inhibition is considerably lower than in other species.

Table XVI

Analysis of double interactions of factors influencing the multiplication index (M)

A	Inc×PS		B	GSH×PS	
	Aerobic	Anaerobic		0	GSH
O	66.5	53.0	O	61.0	61.0
PS	36.5	19.0	PS	31.2	24.2

C	Spec×PS		D	Inc×Spec	
	0	PS		Aerobic	Anaerobic
I	60.6	34.7	I	57.2	37.5
II	62.0	20.7	II	45.7	37.0

M = ratio of final and initial density; I = species detoxifying PS, II = species not detoxifying PS, $I_{P5\%} = d \pm 4.56$; $I_{P1\%} = d \pm 11.46$.

Discussion

The inhibitory action of SA on the multiplication of yeasts may be characterized by changes in three growth kinetic parameters (k, L, M). The effect of SA on the parameters differed and showed alterations according to SA concentration, presence of GSH, mode of incubation, and the species examined.

As regards the aerobic growth of the control cultures, *C. krusei* and *S. carlsbergensis* occupied two extreme positions; on the basis of all growth kinetic parameters the former grew best and the latter worst (Fig. 1). In the present experiments, similarly to the findings of VAS [33] and to the results of our earlier investigations [21], only k and M showed an interaction (Fig. 3). *C. beverwijkii* and *C. claussenii* were characterized by a long lag phase and high growth rate.

As to the mode of incubation, under anaerobic conditions all species grew less readily than in aerobic cultures (Fig. 2). This finding may be explained by a lower effectiveness of anaerobic energy production. The only exception was *S. carlsbergensis*, the examined strain of which (Schwechat brewer's yeast) had been adapted to fermentation and thus grew equally well with both modes of incubation. Considering all growth parameters, under anaerobic conditions *C. krusei* and *Pc. tropicalis* showed the best, while *C. utilis* and *C. claussenii* the worst growth.

No attempts have been made to elucidate the reason for the morphogenetic change in *C. pseudotropicalis*. The change occurred in the presence of sulfhydryl-containing or sulfhydryl-reacting compounds (GSH or SA), which are known to influence oxidoreduction and the proteindisulfide-reductase enzyme systems playing and important role in cell division and morphogenesis [10, 13, 20].

Growth characteristics of the organisms in Sabouraud glucose broth were generally very similar to those revealed in earlier studies, in which various species were compared in glucose and other carbohydrates [21].

The growth-inhibiting activity of SA was evident for all three parameters. The most definite effect was exerted on the lag phase, which was prolonged about four times. The inhibitory effect of SA on k and M was of about the same order (both values decreased approximately to half); this finding indicates again the correlation between the two growth parameters. The results are in agreement with other authors' growth kinetic studies with SA [22–25, 31]. Only HARADA *et al.* [12] state that SA, although it considerably decreased the growth rate and final density, was almost ineffective on the lag phase. According to their paper, they made the first readings at 16, 28 and 42 hours, which was evidently insufficient for an exact determination of the duration of the lag phase.

The effect of SA on growth kinetic parameters was influenced by several factors. Preliminary experiments with *Pc. albicans* [9] have already shown that the inhibitory effect depends mainly on the concentration of SA and the mode of incubation. Studies of other species confirmed these findings and threw light on the difference among the organisms in sensitivity to SA. Statistical evaluation of the results allowed a thorough analysis of the effect of the factors alone or in combination. In evaluating the results, another factor exerting a decisive influence should also be considered, the alteration of the initial concentration of SA during cultivation.

There are a number of data in the literature concerning the decrease of SA concentration in preserved products [1–7, 14, 17, 18, 34]. The decrease occurred mainly in products with high initial counts of microorganism or in products the preparation of which are accompanied with intensive microbial activity (*e.g.* pickles). Thus it has been supposed that the decrease of SA

was due to microorganisms. Certain bacteria [30, 37, 38] and molds [15, 16, 19, 28, 29] were shown to decompose SA. As to yeasts no such findings were available in the literature; COSTILOW *et al.* [3] failed to demonstrate this effect in *Torulopsis holmii*.

From our experiments it is evident that the concentration of SA decreases in aerobic cultures of *C. claussenii*, *C. pseudotropicalis*, *C. utilis*, *Pc. albicans* and *Pc. tropicalis*. Our previous experiments with *Pc. albicans* also demonstrated this effect and suggested that the decrease was associated with a detoxication of SA during aerobic metabolism, which process is accompanied with the ceasing of the inhibitory effect. In the present experiments there was a slight reduction in the amount of SA also in certain anaerobic cultures of *C. claussenii* and *Pc. tropicalis*. This finding might have been due to the fact that incubation in standing tubes ensured only semianaerobic conditions and that for the turbidimetric estimation the tubes had been shaken. Thus the two organisms, especially active in SA detoxication, could decrease the concentration of the agent even under semianaerobic conditions. More extensive studies on the detoxication of SA will be presented in a subsequent paper.

During aerobic incubation, especially at lower initial concentrations, SA decreased markedly or even disappeared from cultures of the above organisms (Table XIV). As the decrease was observed only in aerobic cultures, under anaerobic conditions even lower SA concentrations were enough for marked and durable inhibition and therefore the increase of the amount of SA promoted the degree of inhibition less definitely than in aerobic cultures. This was evident from the effect of SA on the growth rate and the multiplication index (Figs 6 and 9). The effect of increased SA concentrations on the lag phase of anaerobic cultures was more marked (Fig. 8). It should be considered here that at the beginning of the incubation period (during the lag phase) there was almost no decrease in SA concentration even under aerobic conditions; as shown in Table XIV the decrease amounted to a few per cent in the first 16 hours, so that in aerobic cultures SA was present practically at its initial (added) concentration.

The decrease in SA concentration occurred only in aerobic cultures of certain microorganisms. This finding explains the important differences in SA sensitivity among the species, as judged on the basis of all the three parameters. As the decrease was highest at the end of the incubation period, the most definite difference among the species was reflected in the effect of SA on the M value (Tables XV and XVI).

Further experimental results demonstrate the interaction of SA and GSH. The studies of other authors indicate that the inhibitory effect of SA on microorganisms is associated with the inhibition of sulfhydryl group-containing enzymes and it has been demonstrated that this effect can be eliminated *in vitro* with cysteine or GSH [36, 39]. Accordingly, these thiol

compounds were expected to eliminate or at least to decrease the inhibition by SA. However, in agreement with our previous experiments with *Pc. albicans* [9], an opposite effect was obtained, as in the presence of both GSH and cysteine the growth inhibition by SA increased and became more prolonged. A similar synergism has been demonstrated between SA and cysteine acting on the growth of *Aspergillus niger* [27].

According to the present findings, GSH *per se* does not influence significantly the value of any of the growth parameters. Together with SA it exerted an inhibitory effect which became stronger on increasing the concentration of either SA or GSH. As compared to the effect of SA alone, the combined effect of SA + GSH was higher under aerobic but not under anaerobic conditions (Tables IX, XI, XIII and XVI). On the basis of these results the inhibition-promoting effect of GSH can be explained only if it is assumed that GSH stimulates the entrance of SH into the cell by increasing the permeability of the cell membrane, by decreasing the redox potential or by exerting a specific effect on the membrane. Considering that the effect of GSH was more definite for SA-detoxifying species than for organisms not exhibiting such activity (Table XVI) and that in the presence of GSH the decrease of SA concentration was slower and less definite (Table XIV), it may be assumed that the synergistic effect of GSH is an indirect one, hindering or eliminating the detoxication of SA during cultivation. In addition, the effect upon SA detoxication may be secondary itself, when due to the increased entrance of the agent its own detoxication is inhibited. This is indicated by experiments in progress. The problem and elucidation of the role of GSH need further investigations as the above mentioned effect of GSH may be used in practice to prevent the microbial decomposition of SA. On the basis of this possibility a method has been elaborated for increasing the effectiveness of food preservatives [32].

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EFFECT OF SORBIC ACID ON THE GROWTH OF YEASTS ON VARIOUS CARBOHYDRATES

By

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Summary. The growth of *Candida beverwijkii*, *C. claussenii*, *C. utilis*, *Procandida albicans*, *Pc. tropicalis*, *Saccharomyces carlsbergensis* and *S. cerevisiae* was studied in the presence of 0.75 mg/ml potassium sorbate, in aerobic liquid media containing as substrate glucose, galactose, maltose, sucrose or raffinose. By comparing the effect of the inhibitor on given parameters of growth kinetics (growth rate constant, lag phase, multiplication index) with the different substrates added, conclusions were drawn as to the cause of inhibition at the level of carbohydrate metabolism. The results indicated that the cause of inhibition is often in the transport processes which are the first steps of carbohydrate metabolism. Interference with the active transport processes may play an important role in the mechanism of action of sorbic acid.

Changes of growth kinetic parameters of various yeast species with the kind of carbohydrate medium as well as possible relationships of growth parameters in different carbohydrate media with the early phases of carbohydrate metabolism have been described earlier [18]. In other studies [4, 9] it was demonstrated that sorbic acid (SA) as a fungistatic, had a distinct influence on growth parameters. Since, however, growth kinetic studies as a rule had been performed in one given growth medium and previously we had applied exclusively glucose medium ourselves, it seemed worthwhile to examine the effect of SA on yeast growth in other types of carbohydrates with the aim of concluding from comparative studies of various yeasts and various substrates on the mechanism of action of the inhibitor as well as on certain particularities of the carbohydrate metabolism of the yeast species examined.

Materials and methods

The strains examined, maintenance and growth media, incubation, methods of growth measurement as well as the determination of growth kinetic parameters (growth rate constant k, lag phase L, multiplication index M) were the same as described earlier [18]. SA was applied in the form of potassium sorbate (PS), at a concentration of 0.75 mg/ml. Determination of PS was described previously [9].

Results and discussion

To characterize the growth inhibitory effect of SA, values of growth kinetic parameters as compared with controls without SA are shown in Table I. k and M were expressed as per cent inhibition designated with k_{rel} and M_{rel},

the prolongation of the lag phase in per cent as L_{rel}, calculated as averages of the various growth substrates and the examined yeast species. As seen in Table I, SA inhibited the parameters k and M to a similar extent, about 30%, whereas it increased the value of L somewhat more than one and a half times. This correlates well with the earlier findings concerning the effect of SA on growth kinetic parameters [9] and also discloses the correlation between k and M, as demonstrated previously [9, 18].

Table I

Effect of potassium sorbate on the growth kinetic parameters of yeasts

Concentration of inhibitor: 0.75 mg/ml. The values for the growth parameters are in per cents of the inhibitor-free control value, k_{rel} and M_{rel} as per cent inhibition, and L_{rel} as per cent prolongation

Yeast species	Growth kinetic parameters		
	k_{rel}	L_{rel}	M_{rel}
Candida beverwijkii	45	63	43
Candida claussenii	15	66	12
Candida utilis	17	39	25
Procandida albicans	24	30	4
Procandida tropicalis	40	93	9
Saccharomyces carlsbergensis	33	108	65
Saccharomyces cerevisiae	23	63	46
Average	28	63	31

There was a considerable species variation in respect of the individual growth parameters. When all the three parameters are considered, *C. beverwijkii* and *S. carlsbergensis* were most sensitive to SA (Table I). Interestingly, with three species *(C. claussenii, Pc. albicans, Pc. tropicalis)* the value of M showed hardly any inhibition. The reason of this may be sought in the ability of these species to decompose and detoxify SA under aerobic conditions [4, 9]. In fact, determination of SA disclosed a considerable decrease of its concentration which varied with the type of the substrate in the aerobic cultures of the above three yeasts and also in those of *C. utilis* (Table II). The detoxifying action of *C. utilis* appeared to be less distinct as compared to previous examinations. In the cultures of the other strains, the concentration of SA did not change.

The growth inhibitory effect of SA varied with the substrates. To facilitate understanding, values of the growth parameters in identical carbohydrate medium as compared to controls without PS are given in Tables III—VII for each substrate.

Table II

Decrease of potassium sorbate concentration in yeast cultures grown on different substrates under aerobic conditions for 60 hours
Initial concentration of inhibitor: 0.75 mg/ml

Yeast species	Per cent decrease of potassium sorbate concentration			
	Glucose	Galactose	Sucrose	Maltose
C. claussenii	67	53	73	87
C. utilis	19	—	21	42
Pc. albicans	46	68	70	91
Pc. tropicalis	36	66	63	90

—: Substrate not utilized by the species.

Table III

Effect of potassium sorbate on growth kinetic parameters of yeasts grown on glucose substrate under aerobic conditions
For values of relative parameters, see Table I

Yeast species	Growth kinetic parameters		
	k_{rel}	L_{rel}	M_{rel}
C. beverwijkii	45	70	53
C. claussenii	18	60	9
C. utilis	15	39	22
Pc. albicans	23	29	3
Pc. tropicalis	31	80	3
S. carlsbergensis	32	85	57
S. cerevisiae	22	41	28

Table IV

Effect of potassium sorbate on growth kinetic parameters of yeasts grown on galactose substrate under aerobic conditions
For values of relative parameters, see Table I

Yeast species	Growth kinetic parameters		
	k_{rel}	L_{rel}	M_{rel}
C. beverwijkii	26	53	40
C. claussenii	8	70	8
Pc. albicans	22	33	0
Pc. tropicalis	49	94	15
S. carlsbergensis	35	114	71
S. cerevisiae	19	62	35

Table V

Effect of potassium sorbate on growth kinetic parameters of yeasts grown on sucrose substrate under aerobic conditions

For values of relative parameters, see Table I

Yeast species	Growth kinetic parameters		
	k_{rel}	L_{rel}	M_{rel}
C. beverwijkii	43	74	55
C. claussenii	16	81	11
C. utilis	16	42	24
Pc. albicans	24	25	1
Pc. tropicalis	31	84	—3
S. carlsbergensis	32	69	53
S. cerevisiae	28	41	36

Table VI

Effect of potassium sorbate on growth kinetic parameters of yeasts grown on maltose substrate under aerobic conditions

For values of relative parameters, see Table I

Yeast species	Growth kinetic parameters		
	k_{rel}	L_{rel}	M_{rel}
C. beverwijkii	43	50	33
C. claussenii	14	56	15
C. utilis	19	38	30
Pc. albicans	37	32	6
Pc. tropicalis	54	126	15
S. carlsbergensis	36	116	67
S. cerevisiae	20	154	63

Table VII

Effect of potassium sorbate on growth kinetic parameters of yeasts grown on raffinose substrate under aerobic conditions

For values of relative parameters, see Table I

Yeast species	Growth kinetic parameters		
	k_{rel}	L_{rel}	M_{rel}
C. utilis	17	41	21
S. carlsbergensis	31	138	72
S. cerevisiae	22	36	22

If variations in the changes of the SA effect with the substrates are to be evaluated with regard to carbohydrate metabolism, comparison of the parameters estimated on the various substrates with those measured on glucose as a baseline seemed to be the most reasonable approach [18]. It is inferred that in the case of a given substrate the divergence of the SA effect from that estimated on glucose implies an inhibitory action on the step(s) of carbohydrate metabolism different from that affected with glucose substrate. To support this assumption, the data of Tables III—VII were examined by analysis of variance; the values obtained with galactose, sucrose, maltose and raffinose substrates being compared each in itself to those obtained with glucose. In the case of raffinose, the data owing to their low number were evaluated together with those for sucrose. The results shown in Tables VIII—X

Table VIII

Difference in growth inhibition by potassium sorbate between galactose and glucose substrates, expressed as difference of growth parameters vs. glucose

Yeast species	Growth kinetic parameters		
	k_{rel}	L_{rel}	M_{rel}
C. beverwijkii	—19	—17	—13
C. claussenii	—10	10	— 1
Pc. albicans	— 1	4	— 3
Pc. tropicalis	18	14	12
S. carlsbergensis	3	29	14
S. cerevisiae	— 3	21	7
$SD_{5\%}$	14.0	13.3	8.3

Table IX

Difference in growth inhibition by potassium sorbate between maltose and glucose substrates, expressed as difference of growth parameters vs. glucose

Yeast species	Growth kinetic parameters		
	k_{rel}	L_{rel}	M_{rel}
C. beverwijkii	—2	—20	—20
C. claussenii	—4	— 4	6
C. utilis	4	— 1	8
Pc. albicans	14	3	3
Pc. tropicalis	14	46	12
S. carlsbergensis	4	31	10
S. cerevisiae	—2	113	35
$SD_{5\%}$	7.5	8.3	16.7

Table X

Difference in growth inhibition by potassium sorbate between sucrose or raffinose and glucose substrates, expressed as difference of growth parameters vs. glucose

Yeast examined	Growth kinetic parameters					
	k_{rel}	L_{rel}	M_{rel}	k_{rel}	L_{rel}	M_{rel}
	Sucrose—Glucose			Raffinose—Glucose		
C. beverwijkii	—2	4	2	—	—	—
C. claussenii	—2	21	2	—	—	—
C. utilis	1	3	2	2	2	—1
Pc. albicans	1	— 4	—2	—	—	—
Pc. tropicalis	0	— 4	—6	—	—	—
S. carlsbergensis	0	—16	—4	—1	53	15
S. cerevisiae	6	0	8	0	—5	—6
$SD_{5\%}$ (Sucr.—Gluc.)	3.4	5.8	10.3	—	—	—
$SD_{5\%}$ (Sucr.—Raff.—Gluc.)	8.1	27.4	9.7	8.1	27.4	9.7

represent the differences in k_{rel}, L_{rel}, and M_{rel} values of the above substrates relative to glucose as well as the lowest significant difference assessed at the 5% probability level ($SD_{5\%}$).

Effect of sorbic acid on growth in galactose medium. The effect of SA on growth parameters was examined first with galactose as the substrate. Using galactose instead of glucose as substrate, a difference in SA action was found only when one or more of the first steps of galactose metabolism (transport — phosphorylation — transfer — epimerization — phosphomutation) preceding the EMP way and differing from those of glucose, were inhibited in a greater degree than the subsequent steps which are already identical with the corresponding phase of glucose metabolism. Another source of divergence may have been the adaptive nature of any of the enzymes involved in the above five steps which allows SA to inhibit the induction of the appropriate enzyme. In the latter case, primarily the parameter L was expected to differ. As seen in Table VIII, L indeed differed significantly with *S. carlsbergensis* and *S. cerevisiae;* with the former also M was different. The cause of this may have been a delayed induction of galactose transport or, perhaps, an inhibition of galactose transport itself since, at least in the case of *S. cerevisiae*, the inductive nature of active galactose transport has been demonstrated [28, 30].

Growth on glucose and galactose differed considerably also with *Pc. tropicalis* and *C. beverwijkii*, for all the three growth parameters studied (Table VIII). With the former organism, inhibition was greater on galactose than on glucose, while with the latter organism, the opposite was the case. Since the metabolism of these two hexoses differs in several steps, the cause

of the divergence of SA inhibition remains to be clarified. Anyway, it is known that the decomposition of galactose by *Pc. tropicalis* is a slow and prolonged process [13, 22, 25]; this may imply that the growth of *Pc. tropicalis* on that sugar was more liable to inhibition than its growth on glucose. On the other hand, with *C. beverwijkii* active transport processes have been demonstrated [8] and the inhibitory effect of SA on such processes has been described [2, 5, 20]. It therefore seems likely that if both glucose and galactose are transported actively, but each by another system, the inhibitory effect of SA on the latter may be comparatively less pronounced.

Effect of sorbic acid on growth in maltose medium. Evaluating the effect of SA on growth in maltose medium, *C. claussenii* should be treated separately as this organism splits maltose by exomaltase [17, 31] unlike all others which split it by endomaltase [10, 12, 14, 15, 17, 23, 24, 27]. Deviation of the growth rate from that measured on glucose was noted only with *Pc. albicans* and *Pc. tropicalis;* for *Pc. tropicalis* the parameters L and M also indicated a distinct effect of SA (Table IX). For these species and in general for all yeasts with endomaltase activity, uptake and splitting of maltose are the steps which differ from those of glucose metabolism. Since, according to our findings, SA did not affect the activity of the splitting enzyme of *Pc. albicans* [1, 3, 7], it may be supposed that the difference found was related to the inhibition of the transport step. The active nature of maltose transport is well-known [10, 11, 26] and according to our own observations, the active transport processes of *Pc. albicans* were inhibited by SA [5, 6, 19]. The endomaltases of *Pc. albicans* and *Pc. tropicalis* being constitutive enzymes of a similar type [16, 17, 23, 24, 32] the dissimilarity between the two species in their L and M parameters may be attributed to the different sensitivity to SA of their maltose transport systems.

Inhibition of the growth rates of *S. cerevisiae* and *S. carlsbergensis* did not differ from that of glucose, whereas their L parameters and with the latter also the M parameter, were notably affected by SA. Maltose transport of those species is both inductive and active [10, 11, 26] and endomaltase itself may be inductive [10, 14]. Since active transport and inductive protein synthesis are energy requiring processes, they may be highly sensitive to the effect of SA. The dissimilarity noted with the two species in respect of SA inhibition may have been due to the differences in their tranport processes; in addition, it is known that the maltose splitting enzymes of the two species are different, that of *S. carlsbergensis* being structure-bound [15, 16].

The growth of *C. beverwijkii* on maltose was less inhibited by SA than its growth on glucose, to the analogy of galactose. This seems to be in support of the implication that active glucose transport of this species was more sensitive to inhibition by SA than were its galactose or maltose transport mechanisms.

As noted in the foregoing, the maltose metabolism of *C. claussenii* takes place by exomaltase. This enzyme not being sensitive to SA [1], the effects of SA on growth in glucose and maltose media did not differ (Table IX).

Effect of sorbic acid on growth in sucrose and raffinose media. With all examined species which utilize sucrose and raffinose by means of invertase (*C. beverwijkii*, *C. utilis*, *S. carlsbergensis*, *S. cerevisiae;* cf. [16, 32]), the substrate utilized for growth is partially or completely split outside the cells to monosaccharides transported by the glucose uptake system and phosphorylated by hexokinase intracellularly. Accordingly, no difference was expected in the inhibition of growth by SA as compared to glucose. This was proved by the data of Table X which shows that the effect of SA on the growth of the above species in glucose, sucrose and raffinose was uniform for all the three growth parameters. In only two cases was there a significant difference: in the growth rate of *S. cerevisiae* on sucrose, and in the *L* parameters of *S. carlsbergensis* on both sucrose and raffinose, and with raffinose also in the *M* parameter.

As described earlier [18], sucrose metabolism of *S. cerevisiae* can take place not only through an extracellular splitting by invertase and subsequent transport of the resulting glucose and fructose, but also by another pathway consisting of sucrose transport and a subsequent splitting by endosaccharase. The SA sensitivity of the former pathway may be identical with that of glucose metabolism unless SA inhibits the production, activity, or the secretion of invertase. This was proved indirectly by the similarity of growth inhibition on raffinose and glucose. Direct estimation also indicated that the activity of invertase is not inhibited by SA [1, 29]. The postulation is, therefore, feasible that with the species in question the second metabolic pathway also plays an important role in the utilization of sucrose and since the SA sensitivity of active transport may be greater, it is to that circumstance that the greater inhibition relative to glucose can be ascribed.

S. carlsbergensis utilizes raffinose by means of two exoenzymes, invertase and melibiase, synthetized and acting in succession. Since, however, SA affected the growth on sucrose to nearly the same extent as on glucose, only the inhibition of *L* having been greater, a more distinct inhibition of invertase seemed unlikely. It is, therefore, reasonable to suppose that the stronger inhibition by SA of the growth parameters *L* and *M* on raffinose was due either to one of the conditions of splitting by melibiase (synthesis, secretion, splitting) or to the inhibition of the metabolism of galactose produced by splitting. Using galactose as the substrate, the effect of SA on the growth of *S. carlsbergensis* affected the parameters *L* and *M* showing that the inhibition of its growth on raffinose also came into effect through depression of the metabolism — very likely of the transport step — of the galactose formed from raffinose by the action of melibiase.

The species *Pc. albicans* and *Pc. tropicalis* are endosaccharase containing yeasts. While the inhibition by SA of the growth of these two species on maltose differed from that on glucose, no such difference was noted with sucrose as the substrate (Table X), though both disaccharides are decomposed by intracellular enzymes [16, 17, 23, 24, 32]. Endosaccharase, like endomaltase was insensitive to SA [1, 3, 7]. This suggested that in the species in question the transport mechanisms are different in the metabolic pathway of maltose and sucrose. As suggested by the results, the transport of sucrose is less sensitive to SA than that of maltose, while being similar in this respect to the glucose transport mechanism.

Comparative analysis of the effects of SA on the growth kinetic parameters of the yeast species examined with different substrates permitted conclusions as to the causes of inhibition which manifested themselves at the level of carbohydrate metabolism. Apparently, interference with the transport processes is an important element in the mechanism of action of SA. Preliminary examinations in this laboratory [2, 5, 6] disclosed a distinct SA sensitivity of the active transport processes which is largely in support of the above implication. Consistently with earlier results [8, 9, 21] the present findings again stressed the fundamental and definitive role of transport processes in the carbohydrate metabolism of yeasts. Further studies of sugar transport seem to be a proper approach to the better knowledge of both the carbohydrate metabolism of yeasts and the mechanism of action of growth inhibitors.

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STUDIES ON SWINE ENTEROVIRUSES

III. OCCURRENCE OF ENTEROVIRUSES IN HUNGARIAN SWINE HERDS AND IN THE KIDNEY TISSUE OF NORMAL PIGS

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Summary. A total of 505 enterovirus strains has been isolated from 1585 faecal samples of pigs of different age groups. No enterovirus shedding was revealed before one week of age, but subsequently the rate of virus shedders increased gradually up to an average of 75.3% after weaning. Then the rate decreased, and shedding became sporadic beyond one year of age. The spread of infection among litters in a large piggery was connected with isolation circumstances of the litters of individual sows.

With the exception of 16 isolates, the isolated strains belonged to one of 16 serotypes and 2 cytomorphological types. Repeated faecal sampling of individual pigs proved that several enterovirus serotypes were shed one after the other or less frequently simultaneously.

Enterovirus infection was transferred vertically from the sow to the offspring in the farrowing pen, and horizontally among piglets especially after weaning, when mixed to form large groups.

Isolation, serological typing and grouping of enteroviruses of pigs on the basis of cytomorphological lesions caused in pig kidney monolayer cultures was described earlier [12, 13]. A further report described cases of encephalitis in pigs caused by type I-2 enterovirus [14].

Though a great variety of enterovirus strains was isolated from pigs all over the world [2, 8], only a few data are available on the incidence of enterovirus infection in swine herds and the frequency of virus shedders within a herd [4, 15].

This paper reports on the incidence of enterovirus infection in pigs in Hungary and on the connection of virus shedding with age in young pigs of large breeding plants. The incidence of enterovirus infection in kidney tissue of normal pigs is also reported.

Materials and methods

Source of material. I. A total of 1585 rectal swabs were obtained from pigs of different age groups in 52 large plants.

II. In each of 4 additional piggeries rectal swabs were taken of 10 sows in late pregnancy and again at the time of weaning of their litter. Rectal swabs were obtained also from 2 or 3 ear tagged piglets of each of these sows, when 1, 2, 4, 6, 8, 12, and 24 weeks old. On two farms sampling was repeated in some of the experimental pigs at the age of 12 months.

III. Not inoculated pig kidney cultures prepared from freshly processed tissue of 8—12-week-old individual pigs weekly for 10 consecutive years were tested systematically for latent enterovirus infection.

Laboratory technique. Preparation of the tissue cultures used throughout these experiments, processing and inoculation of faecal samples as well as cytomorphological and serological typing of the isolates have been described previously [12, 13].

Results

Experiment I. Enteroviruses were isolated from 505 out of the investigated 1585 rectal swab samples which means an overall frequency of 31.8%. The distribution of positive faecal samples within age groups is shown in Table I.

Table I

Occurrence of enteroviruses in the faeces of pigs of different age groups

Age	Number	Positive enterovirus isolation	
of pigs tested		Number	Per cent
0— 4 weeks	382	26	6.9
5— 8 weeks	328	105	32.0
9—12 weeks	426	321	75.3
13—24 weeks	165	38	23.0
over 24 weeks	284	15	5.3
Total	1585	505	31.8

All 505 isolates were tested for the morphological character of cytopathic changes caused in pig kidney tissue cultures. 206 strains belonged to cytomorphological type I and 285 to cytomorphological type II, described earlier [13]. The remaining 14 isolates were mixtures of strains of both cytomorphological types.

In addition to those used in previous tests [12], 3 additional sera were applied for serotyping the isolates; they have been produced recently with strains not fitting into the formerly established 13 serotypes.

Distribution of the strains according to the type of cytopathic lesion and serotype is shown in Table II.

Experiment II. In each of four piggeries rectal swabs were obtained from 10 sows immediately before farrowing and again when their litter was weaned at 8 weeks of age.

At farrowing only one sow of herd B shedded enteroviruses with the faeces, but at weaning of their litter one sow each of herds A, B and C proved positive.

Table II

Classification of strains isolated in Experiment I according to cytomorphology and serotype

Cytomorphological type	Serotype	Prototype strain	Corresponding foreign strains	Number of isolates belonging to the type
I	1	5-DVIII	Talfan	24
I	2	12-PL	T-80[1] S-180/4[2]	36
I	3	1-AAVI	V-4[2]	2
I	4	21-Sz		6
I	5	25-TVII	41	41
I	6	37-UVI		89
I	14	60-EV	N-11[2]	11
I	15	112-DII		2
I	16	121-EIX	F-7[3]	3
II	7	2-AKIII	U-10[2]	70
II	8	9-KIV		24
II	9	39-V.II		5
II	10	16-SX		21
II	11	20-SIM		6
II	12	26-TXII		62
II	13	27-THI	V-13,[4] N-7[2]	97
I	Not typable			2
Mixed				14
			Total	505

[1] Ref. [1], [2] Ref. [11], [3] Ref. [9], [4] Ref. [10].

Virus isolation results for the same two (herds A and B) or three (herds C and D) piglets of the litters of the sampled 10 sows are summarized in Table III. The relative frequency of virus shedders among these pigs is demonstrated by Fig. 1.

All 133 enterovirus strains isolated in this series of investigations were typed for cytopathic lesions induced in tissue cultures and also serologically. The number and type of the isolates per herd are listed in Table IV.

Experiment III. Monolayer cultures of trypsin digested tissue of the kidneys of individual 8—12 weeks old pigs, prepared weekly for nearly ten years, yielded spontaneous enterovirus infection in 9 cases. This means an overall infection rate of nearly 1%. In all cases spontaneous infection was recovered before the change of the culture medium. In all but one case only

Table III

Enterovirus shedding in ten litters each

Herd	The age of investi-											
	0			1			2			4		
	test positive			test positive			test positive			test positive		
	No.	No.	%	No.	No.	%	No.	No.	%	No.	No.	%
A	20	0	0	18	1	5.1	17	3	17.6	17	7	41.1
B	20	0	0	17	0	0	18	0	0	16	1	6.2
C	30	0	0	27	0	0	26	1	3.8	26	6	23.1
D	30	0	0	29	0	0	28	2	7.1	28	4	14.2
Total	100	0	0	91	1	1.1	88	6	6.8	87	18	23.5

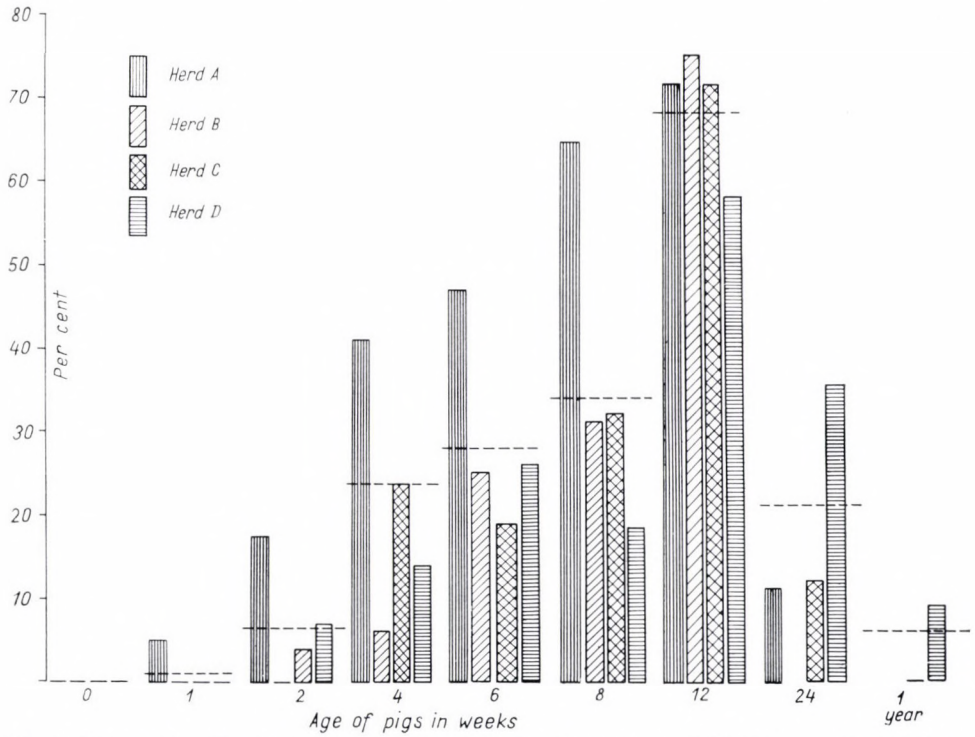

Fig. 1. Proportion of enterovirus shedding pigs in the average of 10 litters each of 4 large piggeries

5—10% of the tube cultures showed a cytopathic effect. In one case, however, massive cytopathic effect was revealed in all cultures prepared from the kidneys of an 8-week-old pig.

In 4 cases type I-1, in 3 cases type I-5, and in 2 cases type I-6 was present in all cultures of the series.

of four pig breeding plants (Experiment II)

gated pigs in weeks												One year		
6			8			12			24					
test positive			test positive			test positive			test positive			test positive		
No.	No.	%	No.	No.	%	No.	No.	%	No.	No.	%	No.	No.	%
17	8	47.0	17	11	64.8	14	10	71.5	9	1	11.1			
16	4	25.0	16	5	31.1	12	9	75.0						
26	5	19.2	25	8	31.9	21	15	71.4	16	2	12.5	6	0	0
27	7	25.9	27	5	18.5	19	11	57.9	17	6	35.1	11	1	9.1
86	24	28.0	85	29	34.1	66	45	68.2	42	9	21.2	17	1	5.9

Table IV

Number and type of enterovirus strains isolated from piglets of four herds (Experiment II)

Herd	Number	Cytomorphological and serotype	Number	Cytomorphological and serotype
	of strains isolated from piglets before weaning		of strains isolated from piglets after weaning	
A	26	I-4, I-4 II-10, ?*	15	I-4, I-5 II-10, II-12
B	9	I-2	10	I-2 II-13
C	28	I-1 II-10, ?*	9	I-1 II-8, II-10, II-11
D	27	I-6 II-10, II-13	9	I-6 II-10, II-13, ?*

* Not neutralized by any of the 16 type sera.

Discussion

Experiments I and II proved that faecal enterovirus shedding is connected with the age of the pig. No enterovirus shedding occurred in piglets younger than one week. However, the results of Experiment II demonstrated in Table III and Fig. 1 prove that housing circumstances are at least so important as age in spreading of the infection. More rapid virus spreading in herd A than in herds B, C and D was due to the circumstance that in herd A piglets

of different litters had access to a common feeding and watering place outside the farrowing pens after the first week, but in herds B, C and D the litter of individual sows was isolated in closed pens until weaning at 8 weeks of age. Therefore the rate of virus shedding piglets in herd A reached already before weaning a value (64.8%) very close to the maximum (71.5%) attained after weaning. But in herds B, C and D the rate of virus shedders showed an abrupt increase from 31.9, 31.1 and 18.5% before weaning, to 71.4, 75.0 and 57.9% after weaning, respectively.

The incidence of enteroviruses in pigs was tested mainly for Talfan (I-1) and T-80 (I-2) types [3, 5, 6], or for unidentified enterovirus isolates [4].

As shown in Table II, there were great differences in the occurrence of certain enterovirus serotypes. Data of Experiment II summarized in Table IV revealed the occurrence of enteroviruses of several serotypes in the same herd. The same piglet shedded more than one serotype one after the other, or less frequently simultaneously. Continuous shedding of the same serotype by an individual pig exceeded 4 weeks in one single case and lasted 8 weeks.

Though enteroviruses could be isolated only sporadically from the faeces of adult pigs in large piggeries, the results of these investigations proved that new-born piglets are infected mostly by the sow. In all cases of isolation of an enterovirus from the sow's faeces the same serotype was soon shed by her piglets.

The investigations proved that practically every pig of a large piggery contracts infection by at least one enterovirus serotype, followed by shedding of this serotype for a few weeks. Most probably, shedding of enteroviruses in small quantities not revealed by the isolation technique used, is more widespread than it has been indicated by these investigations.

In agreement with a former statement [6], Experiment III proved that latent enterovirus infection can be present in the kidney tissue of apparently normal pigs. In 7 out of the 9 cases in which the non-inoculated pig kidney tissue cultures contained enteroviruses, the pigs used as kidney donors originated from herds in which the same type of enterovirus was isolated in simultaneous faecal screening tests. All 4 pigs whose kidney proved infected with type I-1 (Talfan) enterovirus, came from the same herd (herd C of Experiment II) within a few weeks.

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SPECIFIC ORAL PREVENTION OF INFANTILE GASTRO-ENTERITIS

I. EXPERIMENTS IN MICE

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Summary. The possibility of inducing immunity against *E. coli dyspepsiae* was studied in mouse experiments, with the aim of preventing infantile gastro-enteritis. The investigations were performed with freeze-dried Boivin antigen prepared from O111:K58(B4) strain. Immunization was carried out orally, by graduated doses given daily or at 3-day intervals.

The immune response was determined by direct studies of the active protection and on the basis of the protective effect of sera and intestinal extracts of immunized animals. Protectivity afforded actively and the protective effect of intestinal extracts (copro-antibodies) was studied in mice by the mucin technique. The protective value of sera was determined in 10-day old chick embryos.

Immune response was demonstrated in all the three examined groups. A correlation was found to exist between the immunizing dose, the mode and the effect of immunization.

Immunity, as judged from active and passive protectivity, was considerably diminished 31 days after the completion of vaccination. By the administration of small doses of antigen in 5-day intervals, the degree of immunity could be maintained or increased.

Protective copro-antibodies were detected in the intestinal extract. These antibodies, too, disappeared within 31 days, while their effect could be maintained or enhanced by small doses of antigen. The copro-antibodies are indicative of the development of local immunity and justify a study of the immunogenicity of the vaccine in infants.

The problems of live and killed vaccines are discussed.

Since the discovery of enteropathogenic *E. coli dyspepsiae* in Hungary [1], the incidence of infantile gastro-enteritis and the number of hospital outbreaks have been reduced by antibiotic treatment, but later, due to the development of resistance to the antibiotics, there was no further improvement. In the last 5 years, the number of cases was 2000 to 2500 and that of nosocomial outbreaks between 14 and 25 [2]. In 1968, there were 1752 cases, with 16 deaths [3]. For eliminating the disease, active immunization seemed to promise success.

Recently, several authors have studied the possibility of oral immunization against enteropathogenic *E. coli* in mouse experiments. Lodenkämpfer [4] immunized mice orally and found a favourable effect in protectivity tests. Piringer [5] immunized with the O127:K63(B8) strain killed by formalin or acetone; no agglutinins were formed, but protectivity against intraperitoneal challenge could be recorded. Lietz and Raettig [6] demonstrated 73% protectivity against infection with the homologous strain by immuniz-

ing with heat-killed O111:K58(B4) vaccine. No haemagglutinating antibodies were found, but in the blood of animals surviving the challenge haemagglutinins appeared in a high titre. In the opinion of these authors, oral immunization is preparing the antibody producing system to be able to afford protection against the infection. TORIKATA and IMAIZUMI [7] are of the opinion that the decisive factor is not the quantity of antibody elicited by oral immunization but the ability of the immuno-competent cells of the immunized organism to produce antibodies promptly at the time of infection.

OCKLITZ *et al.* reported on the immunogenic effect of O111:K58(B4) extracted by desoxycholate or killed by heat. There was no significant difference between the effect of these two vaccines, showing a 70% effectivity index under the same conditions of immunization and infection as applied by RAETTIG [8—15]. Regarding the appearance of antibodies, all these authors share the above point of view. The duration of immunity was estimated at 6 months.

Isolation of O111:K58(B4) streptomycin dependent strain, showing a ratio of revertants 2 to 592 out of 10^8 germs, has recently been reported by LINDE *et al.* [16—18]. By the use of this live strain (10^{11} germs on 10 occasions) and a vaccine killed at 60°C, massive protection was established in mice, manifesting with 70—80% protectivity against intraperitoneal challenge with 20 to 40 LD_{50} of a wild strain. No difference was observed between the immunogenic effect of live and killed strains. Haemagglutinins developed only in animals surviving the challenge. The superiority of the live vaccine was considered to be due to the fact that a streptomycin resistant strain adhered to the colon of only 18% of orally immunized mice previously "sterilized" with streptomycin, in contrast to 80% of the controls.

The studies were supported by our previous findings. (i) We found that in suckling mice the pathogenic strain *E. coli* O101:K30 behaved in several respects like the human strains of *E. coli dyspepsiae* [19]. (ii) Best protectivity could be achieved in the intestines by immune serum given orally, *i.e.* by antibodies concentrating locally [20]. This model displayed characteristics which reminded of the pathomechanism and immunological behaviour of shigellae. It was therefore attempted to prepare an oral vaccine from the entero-pathogenic strains of *E. coli* most frequent in Hungary, O111:K58(B4), O55:K59(B5) and O86:K61(B7); on the same principle as our oral *Shigella flexneri* and *sonnei* vaccine is developed [21, 22]. The present paper will report on these experiments carried out in mice with the O111:K58 (B4) strain.

Materials and methods

Strain. For preparation of the antigen, the strain O111:K58(B4), labelled 1872/68, isolated in our routine laboratory from a severe case of infantile gastro-enteritis, and regarded as typical in biochemical tests and antigenic structure, was chosen.

Cultivation was carried out in fluid medium containing casein hydrolysate diluted 1:5 (approximately 1.5%), 0.1% of glucose and 0.3% of Cellamin,* under vigorous aeration and stirring for 7—8 hours. The culture was killed with 0.3% formalin, sedimented next day in a centrifuge of continuous flow type, and the bacterial mass was freeze-dried. An amount of about 100 g of dry bacteria was obtained from 30 litres of medium.

Preparation of antigen. The antigen was prepared by the classical procedure of BOIVIN and MESROBEANU. Forty mg of the freeze-dried mass of bacteria was suspended in 1 ml of cold distilled water and mixed with 1 ml of cold *N*/2 trichloroacetic acid. After vigorous mixing the suspension was kept at 4°C overnight, then centrifuged and the sediment was re-extracted in half volume, according to the procedure described above. After uniting both extracts, dialysis in cold tap water for 3 days and in distilled water for 2 days was applied, followed by Seitz filtration, concentration to about 1:10 in a rotating vacuum evaporator and lyophilization. The product was distributed in bottles with close fitting glass stopper and stored in the exsiccator. One ml of extract yielded an average amount of 1.2 mg of freeze-dried antigen.

Antigenicity was examined by the HI test, as described earlier [23]. Our antigens were tested for protective ability in mouse experiments comparing them with the chosen antigen which was regarded as a standard.

Immunization. Using a metal bougie and a syringe, graduated doses of the antigen were injected into the oesophagus [24]. For the dosage and the intervals between injections, see below.

Determination of immunity. (a) *Oral active immunization.* Challenge was performed intraperitoneally with 50—75 LD_{50} doses on the 5th day after the last immunizing dose, by the mucin technique. This allowed the determination of the ED_{50} of the antigen and consequently of the optimal dose. These experiments served also for estimation of the duration of immunity and of the effect of revaccination.**

(b) *Study of the protective effect of humoral antibodies in chick embryo test.* Dilutions of sera of immunized mice were injected into the vein of the chorioallantoic membrane of 10 days old chick embryos by means of a tuberculin syringe and a No. 21 cannula. The germ count of 18-hour slope agar culture was determined by NIH-HUMAN turbidity standard. Challenge was carried out similarly intravenously with 50—75 LD_{50} (50—100 germs). For details, see [25].

(c) *Passive mouse protection test.* The immunized mice were bled through the retrobulbar venous plexus and the sera were stored in the deep freezer. The graduated doses of sera were administered intraperitoneally and the mice were challenged intraperitoneally with 50—75 LD_{50}, with the mucin technique. For infection, a diluted suspension of an 18-hour slope agar culture adjusted by the NIH-HUMAN standard, was used.

(d) *Copro-antibodies.* The entire intestinal tract of bled mice was removed, cleaned, the intestines of animals receiving similar immune doses were pooled, weighed and homogenized in cold distilled water by the aid of quartz sand. After centrifugation and Seitz filtration the suspension was freeze-dried. For immunization, dilutions were prepared, corresponding in concentration to the original wet weight, and graduated doses in 0.5 ml volume were tested in mice intraperitoneally, challenging half an hour later with 50—75 LD_{50}, with the mucin technique.

Results

Active immunization of mice. Corresponding to our previous procedure, 5 mice each were immunized orally by graduated doses of Boivin antigen prepared from the strain O111:K58(B4). The doses of antigen were 2.0, 0.2 and 0.02 mg, as calculated on the basis of previous findings. The mg dose, since repeated extraction removed approximately 70% of the cell-wall antigen [26], contained the cell-wall antigen of approximately 1.5×10^{11} bacteria.

* Yeast hydrolysate prepared by the Institute for Serobacteriological Production and Research Human, Budapest.

** The term revaccination refers in this paper to a prolonged oral administration of small doses of antigen given in order to maintain the immunity induced by priming vaccination.

The mice were divided in two main groups. One was immunized on every 3rd day, the other every day. In each main group 3 subgroups were formed. One of them was challenged 5 days after the termination of immunization, the other was given 0.2 mg as a revaccinating dose on every 5th day, altogether on 5 occasions. The third subgroup was the control; here no revaccination was performed to establish whether during the 31-day period of the experiment any reduction in immunity would occur. The time of immunization was coordinated in order to challenge simultaneously and make it possible to compare the results. These are shown in Table I, which presents only the actual (ED_{50}) and the relative (relative potency, RP) immunizing values.

Table I

Active mouse protection

Challenge dose: 75 LD_{50} of O111:K58(B4), intraperitoneally (mucin technique)

Days after priming immunization	ED_{50} (mg) and RP value*			
	immunized on 5 occasions at 3-day intervals		immunized daily on 5 occasions	
	not revaccinated	revaccinated**	not revaccinated	revaccinated
5th day	0.02 (7.5)+		0.02 (7.5)	
31st day	0.15 (1)	0.02 (7.5)	0.10 (1.5)	0.015 (10)

* RP: relative potency, ** revaccination dose, 0.2 mg of antigen given orally every 5th day, +in brackets: RP.

From the data in Table I the following conclusions were drawn.

1. The result of the immunizing procedure was identical whether this was carried out every day or at 3-day intervals.

2. Thirty-one days after priming immunization the protective titres were significantly decreased. There seemed to be some slight tendency in favour of daily immunization.

3. The favourable effect of revaccination was beyound doubt. In the group immunized at intervals, the immunity elicited by the primary dose was maintained, while in the group immunized daily the protective titre showed a rise, which, however, did not reach significance.

The findings were indicative of the possibility of oral immunization against *E. coli dyspepsiae*, demonstrating also the short duration of immunity and pointing to the efficacy of revaccination, as already observed in our experiments in mice with *Shigellae* [23].

Passive immunization of mice. (a) *Serum antibodies.* Ten actively immunized animals of each group were not challenged while performing the active protection test, but were bled. The protective value of their sera was tested

in 10-day-old chick embryos. This method was chosen because in preliminary experiments the chick embryo test was found to be more sensitive than the mouse protection test. The results are demonstrated in Table II in the same arrangement as in the case of the active protection test, except that within the groups the protective values are indicated along with the immunizing doses. In this way information is supplied, beside the immunization interval, also concerning the time factor, the effect of revaccination and the relationship of immunizing dose and immunity.

Table II

Protective values in sera of immunized mice
Chick embryo test
Challenge dose: 75 LD_{50} of O111:K58(B4)

Days after priming immunization	Dose of active immunization (mg)	ED_{50} (mg) and RP value*			
		immunized on 5 occasions at 3-day intervals		immunized daily on 5 occasions	
		not revaccinated	revaccinated**	not revaccinated	revaccinated
Before immunization	0	0.01 (1)+		0.01 (1)	
5th day	2	0.001 (10)		0.0005 (20)	
	0.2	0.0017 (6)		0.0017 (6)	
	0.02	0.005 (2)		0.005 (2)	
31st day	2	0.0017 (6)	0.0001 (100)	0.0005 (20)	0.00005 (200)
	0.2	0.01 (1)	0.0005 (20)	0.003 (2)	0.00017 (60)
	0.02	>0.01 (<1)	0.0028 (3)	0.01 (1)	0.005 (2)

* RP: relative, potency, ** revaccination dose, 0.2 mg of antigen given orally every 5th day, +in brackets: RP.

The data in Table II are in harmony with those concerning the active protection test shown in Table I, but, due to the higher sensitivity of the test, the differences were more definite. The rapidly decreasing tendency of immunity, especially in the case of lower doses, the efficacy of revaccination and the effect of daily immunization were also more expressed. The latter was especially marked in the revaccinated group where the value was nearly 10 times higher than that conferred by the priming dose. Thus, the better effect of daily immunization was most probably due to the higher frequency of stimuli. The correlation between immunizing doses and immune response was also established; the combined effect of the immunizing and revaccinating dose had the best effect

(b) *Copro-antibodies.* The presence of copro-antibodies in enteric infections of local character is well-known. Protective copro-antibodies were

found by BURROWS *et al.* [27, 28] in guinea pigs infected by *V. comma*. In mice immunized against *Shigellae* and in mouse shigellosis, protective copro-antibodies were demonstrated by RAUSS and KÉTYI [21, 29]. The local character of dyspepsia-like *E. coli* infection in suckling mice was reported by KÉTYI [20, 30]. All this has made it necessary to search for protective antibodies in the intestinal tract of mice orally immunized against *E. coli*, and it was expected that in the case of an immune response in not susceptible mice this would be even more marked in susceptible infants, a fact which would be of decisive importance in prevention. Results of such studies are summarized in Table III; they were carried out in mice, since the extracts were toxic for the chick embryo.

Table III

Protective values in the intestinal extract of immunized mice
Mouse test
Infective dose: 75 LD_{50} of O111:K58(B4)
(mucin technique)

Days after priming immunization	Dose of active immunization (mg)	ED_{50} (mg) and RP value*			
		immunized on 5 occasions at 3-day intervals		immunized daily on 5 occasions	
		not revaccinated	revaccinated**	not revaccinated	revaccinated
Before immunization	0	0.05 (1)+		0.05 (1)	
5th day	2	0.016 (3.1)		0.005 (10)	
	0.2	0.028 (1.7)		0.028 (1.7)	
	0.02	>0.05 (<1)		>0.05 (<1)	
31st day	2	>0.05 (1)	0.05 (10)	0.05 (1)	0.0028 (17)
	0.2	>0.05 (1)	0.016 (3.1)	>0.05 (1)	0.009 (5.5)
	0.02	>0.02 (1)	0.05 (1)	0.05 (1)	0.05 (1)

* RP: relative potency, ** revaccination dose, 0.2 mg of antigen given orally every 5th day, +in brackets: RP.

The most important finding was the presence of copro-antibodies in the intestinal extract of mice. There was little possibility for standardization of the extract, but on the basis of weight ratios the same trends of protectivity were found as in the case of serum. After the completion of immunization, the copro-antibodies decreased more rapidly than did the serum antibodies, to disappear completely in 31 days, when the serum antibodies, especially after a large immunizing dose, were still detectable. The effect of revaccination was marked and the prolonged and the daily administration of antigen enhanced the protective effect, just as in the case of serum antibodies; the increased effectiveness of large immunizing doses was also noted.

Discussion

The prevalence of *E. coli* infections in Hungary has made it imperative to study the possibility of a specific prevention of infantile gastro-enteritis. The basic experiments were carried out in mice, since several data were known concerning similar attempts [5—18]. The investigations were performed by oral immunization, the more so as we had a favourable experience with such immunization against dysentery [21, 29] and, also, as the syndrome elicited in suckling mice by a pathogenic *E. coli* strain has been shown to represent an analogue of infantile gastro-enteritis [20, 30].

In the studies, Boivin antigen was used in view of its excellent immunogenic effect [21]; and oral immunization of humans with *Shigella* Boivin antigen was equally convincing [21, 32].

For the active immunization of mice, graduated doses totalling 2.0, 0.2 and 0.02 mg of freeze-dried antigen were applied, administering identical amounts on 5 occasions. The highest dose contained the cell-wall antigen of approximately 1.5×10^{11} bacteria. RAETTIG [6] administered 8×10^{10} germs in ten doses. OCKLITZ *et al.* [10] the same number, LINDE and KOCH [17] 1×10^{12} of killed or streptomycin dependent live germs similarly in 10 doses. Thus in our experiments the maximum immunizing dose essentially corresponded to those used by other authors. Considering, however, the ED_{50} (0.02 mg) of the antigen applied by us, 1.5×10^{9} germs were already effective, although their comparison with other data was difficult in view of the different methods used by the authors mentioned. They applied identical doses and established the degree of protection in percentage, while we used graduated ones and determined ED_{50} values. They expressed the challenge dose by the germ count, while we did it by the LD_{50} values. LINDE and KOCH [18] used 20—40 LD_{50} for infection, an amount representing 2 to 6×10^{7} germs suspended in egg yolk. In constrast, in our experiments carried out with the mucin technique, protectivity was tested against 75—100 LD_{50}. Thus, the orally administered Boivin antigen proved to be highly effective against parenteral infection.

Instead of a daily immunization performed for 10 days by the authors referred to, we compared the efficacy of immunization carried out on 5 consecutive days with the immunogenic effect of 5 vaccinations performed at 3-day intervals, in both active and passive protection tests. Apart from the similarities of these procedures, the characteristics were more expressed in the sensitive passive protection test in the chick embryo. The properties of immunity are summarized as follows.

1. The relationship between the immunizing dose and the degree of protectivity was obvious.

2. The passive protection test revealed the superiority of daily immunization.

3. Immunity elicited by active immunization ceased completely, according to the direct test, and it was considerably reduced according to the passive protection test, by the 31st day. The correlation between dose and persistence of immunity was distinct.

4. The favourable effect of revaccination came into prominence mostly in connection with passive immunity (serum- and copro-antibodies), manifesting itself with an increase of protective titres, in good agreement with the results of the immunization of mice [21] and man [31, 32] against dysentery; the phenomenon has been attributed to the effect of repeated stimuli.

The above results may be considered the partial manifestations of general immunity and thus one could conclude to the existence in the intestine of immune relations. This was studied by the investigation of protective copro-antibodies, in the passive mouse protection test. This in fact revealed the production of protective copro-antibodies in the course of immunization. The dosage, the intervals between vaccinations, the duration of immunity and the effect of revaccination were identical with those applied in the active and passive immunity test.

According to FRETER and GANGAROSA [33] and our own studies [21, 29] in enteric infections it is the copro-antibodies which serve as the protective factor. As to their presence in infantile gastro-enteritis, KARAKASEVIČ [34] found copro-agglutinins in 46 of 56 patients; protectivity was not investigated.

If it is possible to ensure development of copro-antibodies by means of killed vaccine, it is questionable whether live streptomycin dependent strains should be applied, in view of the risk of reversion. The streptomycin dependent strains used by LINDE *et al.* [16] resulted in 2 to 592 revertants among 10^8 colonies from which a suspension of 10^{11} germs induced enteritis in infants [35]. In respect of general immunity, no difference was found between the live and killed vaccine [17]. The advantage of live vaccine was supported by the observation that in the intestines of mice fed by live vaccine and subsequently treated with streptomycin, a streptomycin resistant strain was eliminated in 2–3 days, while in the control animals protracted excretion was noted [18]. The rapid elimination of the streptomycin resistant infective strain from immunized animals was observed also by OCKLITZ *et al.* [13]. We have shown [36] the dominance of a single type of *E. coli* in the intestines, preventing the colonization of any other strains; the phenomenon was termed antagonism. Exchange of strains may, however, occur after eliminating the resident strain by streptomycin. In this way strain O111:K58(B4) was successfully implanted into the colon of mice, performing the role of the normal flora for a long period. Similar observations were later made by ASHBRUNER and MUSHIN [37]. The question may arise whether LINDE's observation was

also a case of antagonism. Due to the limited number of control tests applied by LINDE *et al.* (3 days observation after immunization, omission of investigation of other *E. coli* strains), this possibility cannot be excluded.

The phenomenon of "implantation" after feeding a great number (100—200) of *E. coli* was demonstrated by LINDE *et al.* According to our investigations, of virulent (keratoconjunctivitis positive) Shigellae, 2 to 3 germs were enough to induce persistent excretion. As for avirulent Shigellae, 200—300 germs were eliminated within 1 to 2 days without preimmunization, while the implantation of virulent Shigellae could be prevented only in immunized animals [24]. Since a reliable procedure for the determination of virulence of *E. coli* is lacking [38], determination of the actual rate of implantation is a difficult problem.

The papers of LINDE *et al.* failed to mention how long the state inhibiting the implantation had persisted. In our experience, the presence of copro-antibodies can be maintained as long as necessary, by the periodical application of small doses of antigen.

In our studies no difference in immunogenic capacity was found between live and killed Shigellae [39]. RAETTIG [40] took a definite stand against the live vaccines.

The present experiments provided a basis for studying the possibility of oral immunization in infants. Such investigation should, however, be performed gradually.

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PROPHYLACTIC USE OF AMANTADINE DURING HONG KONG INFLUENZA EPIDEMIC

By

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Summary. In March, 1969, when Hong Kong influenza was wide-spread in Hungary, amantadine HCl (Viregyt®) and placebo were administered to a total of 2440 and 2133 young men, respectively, in seven military units. Treatment was started as soon as cumulation of influenza-like cases had been recognized as an outbreak and lasted until the outbreak had subsided. The daily dose was 200 mg, divided in two capsules.

The drug failed to influence morbidity, but reduced the peak ($P < 0.01$) and duration ($P < 0.001$) of fever, the period of bed-care ($P < 0.001$), the incidence of complications in general ($P < 0.05$) and, especially, the incidence of cases with lower respiratory complications ($P < 0.01$).

Virological and serological results suggested that in four units (and perhaps also in a fifth one) the outbreaks were caused by a strain practically identical with A2/Hong Kong/1/68. In two or three units an unidentified agent (or agents) other than influenza A or B must have contributed to the outbreaks. The beneficial effect proved significant only in the latter units.

Seroconversion rate was not reduced by the drug, while the postepidemic haemagglutination-inhibiting antibody titres of initially seronegative test subjects tended to be lower in the amantadine-treated than in the placebo-treated group. The difference just failed to reach the 0.05 significance level.

In tests *in vitro* the Hong Kong 1/68 strain proved to be less sensitive to amantadine than was an earlier strain of influenza A2 virus.

1-adamantanamine hydrochloride (amantadine) has proved to be virostatic against influenza-A viruses in tissue cultures [1—8], embryonated eggs [2, 4], and mice [4, 5, 9]. In mice the compound had a therapeutic effect if administered soon after artificial infection [9—11].

In man, trials with artificially induced influenza A2 infection [7, 12—15] and prophylactic trials against influenza outbreaks [16—18] have shown mostly promising, yet variable, results. Therapeutic effect has also been reported [19—22].

In the present report a prophylactic trial is described which was conducted in Hungary with an amantadine HCl preparation (Viregyt®, United Works of Pharmaceutical and Dietetic Products, Budapest) in March, 1969, during the Hong Kong influenza epidemic.

The trial was designed to test the morbidity-reducing effect of the compound, and no special effort was made to follow the clinical course of the disease, consequently, it had shortcomings as compared to controlled clinical trials.

Under the given conditions amantadine failed to reduce morbidity, whereas the analysis of routine case records yielded some features which seemed to justify the publication of the present paper.

Materials and methods

Preparations. Both amantadine (Viregyt®) and the placebo were kindly supplied by the makers. The preparations were distributed in capsules, 100 mg in each. The daily dose was uniformly two capsules (200 mg). Neither the sanitary personnel nor the test subjects knew that a placebo was included in the trial.

Test subjects. The trial was carried out in seven military units. The test subjects were, with few exceptions, young men of 18—21 years. It was aimed to collect for the active substance at least 2000 subjects exposed to the risk of influenza, and the same for the placebo. As some units were expected to evade the epidemic, an excess number of units were involved in the trial.

Timing of the trial. When either of the adopted units had reported cumulation of influenza-like cases, the unit was immediately visited by two of the present authors to investigate the clinical and epidemiological features of the outbreak. If these were consistent with influenza, from the most acute cases throat washings for virological purposes and blood samples for serology were obtained. Medication was started on the same day and continued until the outbreak had subsided, *i.e.*, over 2—3 weeks. For those who were admitted to the infirmary medication was discontinued on the next day.

Allocation. The technical feasibilities excluded the man to be taken as unit. Due guarantee of swallowing of the capsules at prescribed times and excluding errors and misuses were aimed at. This end could best be achieved by considering the dormitory as unit. Consequently, the dormitories were allocated to the treatments and balanced within the buildings. The subjects in the same buildings were among themselves the whole day, independent of their dormitories. In some small buildings this schedule could not be followed rigorously. The number of allocated subjects per unit is shown in Table I.

Paired sera for estimating seroconversion rate. Paired blood samples were taken in each unit from 25 healthy propositi allocated to receive the active drug and from 25 persons in the placebo group. The samples were obtained on zero day and several days after termination of the trial.

Recording. The course of the illness of in- and outpatients was recorded on usual protocols. In calculating morbidity both types of records, while for the incidence of symptoms and complications only the case records were taken into account.

The following data were analyzed: duration and peak of fever, period of treatment in infirmary, and complications, *viz.*, lower respiratory (bronchitis and bronchopneumonia), upper respiratory (tonsillitis, frontal and maxillar sinusitis) and gastrointestinal complications (vomiting and diarrhoea).

Statistical methods used were, for the graded responses: t test; for frequencies: χ^2 test: and — when frequencies were low — Fisher's exact test.

Viruses. The strain influenza A2/Hong Kong/1/68 was kindly supplied by the World Influenza Centre, London; the influenza A2/Hung/4/65 strain was isolated in our laboratory.

Virus isolation. Throat washings were centrifuged at low speed. From the supernatants with added antibiotics 0.1 ml was inoculated into the amniotic sac of each of eight 10-day-old chick embryos and into each of six primary monkey kidney (MK) cell cultures. In positive cases virus was demonstrable in the amniotic and allantoic fluids as soon as in the first passage; in MK cell cultures a second passage was necessary for several strains.

Haemagglutination-inhibition (HI) test. Human sera were pretreated as follows. 0.1 ml serum mixed with 0.05 ml of a 0.25% trypsin solution was heated for 30 minutes at 56°C; then 0.15 ml 0.02 *M* KIO_4 was added and the mixture was left to stand at room temperature for 15 minutes and, after adding of 0.05 ml 6.0% glycerol, for another 15 minutes. Finally, the mixture was made up to 0.5 ml with PBS. Thus the serum was diluted 1 to 5 and the first test dilution was 1:10.

The haemagglutinating (HA) antigen was prepared from an inhibitor-insensitive variant of the Hong Kong 1/68 strain, which had been obtained by transferring the original strain through several passages in embryonated eggs in the presence of heated horse serum. The HI test was performed in the wells of the Microtitrator plate [23]. Twelve HA units of virus, and chicken erythrocytes were used.

In the preliminary experiments it was found that this method of pretreatment and testing resulted in titres highly consistent with those obtained with the original Hong Kong strain after RDE pretreatment of sera.

Testing of antiviral activity in vitro. A hundred mg amantadine HCl was dissolved in 4 ml ethanol and the solution was diluted tenfold in a fluid (Glucosol) containing glucose and 1% eggwhite in buffered saline. Further dilutions were made in the same fluid.

Antiviral activity was estimated in the Virutor apparatus [24]. Each vial contained 0.5 ml Glucosol with or without amantadine HCl, small fragments of pooled chick embryo chorioallantoic membrane made up to 0.5 ml with Glucosol, and virus inoculum in a volume of 0.1 ml. The vials were rolled in a drum at 37°C and 10—12 r.p.hr. for 72 hours. The viral haemagglutinin in the liquid phase was titrated in the Microtitrator apparatus. Geometric means of titres were calculated from 8 parallels; virus inhibition was expressed using the quotients of the geometric means of the titres obtained in the presence and in the absence of amantadine.

Results

Test for side effects. Prior to the trial, amantadine was administered to 50 young males over a period of two weeks. The daily dose was 200 mg. No side effect could be detected.

The 1969 influenza epidemic. The epidemic that had originated in China and appeared as a very large epidemic in Hong Kong in the summer, 1968, reached Hungary late in January, 1969 and caused there an unusually protracted epidemic. It reached its peak at the end of March and came to an end in the first week of May. Morbidity rate showed a considerable variation between regions. According to virological and serological investigations the epidemic was caused by the Hong Kong variant of influenza A2 virus.

The outbreaks. Outbreaks were reported from seven of the elected units. In units 1—6 cumulation of cases was first observed between March 16th and 23rd; the outbreaks lasted about three weeks; the decline was less sharp in unit 5 than in the other five units. In unit 7 cumulation was first observed on March 6th and the outbreak lasted for about five weeks.

In unit 2 the outbreak was sudden and it was on its second day that medication was started. In the other units it took 4 to 9 days before the cumulation of cases was recognized as an outbreak and treatment was started. Consequently, a considerable part of the allocated patients had not been treated or had received only a one-day dose before the onset of illness (Tables I and III). In the following, these patients, together with the 12 patients who became ill after the treatment had ended have been considered untreated.

Evidence of the causative role of the Hong Kong variant. The Hong Kong virus could be isolated from acute cases in each of the seven units (Table II), and in units 1—4 61% of the healthy blood donors (106/173) developed HI antibodies to the same virus. Thus, the outbreaks in units 1—4 may be considered pure Hong Kong influenza outbreaks. For units 5—7 the mean antibody response rate was as low as 14%, and the protractedness of the outbreaks in these offered an opportunity to obtain test material from late cases. From

Table I

Influenza morbidity data based on clinical diagnosis

Unit No.	No. of subjects allocated	Patients admitted to infirmary		Placebo-pretreated		No. of subjects allocated	Patients admitted to infirmary		Amantadine-pretreated		Morbidity data, per cent
		before the trial	after the trial	in-patients	out-patients		before the trial	after the trial	in-patients	out-patients	
1	300	8	2	1	15	300	3	1	—	8	6.3
2	100	19	—	9	—	150	3	1	13	7	20.8
3	110	9	2	3	3	90	25	—	3	6	25.5
4	530	22	—	3	7	500	10	1	4	16	6.1
Total 1—4	1040	58	4	16	25	1040	41	3	20	37	9.8
5	200	—	2	3	10	300	1	3	14	8	8.2
6	410	6	—	2	—	440	22	—	5	3	4.5
7	560	13	—	32	41	750	26	—	30	56	15.1
Total 5—7	1170	19	2	37	51	1490	49	3	49	67	10.4
Grand total	2210	77[a]	6[b]	53*	76	2530	90[c]	6[d]	69+	104	10.2

* Group P; + Group V; [a+b+c+d] Group U.

these no virus could be isolated and some of these patients produced neither HI antibodies to the Hong Kong virus nor complement-fixing antibodies to the S antigen of influenza A and B viruses. Accordingly, in units 5 and 7 the outbreaks were of mixed aetiology. In unit 6, where all the five patients tested became seropositive, the low serological response was consistent with the low morbidity rate (4.5%), so that no other causative agent had to be assumed.

Table II

Virus isolation and HI antibody response

Unit No.	Isolation rate for		Antibody response of		Response rate / morbidity rate
	early cases	late cases	patients	healthy* subjects	
1	3/5	n.t.	5/5	25/49	5.4
2	6/7	n.t.	7/7	33/44	3.6
3	3/3	n.t.	3/3	18/34	2.1
4	5/5	n.t.	4/4	30/46	10.6
5	1/4	0/5	2/5	6/41	1.8
6	4/5	n.t.	5/5	5/44	2.4
7	5/5	0/10	6/12	6/39	1.1

* Healthy at time of taking pre-epidemic sample. n. t. = not tested.

Morbidity. Morbidity rate for the whole period of the outbreaks ranged between 4.5% and 25.5% (Table I). The great scattering of the data and the relatively low morbidity rendered impossible to perform an analysis by units. Considering the virological and serological findings the data for units 1—4 were pooled and so were the data for units 5—7 (Tables I and III).

Table III

Pooled morbidity data

	Units 1—4			Units 5—7			Units 1—7
	No. of subjects	Case morbidity	Morbidity, per cent	No. of subjects	Case morbidity	Morbidity, per cent	Morbidity, per cent
Allocated to placebo group	1040	103*	9.9	1170	109*	9.3	9.6
Allocated to amantadine group	1040	101*	9.7	1490	168*	11.3	10.6
Pretreated with placebo	978	41+	4.2	1149	88+	7.6	6.0
Pretreated with amantadine	996	57+	5.7	1439	116+	7.0	7.1

* During the whole outbreak.
+ During the period of medication.

The morbidity data revealed no significant differences between the amantadine-pretreated and placebo-pretreated subjects. The latter even showed slightly lower morbidity. It has been concluded that preventive treatment with amantadine failed to reduce the morbidity.

Effect of prophylactic treatment on the course of the disease. There was practically no difference in the ratio of outpatients to inpatients. This ratio was 1.5 for the amantadine-pretreated and 1.4 for the placebo-pretreated patients (1.9 *vs.* 1.6 for units 1—4, and 1.4 *vs.* 1.4 for units 5—7).

For further comparison only the inpatients were taken into account, *viz.*, 69 amantadine-pretreated cases (Group "V"), 53 placebo-pretreated cases (Group "P") and 179 patients (Group "U") having received neither amantadine nor placebo. The distribution of these cases between units 1—4 and 5—7 is shown in Table I.

In the analysis of the fever peak and of the complications it seemed permissible to combine the data for Group "P" with those of Group "U". Thus the control patients (Group "P+U") numbered 232. (Of these 122 belonged to units 1—4, and 110 to units 5—7.) Treatment in the different units was approximately the same and symptomatic.

As regards the duration of fever and infirmary days, on the other hand, only the cases in Groups "V" and "P", having occurred synchronously, were

compared to each other, since bed reservations for the epidemic may have urged the discharge of hospitalized patients. For the fever peak values a disqualifying condition like this did not exist.

Within the high-rate antibody units (Nos. 1—4) neither the peak, nor the duration of the fever, nor the duration of bed care showed significant differences between Group "V" and the corresponding control group (Tables IV—VI).

Table IV

Peak value of fever

Peak value °C	Group V		Group P		Group U		Group P+U	
	Frequencies		Frequencies		Frequencies		Frequencies	
	absolute	relative	absolute	relative	absolute	relative	absolute	relative
		Units 1—4						
37.1—38.0	3		2		14		16	
		0.15		*0.12*		*0.13*		*0.13*
38.1—39.0	10		7		67		74	
		0.50		*0.44*		*0.63*		*0.61*
>39.0	7		7		25		32	
		0.35		*0.44*		*0.24*		*0.26*
		Units 5—7						
37.1—38.0	25		3		24		27	
		0.51		*0.08*		*0.33*		*0.25*
38.1—39.0	19		20		33		53	
		0.39		*0.54*		*0.45*		*0.48*
> 39.0	5		14		16		30	
		0.10		*0.38*		*0.22*		*0.27*

In contrast, for units 5—7 all the three features yielded highly significant differences ($P < 0.01$). The difference in fever peak was computed in two ways, *viz.*, having for critical value either 38.0°C or 39.0°C. Both yielded the same result.

On the other hand, Group "P" and Group "U" yielded significant differences with 38.0°C, and a nearly significant one with 39.0°C, Group "P" differing more from Group "V" than did Group "U". Nevertheless, the difference between Group "P" and Group "U" did not invalidate their pooling for analysis.

Table V

Duration of fever

No. of days with fever	Units 1—4		Units 5—7	
	Group "V"	Group "P"	Group "V"	Group "P"
1	3	1	18	2
2	3	2	9	7
3	6	5	13	15
4	4	3	5	7
5	3	1	4	3
6	1	3		2
7		1		
8—				1*
Total	20	16	49	37

Mean values in units 1—4: 3.2 *vs.* 3.9 days; in units 5—7: 2.3 *vs.* 3.4 days.
* 11 days.

Table VI

Infirmary days

Days in infirmary	Units 1—4		Units 5—7	
	Group "V"	Group "P"	Group "V"	Group "P"
3	2	1	11	3
4	4	2	11	3
5	2	2	7	7
6	4	4	6	7
7	7	2	9	10
8		1	3	1
9	1	2		1
10		1	1	1
11		1	1	
12				
13				1
14				2
15				1
Total	20	16	49	37

Mean values in units 1—4: 5.7 *vs.* 6.6 days; in units 5—7: 5.3 *vs.* 6.9 days.

As computed from the pooled data of units 1—7, the fever peak showed a highly significant difference ($P < 0.01$) between Group "V" and Group "P+U", whereas both in duration of fever and in the hospital days there

were very highly significant differences ($P < 0.001$) between Group "V" and Group "P", in favour of Group "V".

Analysis of the cases with complication provided further evidence of the beneficial effect of amantadine. Since double or multiple complications occurred in as few as five cases, it seemed justified to base the analysis on the number of cases with complication. Group "V" was compared to Group "P+U".

Table VII

Complications

Unit No.	No. of inpatients in Group		Lower respiratory complications in Group		Upper respiratory complications in Group		Gastrointestinal complications in Group		Tonsillitis (disregarded)* in Group	
	"P+U"	"V"	"P+U"	"V"	"P+U"	"V"	"P+U"	"V"	"P+U"	"V"
1	15	—	—	—	—	—	1	—	—	—
2	32	13	9	—	1	—	10+	1	1	—
3	39	3	—	—	—	—	1	—	4	—
4	36	4	4	—	1	—	2	1	—	—
5	9	14	1	—	1	1	1	1+	3	3
6	30	5	9	1	—	—	1	—	—	—
7	71	30	8	—	—	—	1	1	5	6
Total	232	69	31	1	3	1	17+	4+	13	9

* See text.
\+ One, suffering also from tonsillitis, disregarded.

As shown in Table VII, the complications occurred at practically the same rate in Group "P+U" and Group "V". However, of the 15 cases with complication in Group "V", 9 were diagnosed as follicular tonsillitis and all these occurred in units 5 and 7, where the outbreak appeared to be of mixed aetiology. Of the 13 tonsillitis cases in Group "P+U", 8 occurred in units 5 or 7. Though systematic bacteriological checking was not done, these tonsillitis cases should not be regarded as influenza cases with complication. Consequently, they were excluded not to disguise the treatment effect, quite like placebo reactors are neglected in analgesic trials.

Omitting the tonsillitis cases, 49 cases with complication remained in Gorup "P+U" and 5 in Group "V". The difference is significant ($P < 0.05$). However, separate analysis in this respect of units 1—4 and units 5—7 revealed no significant difference between the amantadine-pretreated and the control groups.

The difference in cases with complication was due to the different frequencies of the cases with acute lower respiratory complications (bronchitis

plus bronchopneumonia). The 31 cases *vs.* 1 case represent a highly significant difference ($P < 0.01$) as shown by Fisher's exact test.

Seroconversion and postepidemic HI antibody titres. Seroconversion was not influenced by amantadine. We had paired sera from 103 donors who were healthy and seronegative at the time of the first sampling and developed antibodies during the outbreak. Fifty of these had been treated with amantadine and 53 with the placebo. The distribution by postepidemic HI titre of the seronconverted subjects is shown in Table VIII. The geometric mean for the subjects treated with the active substance was 1:68, *i.e.*, lower than

Table VIII

Effect of amantadine on HI antibody response of previously seronegative subjects

Group	Distribution by postepidemic HI titre						Total	Geometric mean
	1:20	1:40	1:80	1:160	1:320	1:640		
Amantadine*	6	20	11	8	3	2	50	1:68
Placebo*	5	10	17	11	5	5	53	1:98
Untreated patients	2	5	5	7	10	2	31	1:136

* Irrespective of clinical illness.

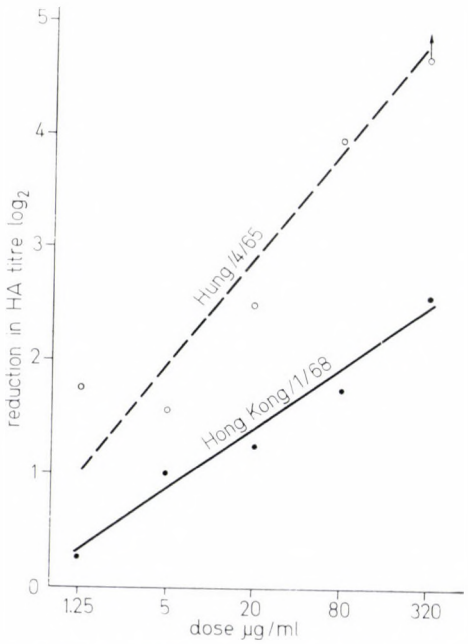

Fig. 1. Inhibition of growth of two strains of influenza A2 virus in rolled tissue fragments as a function of amantadine dose

that for the placebo-treated ones (1:98). The difference just failed to reach the 0.05 significance level calculated by the *t* test. The third row in Table VIII shows the distribution of the convalescent titres of 31 patients whose acute-phase blood samples were negative in the 1:10 dilution. The geometric mean for the convalescent titres was 1:136.

Sensitivity of the Hong Kong 1/68 strain to amantadine in vitro. The data in Fig. 1 represent geometric means calculated from the results of two experiments carried out in the Virutor system. In both experiments the Hong Kong strain proved less sensitive than the Hung 4/65 strain. (Regression lines fitted by eye.)

Discussion

The lack of any prophylactic effect of amantadine on Hong Kong influenza morbidity in the present study was in accordance with the negative results published by GALBRAITH *et al.* [24] and did not agree with the positive results obtained by SMORODINTSEV *et al.* [25] in a trial carried out on 10,053 persons during the 1969 Hong Kong influenza epidemic.

The former authors studied the prophylactic effect of amantadine in the family environment. In their 1968 trial, the same authors [20] demonstrated a definite reduction of clinical influenza in the amantadine-treated contacts of index cases with laboratory evidence of A2 infection. In a similar trial during the 1969 influenza epidemic they failed to demonstrate any protective effect.

GALBRAITH *et al.* [24] attribute the divergency of the results to a difference in the immunological status of the two populations under study. In 1969, 90% of the contacts possessed initial HI titres below 1:12 to the actual pathogen, whereas in 1968 the corresponding figure was 40%. In our study 86% of the subjects tested had pre-epidemic HI titres below 1:10 to the Hong Kong virus.

GALBRAITH *et al.* [24] found the A2 /Hong Kong/ 1/68 strain and an earlier strain of the A2 virus (England 10/67) equally sensitive to amantadine, whereas in our test the former appeared less sensitive than the strain A2/ /Hung/4/65.

Surprisingly, in contrast to the lack of a morbidity-reducing effect, we found a beneficial effect of amantadine on the clinical course of the disease in spite of that medication had been discontinued on the second day of illness.

On the basis of the frequency of serologically evidenced influenza infections the seven units under study could be classed into two groups with an approximately equal morbidity rate. Environmental or habitual factors, or differences in pre-epidemic antibody titres provided no explanation of the divergence. In units 5 and 7 an unknown aetiological agent might have inter-

fered with the spread of the influenza virus. It may be noted that the 1969 Hong Kong epidemic showed two different epidemiological patterns in different countries [27].

The question arose to what an extent the beneficial effect experienced in the present study was due to an effect of amantadine on the influenza virus infection. The question has remained open because the lack of statistical significance for the few cases in the units with pure influenza outbreaks and the significant effect within the units with mixed outbreaks did not exclude an influence upon the Hong Kong virus, for (i) in units 1—4 there was a tendency in favour of the amantadine-pretreated patients, and (ii) a considerable part of the cases in units 5—7 were caused by the Hong Kong virus. On the other hand, there was no reason for excluding an influence upon the supposed other agent(s). According to literary data [17] amantadine may be beneficial also in non-influenzal acute respiratory disease. In this respect it is of interest that, according to serological evidence, influenza B virus, which has consistently proved insensitive to amantadine, played no part in the present outbreaks.

The statistically significant reduction in the occurrence of cases with acute lower respiratory complications was not life-saving in the young and healthy subjects of the present study, but a similar effect in an old and/or chronically ill population may be life-saving. Furthermore, it should be taken into consideration that in the present trial treatment was discontinued early. Treatment for several additional days might have increased the beneficial effect.

Note. During the 1969—1970 influenza epidemic some data were collected on the therapeutic effect of amantadine (Viregyt®). In a unit under conditions like the mentioned ones, 24 amantadine-treated cases were compared to 27 placebo-treated ones. According to a preliminary analysis, amantadine proved to diminish average stay in the infirmary and number of febrile days. In another similar unit, a trial in 57 and 70 cases, no effect could be detected. In the former unit serological tests of all the propositi (amantadine- and placebo-treated as well as clinically healthy) revealed a pure Hong Kong influenza outbreak, whereas in the latter unit serological tests revealed both Hong Kong and influenza B infections.

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STUDIES ON THE PERITONEAL EXUDATE OF ANIMALS EXPERIMENTALLY INFECTED WITH TOXOPLASMA GONDII*

III. COMPARATIVE STUDY ON MOUSE PERITONEAL EXUDATE INDUCED WITH COMPLETE FREUND ADJUVANT

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(Received March 16, 1970)

Summary. Peritoneal exudate was induced in mice by means of complete Freund adjuvant. The intravenous injection of the supernatant of this fluid after heating it at 56 °C or 65 °C for 30 minutes reproduced the syndrome which develops after the intravenous injection of the supernatant of peritoneal exudate of toxoplasmic mice (SPET). The lethal dose varied between 0.006 to 0.012 ml per g body weight. Intraperitoneal and subcutaneous injections produced no apparent ill effects. The activity of this heated supernatant was abolished by hyaluronidase and reduced by trypsin. Intravenous injection of 0.05 ml per g body weight of unheated or heated mouse serum was tolerated by mice. The syndrome developing after the intravenous injection of SPET was also reproduced by 0.01 ml per g body weight of 4% starch or of saturated gum arabic solution. Mouse SPET, supernatant of mouse peritoneal exudate induced with Freund adjuvant and mouse serum are apparently qualitatively similar. In disc electrophoretograms a thick ring formed at the starting zone at the expense of others which became paler, thinner or disappeared in the three fluids after heating at 56 °C for 30 minutes. The significance of these findings is discussed with reference to SPET.

The supernatant of the peritoneal exudate of toxoplasmic mice (SPET) in small doses induces dramatic symptoms and death in mice [1—13]. Mice, however, tolerate up to 0.7 ml mouse ascitic fluid induced by lactic acid, killed streptococci, or a cutaneous graft and hamster ascitic fluid produced by infection with *Leishmania donovani* [11]. Mice also tolerate up to 1 ml of mouse serum stored at 37 °C with or without added tissue [11], and of heated and unheated human ascitic and pleural fluids and isotonic phosphate saline buffer [13]. This is regarded as evidence of the toxic nature of SPET.

Freund adjuvant was used for the production of large amounts of antibody-rich mouse ascitic fluid [14]. Such a fluid is qualitatively similar to mouse serum [15], and the effect of heating of sera was studied by electrophoresis [16].

The aim of this communication was to present a comparative study of the supernatant of mouse peritoneal exudate induced with complete Freund adjuvant with reference to SPET as regards the syndrome developing after its injection, the effective route, the effect of heating at 56 °C and at 65 °C on its activity, its possible chemical nature and the difference between unheated

* This work is part of a Thesis submitted to the Hungarian Academy of Sciences, 1969.

and heated SPET, the supernatant of exudate induced with adjuvant and serum in disc electrophoresis. Attempts were made to reproduce the syndrome with starch and gum arabic.

Materials and methods

Preparation of large volumes of mouse peritoneal exudate using complete Freund adjuvant. This is essentially the same method as that of MUNOZ [14]. Healthy female swiss mice were injected intraperitoneally each with 0.25 ml of complete Freund adjuvant (Difco) twice at an interval of 14 days and ascitic fluid was allowed to develop for at least 3 weeks.

The skin of mice displaying ascites was swabbed with iodine solution, the peritoneal cavity tapped with an 18 gauge needle and the fluid was collected in test tubes. Batches were tested for sterility on blood agar and left to clot overnight in a refrigerator. Sterile lots were pooled and centrifuged at 2000 r.p.m. After centrifugation, the supernatant (SPEF) was collected by a sterile Pasteur pipette fitted with a rubber teat to avoid collecting the oil droplets that may be present on the surface. Fluids were examined in two parts, one unheated (F_u) and the other after heating at 56 °C for 30 minutes (F_{56}) by the intravenous injection into the lateral tail vein of normal mice.

Intraperitoneal and subcutaneous injection of mice with F_u and F_{56}. SPEF was injected into four groups of 10 mice each; in the first two groups intraperitoneally, in the other two groups subcutaneously with F_u and F_{56} respectively, in a dose up to 0.05 ml of the former and of 0.006—0.012 ml of the latter per g body weight.

Effect of trypsin on the activity of SPEF. Experience showed that tryptic activity could be demonstrated on SPET whose original pH is 7.4 and which has been heated at 65 °C for 30 minutes after the addition of trypsin crystals [8]. With SPEF the experiment was carried out as follows. It was heated at 65 °C for 30 minutes and then divided into two parts. One served as control. The other part was poured in a test tube containing 1.3 mg of trypsin crystals for each ml SPEF; the tube was thoroughly shaken to mix enzyme and substrate, and incubated in a water bath for 3 hours.

Effect of hyaluronidase on the activity of F_{56}. SPEF was incubated at 56 °C for 30 minutes. It was then divided into 2 parts. One part served as control. The IU of hyaluronidase for each ml F_{56} were mixed to the second part. Both parts were incubated in a water bath at 37 °C for 4 hours.

Intravenous injection of normal mouse serum (M). Pooled mouse serum was tested in two parts, one unheated (M_u) and the other after heating at 56 °C for 30 minutes (M_{56}), by intravenous injection of a dose up to 0.05 ml per g body weight.

Intravenous injection of starch and gum arabic solutions. A 4% solution of starch and a saturated solution of gum arabic in saline were prepared. The effect of intravenously injecting 0.01 ml per g body weight of each into normal mice was tested.

The assay was done by establishing the difference in the number of dead animals in groups of mice receiving the same dose of SPEF treated in different ways. Since F_{56} was not as viscous as S_{56}, it was not diluted. This is to be considered when lethal doses of these two fluids are compared.

Disc electrophoresis of mouse SPET, SPEF and serum. Mouse SPET was prepared as described previously [6], and SPEF as described above. Pooled mouse serum was prepared from blood obtained from the tail vein of mice. Each fluid was examined in two parts, one unheated, the other after heating at 56 °C for 30 minutes [17]. The volume of the small-pore gel was 2 ml and its length 66 mm, onto which 0.15 ml of spacer gel was layered. Then one drop of the sample fluids was added by means of a Pasteur pipette. A voltage of 300 V and a current of 4 mA per tube was applied and electrophoresis was continued for 120 minutes. After electrophoresis, gel columns were stained for 60 minutes with 1% Naphthaline black 10 B (c. T. 20 470 Reanal, Budapest) in 7% acetic acid for 3 days. Excess dye was removed from the analytical gel by elution with 7% acetic acid. Gel columns were stored in acetic acid.

Results

Character of SPEF. Only a small number of mice inoculated by the adjuvant responded by the production of exudate which collected slowly but

in amounts much larger than after toxoplasmic induction. Peritoneal fluid induced by Freund adjuvant is a straw coloured fluid and is less viscous than toxoplasmic peritoneal exudate. The former tends to form a cobweb coagulum which is removed with the cells by centrifugation. Like SPET, SPEF has a pH of 7.4 and tends to form a tough mucous precipitate when stored at 5 °C.

Symptoms in mice following the intravenous injection of SPEF. No or mild dyspnoea, leaning on the side or hind quarter paralysis were observed after the intravenous injection of 0.05 ml per g body weight of F_u. The syndrome following the intravenous injection of S_u or S_{56} including symptoms such as "frog-like movement", "circus movement", "barrel rolling movement" [6] and haemoglobinuria [8], were exactly reproduced by F_{56} and F_{65}. The lethal dose varied between 0.006 and 0.012 ml per g body weight and death occurred within 1—3 minutes.

Intraperitoneal and subcutaneous injection of F_u und F_{56} were without effect.

Effect of hyaluronidase and trypsin on the activity of SPEF. The activity of F_{56} was completely abolished by hyaluronidase and the activity of F_{65} was reduced by trypsin.

Effect of intravenous injection of M_u and M_{56}. These were without apparent ill effects in a dose up to 0.05 ml per g body weight.

Effect of intravenous injection of starch and gum arabic solution. We were able to reproduce the syndrome following the intravenous injection of SPET

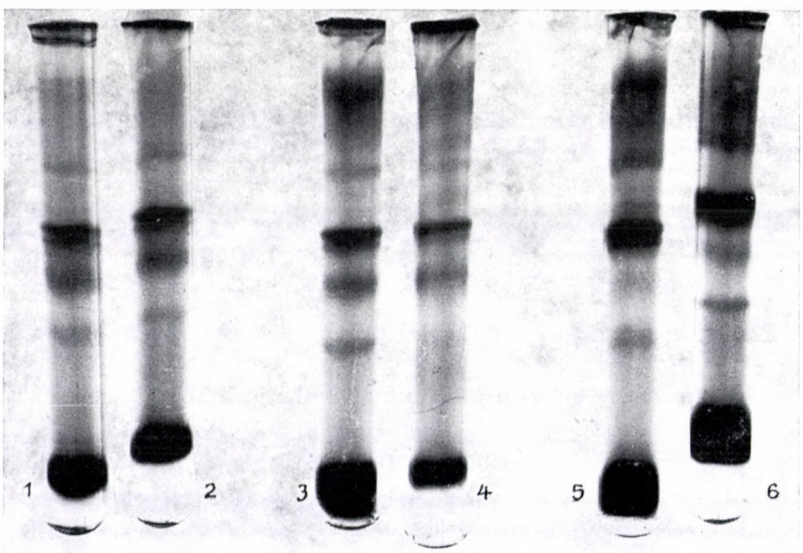

Fig. 1. Electrophoretic pattern of mouse SPET (1, 2), SPEF (3, 4) and serum (5, 6). As compared with unheated fluids (1, 3, 5), heated ones (2, 4, 6) show a thick ring at the starting zone at the expense of the others

and SPEF by the intravenous injection of 4% starch solution or saturated 33% gum arabic solution.

Disc electrophoresis of unheated and heated mouse SPET, SPEF and serum. This showed that these fluids are qualitatively similar (Fig. 1). As compared with unheated fluids (tubes 1, 3 and 5), heated fluids (tubes 2, 4 and 6) showed more dense rings at the starting zone while the others were paler, thinner or disappeared.

Discussion

Since the first report on "toxotoxin" by WEINMAN and KLATCHKO [12] only a few substances were reported that could reproduce the syndrome appearing after its intravenous injection. FULTON [2] has shown this for calf synovial fluid and sodium alginate, while we [6—8] found that starch and gum arabic solution and the heated supernatant of peritoneal exudate induced by complete Freund adjuvant, gave rise to the syndrome, too. All these substances are colloid in nature, have some common properties and the syndrome is most probably anaphylactoic in all cases. Of the above substances, the supernatant of mouse peritoneal exudate induced with Freund adjuvant in the unheated state was tolerated up to 0.05 ml per g body weight, but after heating at 56 °C for 30 minutes it induced respiratory and nervous disturbances with the same manifestations as those observed after the intravenous injection of SPET. The lethal dose of F_{56} varied between 0.006 and 0.012 ml per g body weight. Compared with SPET, this dose is a little more than that of S_u or about one fifth of that of undiluted S_{56}. It caused death in 1—3 minutes. SPEF is the most significant of the fluids that gave rise to the syndrome which follows the intravenous injection of SPET because it corresponds to the latter in several properties. Thus, it is of mouse peritoneal origin, it produces anaphylaxis-like symptoms in mice after single and after each of repeated sublethal intravenous but not intraperitoneal or subcutaneous injection, it is qualitatively similar electrophoretically, it is thermopotentiated at 56 °C and 65 °C for 30 minutes, and its activity is abolished by hyaluronidase and reduced by trypsin, *i.e.* its active substance is a mucoprotein. Moreover, it represents the first peritoneal fluid with properties similar to those of SPET. Other mouse and hamster unheated peritoneal fluids were tolerated [11], but no reports are available on their effect after heating. SPEF is a local reaction of the mouse peritoneum to the non-living content of Freund adjuvant, *i.e.* killed mycobacteria, lanolin and paraffin oil, while SPET is a local reaction of the peritoneum to *T. gondii*. The production of the active substance by means of a non-living material is against its toxoplasmic nature.

Electrophoretically, SPET, SPEF and mouse serum are qualitatively similar although not identical and this is consistent with similar findings con-

cerning SPEF and mouse serum [15]. Most probably, the difference between these fluids is quantitative: SPEF contains smaller amounts of the active substance which forms larger aggregates after heating and serum contains little or none of it.

Heating of SPET and SPEF enhances their activity and the mechanism of thermopotentiation is probably the same [6]. The slow moving fraction at the starting zone of electrophoretograms probably corresponds to the fast sedimenting fraction [2] in the ultracentrifuge and is in harmony with the results of electrophoretic studies [16] on heated horse serum.

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EFFECT OF PHYTOHAEMAGGLUTININ AND TUBERCULIN ON MACROMOLECULE SYNTHESIS IN HUMAN LYMPHOCYTE CULTURES

By

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(Received May 26, 1970)

Summary. Macromolecule synthesis induced by phytohaemagglutinin and tuberculin in lymphocyte cultures from Mantoux positive subjects has been examined. Phytohaemagglutinin stimulation increased histone methylation after 30 minutes and induced RNA and protein synthesis after 6 hours. Uridine and leucine incorporation showed maximum values at 48 hours, DNA synthesis reached the peak at 72 hours.

Tuberculin exerted a similar effect, but the maximum values were reached 24 hours later than with phytohaemagglutinin. Lymphocyte cultures from drug sensitive patients reacted similarly with the specific antigens. Actinomycin D inhibited both phytohaemagglutinin and tuberculin-induced uridine incorporation.

Hungerford *et al.* [1] and Nowell [2] were the first to show that as an effect of phytohaemagglutinin (PHA), an agent agglutinating erythrocytes and leukocytes, the small lymphocytes in peripheral blood develop into blast-type cells. Lymphocytes cultured *in vitro* in the presence of PHA show a gradual enlargement in 36—48 hours and a newly arranged chromatin structure in the nucleus. Nucleoli and mitochondria increase in number and exhibit a structural alteration. Part of the blast-type cells divide in the 48th—72nd hour of cultivation [3—5]. Blastic transformation by PHA is aspecific, as lymphocytes of all normal humans give the reaction.

In 1963, several authors have observed that the purified protein derivative of tuberculin (PPD) induced blastic transformation in part of lymphocytes obtained from Mantoux positive donors [6—9]. Other specific antigens may also exert mitogenic activity, for example, tetanus and diphtheria toxoid tetanus anatoxin, Salk's poliovirus, *Treponema* antigen, cardiolipin, different tissue antigens and drugs [10—14]. A common property of these antigens is that they are able to stimulate lymphocytes originating from previously sensitized individuals. Blast-type cells developing as an effect of PHA or specific stimulators are very similar in appearance [15]. On the basis of their light and electron microscopic picture many authors regard them as lymphoblasts or immunoblasts [16].

In the present experiments we compared PHA and tuberculin-induced histone methylation in lymphocyte cultures from Mantoux positive donors and the degree and time course of subsequent RNA, protein and DNA synthesis.

Materials and methods

Lymphocytes from 5 Mantoux positive non-tuberculous and from 3 drug sensitive (promethazine, procaine and streptomycin) patients were used. The degree of histone methylation was determined by measuring the incorporation of ^{14}C-labelled methyl group. The synthesis of DNA, RNA aud protein was estimated on the basis of ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine incorporation.

Lymphocyte cultures. Citrated blood was taken from the cubital vein and left to stand at room temperature for 1 hour. Then the plasma containing the lymphocytes was removed and centrifuged at 800 r.p.m. for 5 minutes, and the deposit was resuspended in the autologous plasma to 4×10^6 lymphocytes per ml. Lymphocytes were not separated from other white blood cells. Cultures were prepared by adding 2 ml lymphocyte suspension to 8 ml antibiotic-free Parker 199 solution. Stimulating antigens were added to the cultures at 0.1 ml aliquots at the beginning of the experiment. Cultivation was performed at 37 °C.

Mitogens. For aspecific stimulation 0.1 ml PHA (Phytohemagglutinin M, Difco) was added. Specific stimulation was carried out with 0.3 U PPD (Human, Budapest) per ml or, for lymphocytes of drug sensitive patients, with promethazine (5 μg/ml), streptomycin (100 μg/ml) or procaine (200 μg/ml).

Histone methylation. Simultaneously with the stimulators, methyl-^{14}C-labelled methionine (0.1 μC/ml) was added to the culture. These experiments were carried out only with lymphocytes taken from tuberculin positive subjects. Three parallel cultures were prepared from each subject: one of them was stimulated with PHA, the other with PPD and the third served as control. At the beginning of the experiment and at intervals indicated in the Figures, 2×1 ml samples were taken and the histones were extracted from each sample separately. During the 4 hour experiment the lymphocyte count remained at the same level in all cultures. Histone extraction was performed as described by Pogo *et al.* [17]. The nuclei were isolated then resuspended in 0.5 ml 0.2 *N* HCl solution at 4 °C for 20 minutes. The suspension was then centrifuged and the extraction of the deposit was repeated. After uniting the two fractions, the histones were precipitated from the acid solution with 9 ml 4 °C acetone. The deposit was purified by performing two subsequent 20 minute extractions as decribed above. Radioactivity of the hydrochloric acid extract was determined with a Packard Tri-Carb scintillation counter.

RNA, protein and DNA synthesis. Six parallel cultures were prepared from each donor: 3 of them were stimulated by specific antigen, while the other 3 served as controls. Samples were taken at intervals shown in the Figures. To 1 ml sample, 1 μC ^{3}H-uridine, ^{3}H-leucine or ^{3}H-thymidine were added in 0.1 ml sterile isotonic solution. After 6 hours cultivation the samples were precipitated with 5% trichloracetic acid. Acid-soluble precursors were removed by repeated washing in trichloracetic acid. The amount of incorporated uridine, leucine and thymidine was determined by liquid scintillation counting. Because of alterations in the number of cells taking place during incubation, the results were calculated for 100 000 lymphocytes.

Effect of actinomycin D. In all experiments two PHA and two tuberculin-stimulated and one untreated control were prepared and labelled with ^{3}H-uridine. One PHA and one tuberculin-stimulated culture were supplemented with actinomycin D (10 μg/ml) while the corresponding parallel cultures were used as controls. After 6 hours incubation the amount of uridine incorporated was determined as described above.

Results

Histone methylation. In lymphocyte cultures not treated with specific or aspecific antigen, ^{14}C incorporation into the histones did not increase in 4 hours as compared to the initial value. When the culture was stimulated with PHA, a marked histone methylation was observed as soon as after 30

minutes. ^{14}C uptake in the next 30 minutes decreased rapidly, then from the second hour methylation increased continuously till the end of the experiment. By the end of the 4th hour, radioactivity had reached the peak measured after 30 minutes.

Essentially similar results were obtained when PPD was added to lymphocytes of Mantoux positive subjects. In these experiments the kinetic curve for histone methylation reached the first peak in 60 minutes instead of 30 minutes and minimum values were observed 2 hours after the beginning of the experiment. The radioactivity of histone extracts was considerably lower after PPD stimulation than after PHA treatment (Fig. 1).

Time course of RNA, protein and DNA synthesis. In cultures stimulated with PHA, intense uridine and leucine incorporation was evident 6 hours after beginning of the experiment. This increased continuously and reached the peak at the 48th hour of cultivation (Fig. 2), then the degree of RNA and protein synthesis decreased gradually till the end of the experiment.

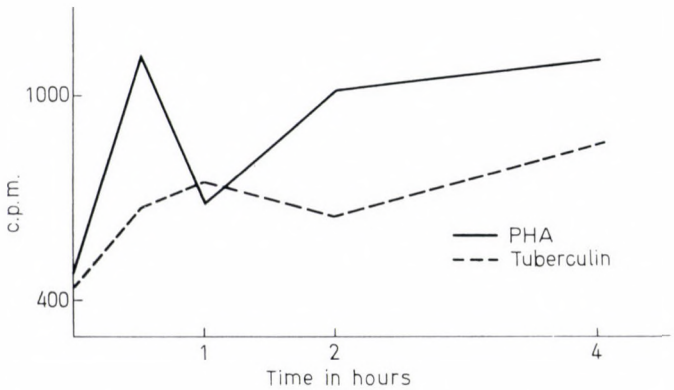

Fig. 1. Effect of phytohaemagglutinin and tuberculin on histone methylation

The course of DNA synthesis after PHA stimulation was essentially different from the course of protein and RNA synthesis. On the first day of cultivation thymidine incorporation was minimal, but on the next days the rate of DNA synthesis increased rapidly to reach the peak on the third day. Subsequently, similarly to uridine and leucine incorporation, thymidine uptake decreased gradually.

Fig. 3 presents macromolecule synthesis induced by PPD in lymphocytes from the same donors. In the first 6 hours of transformation leucine and uridine incorporation began and increased continuously till the third day of cultivation, then the degree of RNA and protein synthesis decreased. DNA synthesis commenced as an effect of specific stimulation only after a longer latency

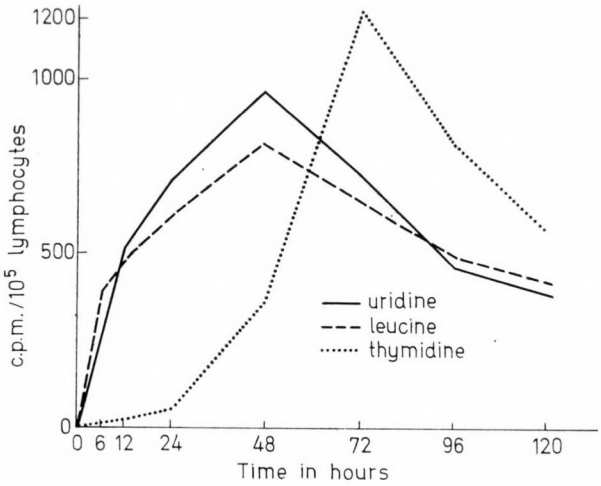

Fig. 2. **Effect of phytohaemagglutinin on uridine, leucine and thymidine incorporation. Radioactivity of non-stimulated control cultures was subtracted from values measured in the test cultures**

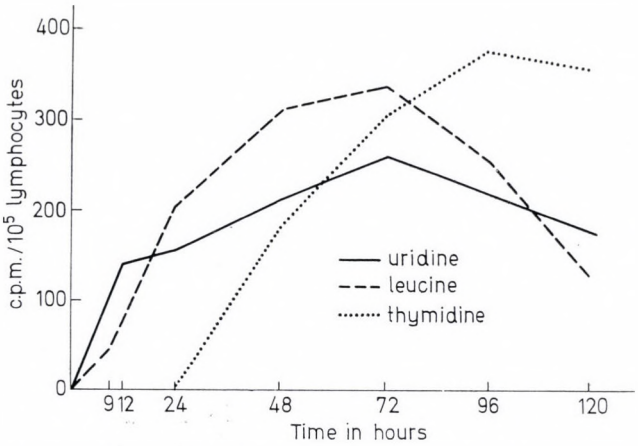

Fig. 3. **Effect of tuberculin on uridine, leucine and thymidine incorporation. Radioactivit of non-stimulated control cultures was subtracted from values measured in the test culture**

period and reached its maximum on the fourth day. In promethazine, procaine and streptomycin-stimulated cultures the course of uridine, leucine and thymidine incorporation was identical with that observed in PPD-treated cultures.

Uridine uptake was always parallel with leucine incorporation in cultures stimulated with PHA or with specific antigen. The synthesis of the two macromolecules commenced in the early phase of the experiments and took an identical course. In contrast, DNA synthesis induced by the two kinds of

antigens began after a longer latency period and reached maximum values one day later. After PHA stimulation, uridine, leucine and thymidine incorporation reached maximum values 24 hours earlier than after treatment with specific antigens and the degree of uptake was considerably higher. As shown in Table I, PHA and PPD-induced RNA synthesis was completely inhibited when cultivation was performed in the presence of actinomycin D (10 μg/ml).

Table I

Inhibitory effect of actinomycin D

Antigen	Actinomycin D	Increase in radioactivity, cpm/ml culture
Nil	0	135
Tuberculin	0	758
Tuberculin	10 μg/ml	93
PHA	0	2069
PHA	10 μg/ml	210

Five parallel cultures were prepared from lymphocytes of Mantoux positive donors and stimulated with tuberculin and PHA. Actinomycin D was added in the beginning of the experiment. The increase in radioactivity was calculated by subtracting the initial counts from values measured after 6 hours incubation.

Discussion

ALLFRAY *et al.* [18] have assumed that acetylation of histones (basic proteins linked to DNA) induces an alteration in the chromatin which allows the starting of DNA-dependent RNA synthesis. This consideration has been based on observations showing that there is a direct association between histone acetylation and the starting of RNA synthesis and that acetylated histones only slightly inhibit the activity of calf thymus and *E. coli* RNA-polymerase [18, 19].

Histone hydrolysates usually contain different methyl-lysines. According to ROBBINS and BORUN [20] methylation of the free lysine groups of histones occurs in the nucleus as a subsequent step after the histone synthesis. Therefore, in order to study histone alteration in the chromatin we examined methylation processes.

POGO *et al.* investigated the effect of PHA in human lymphocyte cultures and showed that acetylation of histones began in a few minutes after the stimulus and reached a constant value after 30 minutes [17]. We also observed that both tuberculin and PHA treatment induce an increase in ^{14}C uptake by histones (Fig. 1). On the basis of this finding we assumed that the derepressive processes needed for the starting of macromolecule synthesis are commenc-

ing within minutes after the specific stimulation. In our experiments, 60 minutes after the addition of PHA the uptake of ^{14}C by histones decreased considerably, but after this transitional decrease methylation began to increase again. Similar changes were observed after PPD stimulation, but ^{14}C incorporation after the specific antigenic stimulus was lower in degree and the transitional decrease occurred later than with PHA.

It has been described that uridine incorporation in PHA-induced lymphocyte cultures begins 30 minutes after derepression. The process was attributed to messenger RNA synthesis [17]. In our experiments 6 hours after PHA stimulation a marked uridine incorporation occurred, which increased till the second day of cultivation, then RNA synthesis decreased gradually (Fig. 2). The course of RNA synthesis after the specific stimulus was essentially similar to that after PHA induction in the lymphocyte cultures of both Mantoux positive and drug sensitive patients but uridine incorporation increased slowly and reached the peak later (Fig. 3).

RNA synthesis starting after PPD treatment, similarly to that after PHA, is inhibited by actinomycin D (Table I). This means that in the first step tuberculin probably induces also messenger RNA production.

Leucine incorporation changed — in the trichloracetic acid-precipitable protein fraction — parallel with uridine incorporation after treatment with PHA or specific antigens. DNA synthesis took a different course after the antigenic stimulus. After the addition of PHA, thymidine incorporation began after a 24 hour latency period and reached its maximum at the 72nd hour (Fig. 2). Specific antigens induced DNA synthesis also after one day, but the maximum appeared at the 96th hour (Fig. 3). Similar results were obtained by CARON *et al.* who investigated DNA synthesis after specific and aspecific stimulation [21]. According to AMBS, thymidine incorporation and blastic transformation after PHA treatment showed a close correlation [22].

As to the effect of PHA and specific antigens on macromolecule synthesis, it may be assumed that the histone methylation, RNA, protein and DNA synthesis induced by the two kinds of mitogen are very similar in course. Quantitative differences may be explained by the fact that PHA is capable of stimulating a much larger portion of lymphocytes than the specific antigen. This assumption has been confirmed by the difference between the mitotic indices of cultures transformed with the two kinds of antigen [23].

In both types of stimulation, first histones are methylated, then messenger RNA and protein synthesis and finally DNA synthesis and mitosis are induced. In transformation by specific antigens the processes take a slower course and RNA, protein and DNA syntheses reach peak values at least 24 hours later than with PHA stimulation. In our opinion these kinetic differences — despite the close similarities — indicate some delicate differences in the mechanism of stimulation.

In human lymphocyte cultures the increase of histone methylation starts within a few minutes after the stimulation, since according to TIDWELL *et al.* [24] after partial hepatectomy the methylation of histones of the liver cells occurs much later than the acetylation of histones.

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ESTIMATION OF ENDOTOXIN-INDUCED INCREASE IN RESISTANCE BY MEANS OF TRYPANOSOMA EQUIPERDUM GROWTH CURVES

By

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Summary. In rats pretreated with *Serratia marcescens* endotoxin, the logarithmic growth of intravenously administered *Trypanosoma equiperdum* started only after a lag phase of several hours.

The length of the lag phase depended on the time elapsed between endotoxin treatment and infection. With an endotoxin dose of 200 μg/100 g, the optimal time interval between treatment and infection was found to be 24—72 hours.

Other factors influencing the length of the lag phase of the trypanosomal growth curve were the body weight of the experimental animals and the germ count of the dose used for infection.

Infecting rats of identical body weight with identical germ count at identical time interval after endotoxin treatment, a linear regression was demonstrable between the length of the lag phases of the growth curves and the applied doses of endotoxin. For mathematical-statistical analysis, rats of 100 g body weight were infected 40 hours after endotoxin treatment with *T. equiperdum* doses so adjusted as to make an initial germ count of $2 \cdot 10^7$ ml.

On the basis of the phenomenon, a method has been elaborated which allows a quantitative comparison of the resistance-increasing effect of endotoxin preparations.

It is well known that experimental animals treated with endotoxin show an increased resistance to injuries [1], ionizing irradiation [2, 3] and infection by parasites [10]. This favourable effect of endotoxin cannot be utilized for prophylactic purposes, as the dose enhancing the non-specific resistance is already toxic. Efforts have therefore been made to develop such endotoxin preparations which, though not toxic, would still be capable of increasing the non-specific resistance [11]. One and the same endotoxin confers various degrees of resistance against the different pathogenic agents. Therefore, testing of certain endotoxins for their resistance-increasing effect has been carried out against several pathogenic agents simultaneously. This is a time consuming procedure, as evaluation has been made as a rule on the basis of post-infection survival of the experimental animals.

Previous studies in this laboratory have shown that in rats infected intravenously with *Trypanosoma equiperdum*, the trypanosomes immediately started to grow logarithmically and in the body weight group of 100—120 g, a lag phase took place only after preceding endotoxin treatment [12]. Elevation of the endotoxin dose resulted in a prolongation of the lag phase [13].

In the present paper, analysis of trypanosomal growth curves as a method of quantitative assay of the resistance increasing effect of endotoxin is reported.

Materials and methods

Experimental animals. Male Wistar rats weighing 100—300 g were used throughout.

Endotoxin. Serratia marcescens endotoxin, prepared with Boivin and Mesrobeanu's method, was used for pretreatment. Doses were expressed in terms of dry material content. The preparation's LD_{50} was 460 μg/100 g for the rat.

T. equiperdum growth curves. A *T. equiperdum* strain maintained in this laboratory in mouse passages was used for infection. As infectious material, blood from rats infected 48 hours before the experiment was used. In the knowledge of the germ count of the blood used for infection and of the circulating blood volume of the rats, the desired initial germ count, 7.45 ml/100 g in the present case, could readily be adjusted, since intravenously administered trypanosomes are uniformly distributed in circulating blood. The blood trypanosomal count of the infected animals was estimated from a 200-fold diluted blood sample in Buerker's chamber. Counting error was $\pm 5\%$. In rats not treated previously with endotoxin, the trypanosomes started logarithmic growth immediately, with a duplication time of 5.5 hours, which correlated well with the relevant literary data [14]. In contrast, in the endotoxin-treated rats, logarithmic trypanosomal growth started only after a certain lag phase. With post-lag phase growth, duplication time was again 5.5 hours, like in the control rats. The length of the lag phase was determined by graphical extrapolation, according to HINSHELWOOD [15]

Results

The first series of experiments was undertaken to clarify which time interval between endotoxin treatment and infection was optimal to study the increase in resistance to infection. Rats weighing 100—120 g were given 200 μg/100 g endotoxin intraperitoneally. Infection with trypanosomes, adjusted to an initial germ count of $2 \cdot 10^7$/ml, was made simultaneously with endotoxin treatment and 24, 48, 72, 96 and 120 hours after it. The length of the lag phases of the growth curves, determined as specified above, are shown in Table I.

Table I

Length of lag phase of trypanosomal growth curves as assessed in rats infected at different time intervals after treatment with 200 μg/100 g endotoxin

Time of infection, hours*	No. of animals	Lag phase (hours)	Standard error
Simultaneously	4	0.25	0.08
+ 24	10	5.9	0.52
+ 48	10	5.6	0.64
+ 72	8	5.7	0.58
+ 96	5	3.2	0.21
+120	5	0.6	0.10

* After endotoxin administration.

The lag phases measured in the rats infected 24—72 hours after endotoxin treatment lasted 5.6—5.9 hours. An abrupt decrease of the lag phase occurred first in those rats which had been infected 96 hours after the endotoxin. When given simultaneously with the infection, the endotoxin did not modify the trypanosomal growth. These findings suggested that the infection time optimal for the study of resistance-increasing effect was 24—72 hours after endotoxin. In further experiments, infection was always performed 40 hours after endotoxin pretreatment.

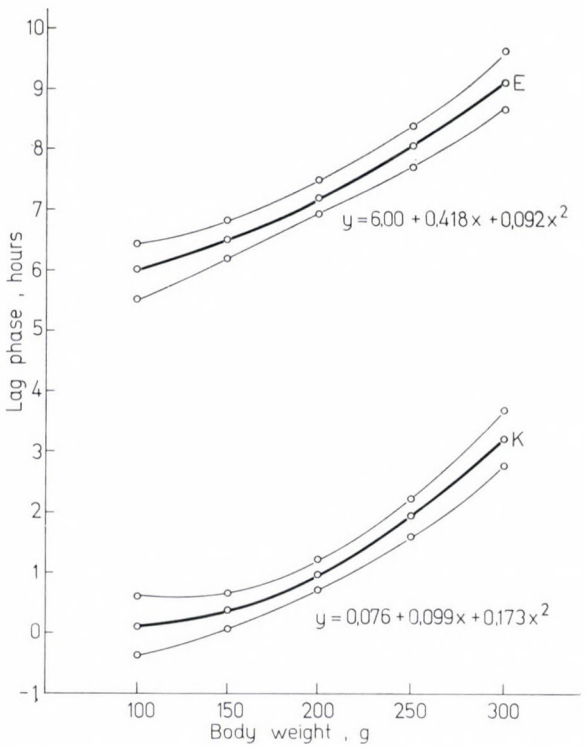

Fig. 1. Relationship between length of lag phase of trypanosomal growth curves and body weight of rats previously treated (E) and not treated (K) with 200 μg/100 g endotoxin. The 95% reliability limits are also shown

Since with rats weighing 200—250 g, short lag phases were noted also in the control group, in subsequent experiments the duration of the lag phase was determined in rats differing in body weight treated and not treated previously with endotoxin. Infection with trypanosomes, adjusted to make an initial germ count of $2 \cdot 10^7$/ml, was always carried out 40 hours after endotoxin treatment. The relationship between the length of the lag phase and body weight in endotoxin-treated and control animals is shown in Fig. 1.

As can be seen from Fig. 1, with higher body weights the lag phase was prolonged in both experimental and control rats. The regression between body weight and the length of the lag phase could be represented graphically as a second order curve. Excluding the 300 g weight group from the analysis, in both cases a linear regression was obtained, with coefficients of 0.56 and 0.66 for the control and experimental groups, respectively. The difference between the coefficients not being significant statistically, it follows that up to a weight category of 250 g, rats of any weight can be used provided the results are appropriately converted or animals of identical body weight are used in the experimental and control groups. Yet, preference should be given to rats of 100—200 g weight, as in this group there never occurred a spontaneous lag phase. Another factor to be considered in the course of the elaboration of the method was the eventual modification of the length of the endotoxin-induced lag phase by the change of the germ count used for infection. Rats weighing 100—120 g were therefore infected 40 hours after endotoxin treatment with various trypanosome counts by the intravenous route. Lengths of the lag phases of the trypanosomal growth curves thus obtained are shown in Fig. 2.

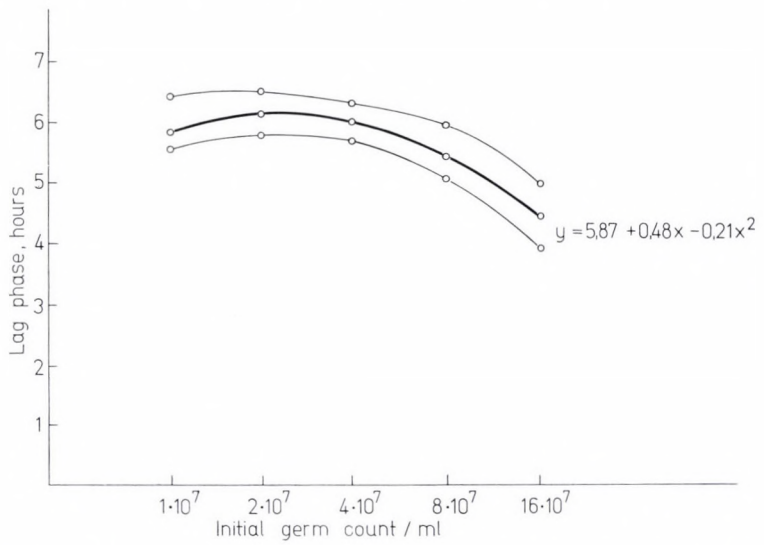

Fig. 2. Relationship between length of lag phase of trypanosomal growth curves and initial germ count in rats pretreated with 200 μg/100 g endotoxin. The 95% reliability limits are also shown

In this case, too, the correlation between the germ count of the infectious dose and the length of the lag phase could be characterized by a second order curve. It shows that with an initial germ count of $2-4 \cdot 10^7$/ml, there was no change in the length of the lag phase, while with higher germ counts there

was a definite shortening. Knowing the animal's body weight the precise adjustment of the initial germ count involves no technical problem. To ensure the exactness of counting in Buerker's chamber, we adjusted the inocula so as to keep the initial germ count at $2 \cdot 10^7$/ml, the lower limit of the optimal range.

Subsequently, the nature of the correlation between the endotoxin dose and the length of the lag phase was scrutinized. In this series, rats of strictly 100 g body weight received various doses of endotoxin and were infected 40 hours later with trypanosome material adjusted to make an initial germ count of $2 \cdot 10^7$/ml. The length of the lag phases of the trypanosomal growth curves thus obtained is shown in Table II.

Table II

Length of lag phase of trypanosomal growth curves as assessed in rats infected 40 hours after treatment with various doses of endotoxin

Endotoxin dose, μg/100 g	No. of animals	Lag phase, hours	Standard error
50	6	1.67	0.53
100	6	4.17	0.57
200	6	5.97	0.61
400	6	7.85	0.54

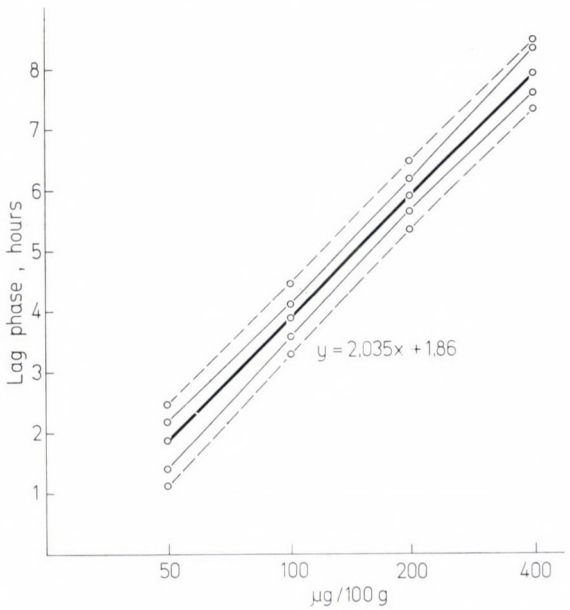

Fig. 3. Relationship between length of lag phase of trypanosomal growth curves and the dose of endotoxin. The 95% reliability limits of the regression line (————) and of the 6 parallel measurements (- - - - - - - - - -) are also shown

The applied endotoxin doses and lengths of the lag phases were shown to be related by linear regression (Fig. 3). The regression coefficient (b = 2.035) was highly significant statistically.

In addition to the 95% reliability limits of the regression line, the reliability limits of the mean of 6 parallel measurements are shown. The reliability limits could further be narrowed down by increasing the number of parallel measurements.

Thus, the method appears to be suitable for the quantitative estimation of the resistance increasing effect of endotoxin preparations. In possession of a reference standard, the procedure allows the determination of the relative potency of any endotoxin preparation by means of the six-point assay.

Discussion

The mechanism of the resistance-increasing action of endotoxin is unclear. It has been shown that treatment with endotoxin was followed by an increase in RES activity [16], and phagocytosis [17], elevation of serum bactericidin [18], opsonin [19] and interferon [20] levels.

The numerous, often controversial literary data suggest that depending on the host organism and the pathogenic agent selected for the study of the phenomenon, one or another component of the non-specific protective mechanism will become predominant.

The mechanism of the lag phases in trypanosomal growth curves is explicable from relevant *in vitro* and passive transfer experiments. In the sera of endotoxin-treated rats, a trypanosomicidal factor was demonstrated which exerted its action on the isolated trypanosomes during the 2nd—3rd hours of incubation and could not be inactivated by the procedures tested [21]. This trypanosomal growth curve modifying action was transferable to susceptible rats by the serum of endotoxin-treated rats [22]. Since the blood plasma of endotoxin-treated rats had no such effect unless transfer was performed in platelet-rich serum, a decisive role of platelets in the development of the lag phase was supposed. It is known from reports of DES PREZ and his group that endotoxin damages the platelets, which then release serotonin, phospholipids and bactericidal substances, *in vitro* into the medium and *in vivo* into the circulation [23, 24]. These bactericidal substances are thermostable and appear to be identical with the bactericidal serum components I and II of MYRVICK, known to be acute phase proteins [25, 26]. PATTERSON [27] has convincingly shown that in the mouse, elevation of the C reactive protein level in response to endotoxin injection and resistance to *Staphylococcus aureus* were related.

According to LANDY and PILLEMER [28], the resistance-increasing action of endotoxin depended primarily on the applied dose and on the time interval between endotoxin treatment and infection. Using endotoxin doses of 100 μg,

10 μg and 1.0—0.1 μg, they found maximum protection in mice infected 24—28, 12 and 6 hours, respectively, after endotoxin treatment, as assessed from the survival of *Salmonella typhi* infection. The resistance-increasing potency of a given endotoxin preparation can be estimated on the one hand from the time when it confers maximum protection against infection, and on the other hand from the comparison of the degrees of protection conferred on animals pretreated and infected at identical times. In the present studies the latter way was followed. Based on experiments with 200 μg/100 g endotoxin, a time interval of 40 hours was chosen for subsequent infection to compare the effect of the various endotoxin doses on the lag phase of trypanosomal growth curves. Though LANDY and PILLEMER [28] drew no such conclusions from their experiments, their findings clearly show that after infecting the mice 48 hours after pretreatment with 100 μg, 10 μg and 1.0—0.1 μg endotoxin, the survival ratios were 90%, 40% and 18%, respectively.

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VITAMIN B_{12} PRODUCING FERMENTATIONS OF SEWAGE SLUDGE ORIGIN WITH A MIXED BACTERIUM POPULATION

I. ROLE OF INDIVIDUAL BACTERIUM SPECIES IN VITAMIN B_{12} PRODUCTION

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Summary. The mixed bacterium population of vitamin B_{12} producing fermentations inoculated with 20% domestic sewage sludge remained unchanged during several years, when the charging of sewage sludge was discontinued and the population was adapted to a sludge-less medium. The analysis of the morphological types was difficult because of the polymorphism of the individual bacterium species. This could, however, be promoted by the fact that it was possible to separate these bacteria by centrifugation into three typical morphological groups. By determining the B_{12} vitamin/analogue content in the three separated morphological bacterial groups, it was possible to clarify their special role in the production of the total vitamin yield. This allowed to influence the bacterial spectrum of the fermentation and therewith its vitamin/analogue content.

The sludge of domestic sewage is well known to contain some vitamin B_{12} [1, 2, 3]. Its amount can be increased by fermenting it for several months at a temperature of 30 °C. The yield is raised further on multiplying the number of bacteria in the sludge by the addition of certain nutrients. It was reasonable, therefore, to start fermentation with a culture medium favourable to these bacteria, composed of cheap nutrients and precursors, with 20% sewage sludge as inoculum. This procedure in batch fermentation was repeated with added sludge until an intensive multiplication of the bacteria had been reached together with a good yield of vitamin B_{12}.

The disadvantage of the process consisted in that starting of fermentation in a 500 cu.m. capacity fermentor needed 100 cu.m. of fermented sewage sludge. Obtaining and transport to the plant of such a large amount of sludge encountered with difficulties. Further difficulties were that the composition of domestic sewage sludge changed season-dependently and its bacterium flora was also varying. Moreover, the so-called industrial wastes flowing into the sewage canals might contain substances toxic for the bacteria. Thus, when using sewage sludge in the fermentations no standard bacterium population had formed; yet, this was a condition of stable production.

Therefore, the sewage sludge was omitted. The well-producing bacterium population formed during several years in the culture medium containing sewage,

was adapted to a culture medium containing no sewage sludge [4]. At first lower amounts of vitamin B_{12} were obtained but by means of transfers repeated every five days, after several generations, a yield was obtained reaching and even surpassing the yields of fermentations performed by the addition of sludge. The bacterium population maintained by serial weekly transfers for more than three years produced 5000—7000 μg/l (and sometimes more) of cyanocobalamine and about the same amounts of analogues in laboratory fermentors.

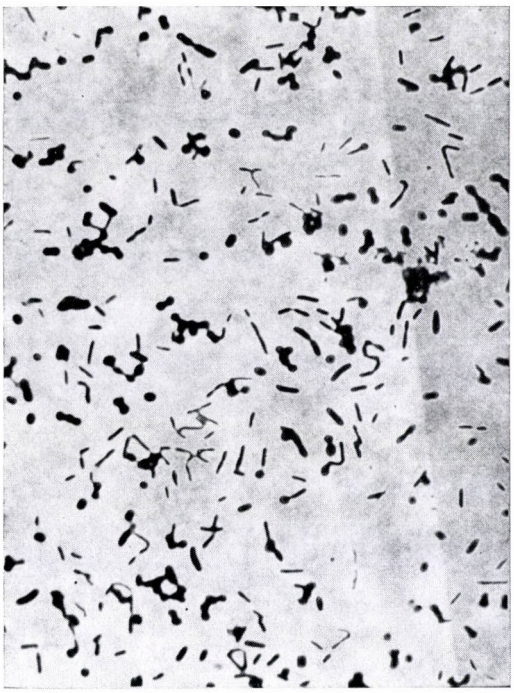

Fig. 1. Mixed bacterium population of 5 days old fermentation (×1250)

Vitamin B_{12} and its analogues are in the form of coenzymes in the bacterial cells. From these the corrinoids are extracted in the presence of cyanide and so the extracts contain the cyanides of corrinoids. Their amount determined in the experiments to be described relates to the quantitative content of the different corrinoid-coenzymes in the bacterial cells. For brevity's sake we shall use the following nomenclature: cyanocobalamine for 5,6-dimethylbenzimidazolyl cobamide cyanide; Factor III for 5-hydroxybenzimidazolylcyanide; Factor B for cobinamide; total B_{12} for vitamin B_{12} plus all the analogues. Summa factor (Σ factor) is obtained by subtracting the amount of cyanocobalamine and Factor B from the total amount of B_{12}.

The corrinoid yield was produced by a mixed bacterium population. Its morphological analysis caused difficulties because of the polymorphism of the individual bacterium species. Roughly, three predominant types were recognized (Fig. 1): (a) Gram-positive cocci-diplococci with 1.0—1.2 μ in diameter (in the following, *c—dc*); (b) Gram-positive small rods measuring 0.3—0.5 $\times$ $\times$1.0—2.5 μ, met with one by one or in the form of a V, or forming chains (in the following, *sr*). A small coccoid form of these was also frequent. (c) Thicker, larger, Gram-positive or Gram-variable polymorphous rods measuring 0.6—0.8$\times$2—4 μ *(thr)*. Occasionally, some other bacteria were also found, *e.g.* several large, Gram-positive streptococci. The taxonomic determination of these bacteria will be given in another paper.

The relative number of the three predominant bacterium types may differ according to the composition of the culture medium or fermentation conditions; sometimes, however, it changed uncontrollably *per se* and this process caused changes in the quantity and quality of the yield. It was therefore desirable to elucidate the role of the individual bacterium species relative to the quantity and quality of B_{12} and analogues in the yields, in order that the composition of the population and so of the yield could be directed on the basis of experience.

A method for clarifying this problem could have been to isolate the bacterium species in pure culture and to examine its quantitative and qualitative production capacity at first separately and then in combinations. These investigations should be completed with fermentation experiments where relative quantities of the bacterium species used as inoculum were varied. This was, however, impracticable since *c—dc* could not be obtained in pure culture. Moreover it was thought that several variants of the bacterium species might occur in the fermentations and hence several strains should have been examined.

It would offer deeper insight into the question if we could separate the individual bacterial species from a high-producing bacterium population at the peak of production and so to determine separately their participation in the total vitamin yield. This approximation of the problem would be desirable, since the life, multiplication, metabolism, and producing capacity of the microorganisms of various species in a mixed population depend to a certain extent upon one another (commensalism, symbiosis, synergism, antagonism; the phenomenon described by us and termed combined fermentation could be also mentioned in this connexion [5]).

Such an examination of the individual role of the different bacterial species in producing the vitamin would only have been possible if B_{12} and the analogues had accumulated in the bacteria and had not been excreted into the fluid up to the moment of examination.

The elucidation of these problems was the aim of the present study.

Materials and methods

The bacterium population used in the experiments was partly the culture of laboratory 10 litre fermentations and partly the culture of well-producing plant fermentations of 500 cu.m.

The composition of the culture medium used most frequently in fermentations has been described in a Hungarian Patent [4]. Culture media with different composition eventually used will be mentioned in the experimental part.

The laboratory fermentors were wide-mouth cylindrical glass vessels of 10 litres filled up to the neck with culture medium and thermostated at 28—30 °C with a thick rubber sheet cover. The plant fermentors were constructed of steel, with a narrowing opening on the top. The contents were mixed after each addition of materials (*e.g.* methanol) and before sampling. Moreover, the liquid was continuously kept in motion by the intensive gas production.

Vitamin B_{12} and its analogues were determined quantitatively by paper chromatography and electrophoresis. The fermentation brew was heated to boiling with cyanide, precipitated with iron chloride, and the precipitate was discarded. From the clear supernatant the corrinoids were extracted with a 1 : 2 v/v mixture of phenol and chloroform. From this solution, after the addition of acetone and chloroform, all the cyano-corrinoids were extracted quantitatively with water. An aliquot of the solution was chromatographed on Macherey—Nagel No. 214 paper in secondary butanol saturated with cyanide containing water. The spots on the paper were cut out, extracted in distilled water containing 0.1% sodium cyanide and compared in a colorimeter to a vitamin B_{12} standard. The same solution was used for electrophoretic determination. The paper was soaked with 0.5 *N* acetic acid to which 0.1% sodium cyanide was added. Electrophoresis was done at 700 V, 10 mA, for 4—5 hours.

Experimental

1. It was studied whether B_{12} and its analogues were to be found in the body of bacteria or were secreted into the surrounding liquid.

The amount of vitamins was determined in 500 ml of well-producing fermentation brew (control). Another 500 ml of this liquid was freed from bacteria by centrifuging and often by subsequent filtration through a Jena G-5 glass filter, and the vitamin content was estimated.

In pure bacterium-free liquid originating from batch fermentations, an average of 2.0%, in liquids from semicontinuous fermentations an average of 7.1% of the total B_{12} content were found. The balance was in the bacteria. The above difference was easy to understand for part of the bacteria in the semicontinuous fermentations was more aged than those in the batch fermentations. The older bacteria died successively. Therefore, the following experiments were generally carried out on materials of batch fermentations.

2. The next experiment was promoted by the following observation: when *Mrs.* SZABÓ (Phylaxia State Serum Institute, Budapest) tried to settle the bacteria from 200 litres of our fermentation brew by means of an O.F. 31 separator at 5000 r.p.m., the success was only partial even at slow flowing velocities. Although the precipitate was abundant, the supernatant still displayed a strong turbidity. On microscopical examination of the two specimens much *sr* were found in the supernatant and all the *c—dc* were in the sediment together with few *sr*.

Based on this observation, sedimentation of the individual bacterium species in fermentation liquids was examined in different laboratory centri-

fuges at different r.p.m. and different duration of spinning. In the upper part of the centrifuged material, in the turbid supernatant, a great number of *sr* with different lengths and usually a few *thr* were found (Fig. 2a). In some cases some Gram-negative, short, thin, sometimes bent rods (similar to vibrio) were also found. After this layer had been poured out of the centrifuge tube, a much more dense, so-called middle layer followed (Fig. 2b). This contained many *sr*, fewer *thr*, and sometimes a small number of Gram-positive, bulky streptococci. When this middle layer was filled up with water, 2.5% sodium chloride dissolved and centrifuged, the *thr* were agglomerated on the bottom together with a small number of *sr* (see Fig. 2).

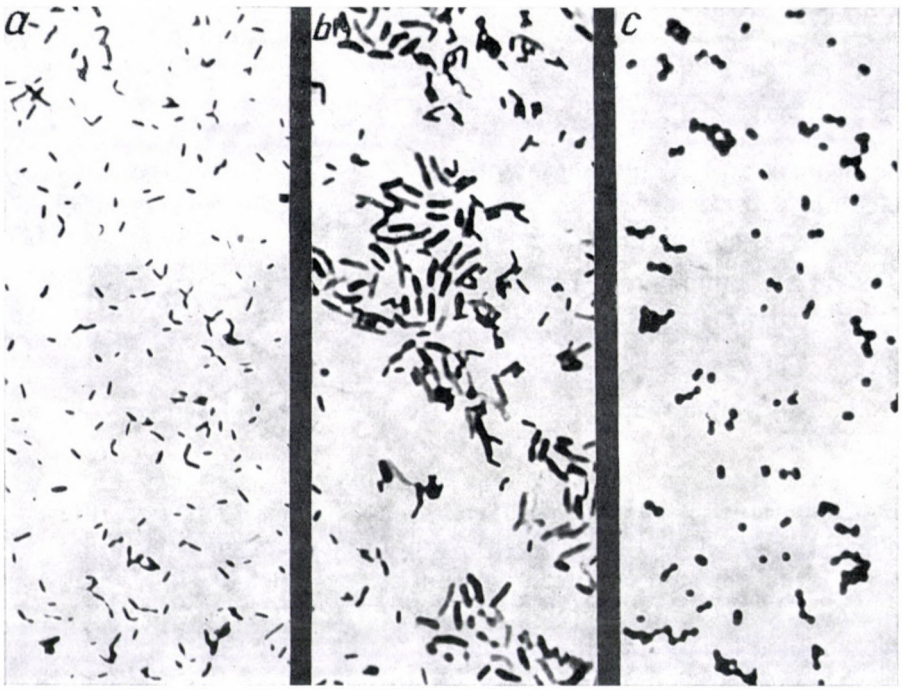

Fig. 2. Bacterium population separated by centrifugation into three layers (×1250). (a) Small rods in supernatant; (b) thicker rods, partly Gram-negative, in middle layer; (c) Gram-positive cocci-diplococci in sediment

After the middle layer had been poured out a thick sediment (Fig. 2c) remained in the tube; this could be removed only by the use of a spoon. In this layer, a large amount of *c—dc* and very few *sr* were found. After the roughly separated bacteria of the different layers had been suspended in saline, the individual species could further be isolated by repeated centrifugation.

Thus we have succeeded in separating the mixed bacterium population into predominant types by centrifugation or in a separator. This allowed to study the main components of population separately.

3. It was attempted to determine the numerical correlations of the predominant bacterium species in the mixed population and to establish their role in vitamin production.

As only part of these species could be cultured, this procedure could not be used for the above purpose. Simple counting yielded no practical data because the dimensions of the *sr* were greatly varying. The 2 μ long rods of *sr*, its coccoid forms 0.3—0.4 μ in diameter, or the long chains of these rods or coccoids could not be considered equivalent from the point of view of vitamin production.

On the other hand useful values were obtained by determining the bacterial masses. For this purpose, bulb-form centrifuge tubes of a capacity of 10 ml with a wide mouth were used which continued below in a closed narrow tube several cm long. The tubes were calibrated and the liquid was centrifuged in these tubes at 10 000 *g* for three to four minutes.

When a completely clear supernatant was obtained, the amount of settled bacteria was 6 to 10% of the centrifuged volume, depending upon the quality of medium and the age of the culture. In this zone, layers varying in colour and hue could be recognized from which samples were taken by means of a fine capillary. After staining it was found that the lower ochre-yellow layer, amounting to 8—10% of the total bacterial sediment, consisted nearly exclusively of *c—dc*. Above this layer there was a thin black streak containing lumps of iron sulfide and higher up, a pale grey layer contained a large amount (90—92%) of *sr*. In culture containing many *thr*, the lower part of the grey layer was greyish-pink.

4. Based on the above findings the problem was studied which of the above bacterium species was producing and in what proportion, the cyanocobalamine and its analogues found in the fermentation brew.

For this purpose, cyanocobalamine, Factor B and the Σ factor were determined in 500 ml of a well-producing, ferment tion fluid ("original") by paper chromatography. The bacterium populationaof further 500 ml of the same fermentation liquid was separated into two layers. After centrifuging at 1000 *g* for 25 minutes there were a large amount of *c—dc* and very few *sr* in the sediment. The upper and the middle layer consisted mainly of *sr*, a few *thr*, and a very few bulky streptococci. The vitamins of this material were determined separately in the single layers by paper chromatography. The results are shown in Table I.

The value for total B_{12} found in the centrifuge-separated bacteria was practically identical with the values for the original (non-centrifuged) material. Thus, the procedure allowed to establish the qualitative and quantitative vitamin production by the bacterium species in the single layers.

Further information was obtained from a study of the material of the centrifuged layers by electrophoretic separation (Table II).

Electrophoresis yielded practically the same total vitamin value as paper-chromatography did. Thus, the *c—dc* in the sediment produced more B_{12} than did the *sr* of the middle layer and the supernatant. Factor III was produced only by *c—dc*.

This experiment was repeated and practically the same results were obtained.

Table I

Cyanocobalamine analogue content in originai and in centrifuged fermentation material, as estimated by chromatography

Material	μg/l of			
	Cyano-cobalamine	Factor Σ	Factor B	Total B_{12}
Original	5630	4930	845	11,405
Centrifuged:				
sediment	3760	3670	—	7,430
middle + upper layers	2040	1610	465	4,115
				11,545

Table II

Cyanocobalamine analogue content in the layers of the centrifuged material as estimated by electrophoresis

Material	μg/l of				
	Cyanocobalamine	Factor III	Factor B	Other factors	Total B_{12}
Centrifuged:					
sediment	3410	2555	205	1010	7,180
middle + upper layers	2040	0	465	1440	3,945
					11,125

5. By changing the composition of the culture medium it was attempted to alter the relative amounts of the three bacterium species predominant in the population, to reach the preponderance of one or of the other, or to decrease their number to the minimum. In the fermentation liquid so obtained, the amount of B_{12} or its analogues was determined by electrophoresis.

The characteristic bacterial population of the fermentation obtained in the culture medium containing sugar, used in our plant fermentations [4] is shown in Fig. 1. Many *sr*, a fair number of *c—dc* and a few *thr* were found here. Electrophoretic separation of the total B_{12} yielded the substances shown in Table III/a. After molasses and sulphite liquor (*i.e.* sugars) had been omitted from the culture medium and urea and ammonium sulphate were added, the *c—dc* and *sr* disappeared or were found in a minimum amount in the fermentation after seven to eight generations, while polymorphous *thr* were prepon-

derant (Fig. 3a). The substances obtained on electrophoresis are shown in Table III/b. Beside much cyanocobalamine, Factor III was present in low amounts or absent.

On increasing the number of *c—dc* beside *thr* and on decreasing simultaneously the number of *sr* (Fig. 3b) the values seen in Table III/c were obtained. Thus, in this case the value of cyanocobalamine was very high, much Factor III was present and Factor B was absent.

Table III

Corrinoids found by electrophoresis when changing the composition of the culture medium

Corrinoids found	μg/l, when the culture medium was		
	type a	type b	type c
Cyanocobalamine	4042	8460	15,440
Factor III	1104	552	4,600
Factor B	0	0	0
Other factors	2944	2200	2,780

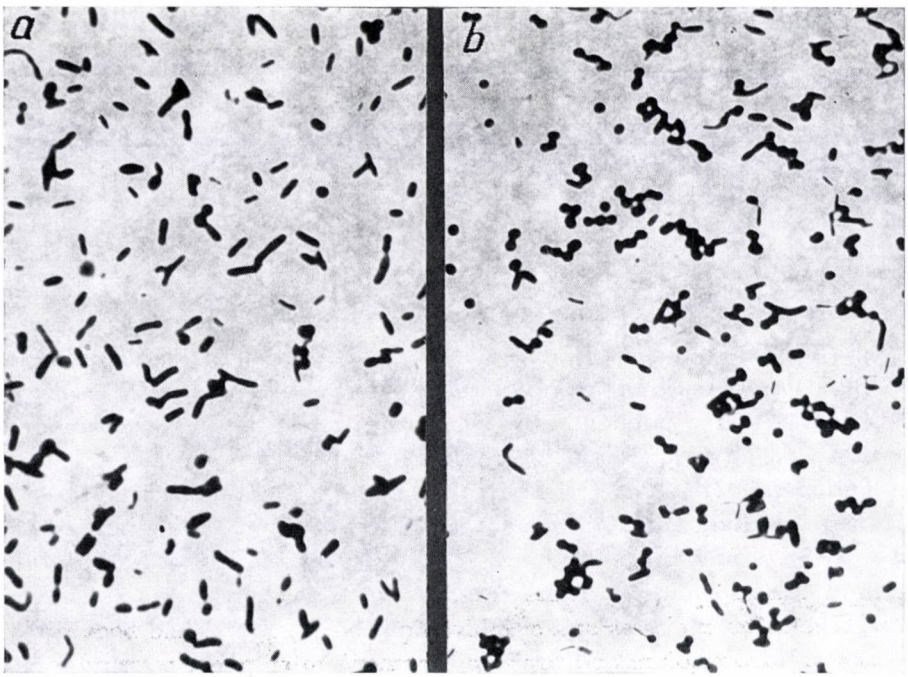

Fig. 3. (a) Prevalent thicker rods in a culture medium; (b) cocci-diplococci and thicker rods in another culture medium (×1250)

Discussion

It has been shown that vitamin B_{12} and its analogues (*i.e.* their coenzymes) are within the cells of the bacterium population originating from sewage sludge. Only a little part of it passed into the liquid probably with the ageing and breaking up of the cells.

A microscopic study of such mixed bacterium populations particularly in the presence of bacteria tending to polymorphism is difficult. As verified by later investigations, three types of Gram-positive bacteria were predominant in the fermentations: a coccus-diplococcus; a thinner, smaller rod; and a thicker, larger rod.

In culture media containing sugars, the overwhelming Gram-positive small rods and the cocci-diplococci were so preponderating that they must be considered the producers of B_{12} vitamin and analogues. Gram-negative bacteria were usually in a negligible minority; thus, they could not play a role in the production of the large amount of vitamin.

The dominating bacteria could be separated by cautious, eventually repeated differential centrifugation. In this way the morphological features of the bacterium population were easy to interpret. By differential centrifugation in a calibrated tube with the same *g* value and spinning time it was shown that the total volume of bacteria in the different batches of the fermentation medium was 6—10%. The sedimented masses of the individual bacterium species were in different layers and differed in colour. In media containing sugar, cocci-diplococci amounted to 8—10% of the total bacterium volume and the remaining 90 to 92% consisted of small rods; a few thick rods were only present. In culture media containing urea but no sugar, after several generations nearly the whole bacterial mass consisted of thick rods and cocci-diplococci. By means of differential centrifugation of a larger amount of fermentation brew, and analysis by paper chromatography or electrophoresis, the bacterial mass in the different layers, the quantity of vitamin B_{12} and its analogues produced by the individual bacterium species could be determined. Cyanocobalamine was produced by all the three predominant species. Factor III was produced only by cocci-diplococci, in a smaller amount by thick rods and none by the small rods. Much Factor III and simultaneously much cyanocobalamine were produced by cocci-diplococci and by thick rods. Under certain circumstances, however, a high yield of cyanocobalamine and a small amount of Factor III were produced when beside an excess of cocci, a fair number of small rods, and an insignificant number of thick rods were present.

Based on the results of these studies, it is possible to enhance the multiplication of the desirable bacterium species and to restrict the propagation of another species by adding suitable ingredients to the culture medium. In

this way the yield of cyanocobalamine and possibly of Factor III can significantly be increased.

The product of these fermentations is not simply the sum of the individual production of the single bacterium species present, because the participating bacterium species are influencing each other's metabolism.

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INTERFERON PRODUCING CAPACITY OF WHITE BLOOD CELLS FROM PATIENTS WITH POLYGLOBULIA

By

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Summary. Interferon production *in vitro* by leucocytes from patients with polyglobulia of various origins and by those from healthy subjects was studied. As inducer, parainfluenza-1 (Sendai) virus was used. Interferon was determined in cultures of a continuous line of human amnion cells with vesicular stomatitis virus as indicator.

Leucocytes were examined from 33 patients suffering from polycythaemia vera. The geometric mean for the interferon titres was 1 : 31, approximately 6 times lower than the geometric mean for the control leucocytes.

The leucocytes from patients with polyglobulia other than polycythaemia vera (8 cases) were as good interferon producers as the control cells.

The possible pathogenetical importance of the altered interferon-producing capacity of leucocytes in various haematological diseases is discussed.

In a previous work [1] the interferon-producing capacity of the white blood cells was found to be decreased in some patients with polycythaemia vera (PV). Since then we have investigated further samples from the same patients and included new patients in the study. Some of these suffered from PV, others from polyglobulia other than PV [2].

Materials and methods

Donors. Patients with PV were divided into two groups: those showing exacerbation are signed by E, those showing remission by R. The patients suffering from secondary polyglobulia or primary benign erythrocytosis were all in the exacerbation stage. Blood samples taken from healthy subjects served for control. In each experiment, samples from sick and healthy subjects were taken at the same time and were processed simultaneously in the same manner.

Interferon was produced according to the method of STRANDER and CANTELL [3]. As inducer the Sendai strain of parainfluenza-1 virus was used. Interferon was determined in human amnion cell cultures, using vesicular stomatitis virus as indicator [1, 4].

Results

The results of 47 experiments carried out with leucocytes from 33 patients with PV and the appropriate control results are shown in Table I.

In 32 of the 47 cases there was a fourfold or greater difference in interferon production in favour of the control leucocytes. The geometric mean of the interferon titres was 1 : 31 for the PV patients and 1 : 182 for the controls, the quotient being approximately 6.

Of the 32 cases with reduced interferon production 27 were in the exacerbation stage and 5 in remission. For the 35 samples obtained from patients in exacerbation the geometric mean was 1 : 23 as compared to the 1 : 174 for the corresponding controls. It may be concluded that the reduced interferon production of leucocytes from PV patients is characteristic, first of all, of those being in the stage of exacerbation.

Table I

Interferon-producing capacity of leucocytes from patients with polycythaemia vera and of normal leucocytes

Experiment No.	Initials of patients	Phase of illness*	Interferon titre (reciprocals)	
			patient	control
1	O. J.	E	<4	64
2	B. A.	E	16	32
3	B. A.	E	16	256
	B. B.	E	16	
	B. V.	E	64	
4	Sz. G.	E	256	128
	C. M.	E	64	
	M. M.	R	128	
5	O. J.	E	32	512
	Ü. L.	R	64	
	Ó. J.	R	64	
6	L. J.	R	32	64
7	P. I.	R	256	128
	V. Gy.	R	128	
8	S. S.	E	256	1024
9	S. J.	E	32	128
10	V. J.	E	64	128
	O. M.	E	32	
11	V. D.	E	32	128
12	H. G.	E	<16	128
13	K. J.	E	64	128
14	K. I.	R	64	256
15	M. F.	R	32	512
	P. L.	E	32	
16	Sz. K.	E	8	128
		R	64	96
		E	16	128
		E	<16	256
		E	16	128
17	Zs. I.	E	< 4	32
		E	< 4	128
18	M. L.	R	64	512
		E	128	512
19	V. I.	E	64	64
		E	512	256
20	A. I.	R	256	256
		E	64	96
21	G. F.	E	128	512
		R	96	96
22	M. G.	E	<16	512
		E	32	256
		E	<16	128
23	K. J.	E	32	128
		E	32	128
24	K. P.	E	16	256
		E	16	128
		E	128	512

* E = exacerbation
R = remission

Leucocytes from 8 patients with polyglobulia other than PV were tested in 10 experiments (Table II). Here there was no difference in interferon production *in vitro* between leucocytes from patients and those from control subjects. The respective geometric means were 1 : 88 and 1 : 99.

Table II

Interferon-producing capacity of leucocytes from patients with polyglobulia other than polycythaemia vera, and of normal leucocytes

Initials of patient	Character of polyglobulia	Interferon titre (reciprocals)	
		patient	control
B. J.	Endocrine (Cushing)	16	32
Cs. Z.	Endocrine (hyperthyreosis)	64	96
A. S.	Hypoxic	32	32
H. F.	Hypoxic	256	128
		128	256
Sz. J.	Hypoxic	64	96
		64	64
J. A.	Hypoxic	512	512
Sz. M.	Primary benign erythrocytosis	96	96
K. J.	Primary benign erythrocytosis	128	96

Discussion

The cause of the reduced interferon production by leucocytes from patients with PV is unknown. It may be mentioned that PV is usually characterized by panmyelopathy; the bone marrow is hyperplasic and hypercellular, showing enhanced erythro-, myelo- and thrombopoiesis (megakaryocytosis) with the predominance of erythropoiesis [5—8]. DAMESHEK [9] classified the disease into the group of myeloproliferative diseases. Thus, during the illness both erythropoiesis and leucopoiesis as well as the peripheral counts are altered; in addition to erythrocytosis, leucocytosis and a slight shift to the left [10, 11] and a functional weakness of the lymphatic tissues can be observed [11]. The pathological leucopoiesis might be related to the reduced interferon-producing capacity observed by us. This is supported by the present observation that in patients with secondary polyglobulia, in whose bone marrow only the erythropoiesis was altered and there was no peripheral lymphopenia [11], the interferon-producing capacity of the leucocytes was normal.

Like in the case of PV, the interferon-producing capacity of leucocytes in chronic lymphoid leukaemia is reduced [4, 12, 13, 14], whereas in chronic

myeloid leukaemia it is sometimes increased [4, 13]. The importance of these observations is unknown. We suppose that, if any virus plays a role in the aetiology of the myeloproliferative diseases or any kind of leucosis, the abnormal interferon-producing capacity might influence the pathomechanism of the disease.

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SPECIFIC ORAL PREVENTION OF INFANTILE GASTROENTERITIS

II. EXPERIMENTS IN INFANTS

By

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Summary. (i) *Escherichia coli* Boivin antigens producing effective immunity in mice were tested by oral administration for reactivity and immunogenicity in 62 mostly dystrophic infants 1 to 8 months of age. The infants received O111 : K58(B4), O55 : K59(B5) and O86 : K61(B7) extracts in tablets containing 0.5 mg amounts of antigen each.

(ii) Antigen doses increased gradually up to 12 mg (8 tablets) per kg body weight exerted no harmful effect. Higher doses were not given.

(iii) Antigenicity of the vaccine was studied by passive immunization of 10-day-old chick embryos with the infants' serum, and by passive immunization of mice with copro-antibodies.

(iv) Suboptimum doses ($2-3\times3$ tablets) increased the level of serum antibodies about five times. The effect was significantly increased by weekly revaccination with one tablet. The immune response was polyvalent. Larger doses ($2-3\times8$ or 8×3 tablets) produced significantly higher (18- to 55-fold) titres as compared to those in the above group of infants, but weekly revaccination failed to raise the antibody level significantly. Determination of the optimum interval between vaccinations requires further studies.

(v) The oral antigen induced copro-antibody production characterized by parameters similar to those of serum antibodies.

(vi) As shown by the development of serum and copro-antibody titres, revaccination after priming is essential to maintain an effective immunity.

(vii) The role of serum and copro-antibodies in immunity is discussed. The favourable immune response, especially the development of copro-antibodies, suggests the need for a large scale epidemiological survey of the vaccine's prophylactic effect.

In a previous paper [1] we reported on mouse experiments indicating that repeated oral doses of Boivin antigen protect the animals against challenge with the corresponding organism injected intraperitoneally with mucin. There was a correlation between the degree of protectivity and immunizing dose. The mode of immunization also influenced the results in that immunity was better after daily vaccination than if the vaccine was given at intervals. Immunity ceased one month after discontinuation of the vaccine, but it could be maintained and even increased by small antigen doses given at 5 day intervals. Development of protective copro-antibodies was promoted by small antigen doses.

These favourable results have stimulated us to perform laboratory investigations with the further aim of elaborating the oral vaccination of

infants. Concerning this problem, few data are available. MOCHMANN *et al.* [2] and OCKLITZ *et al.* [3] immunized 300 new-born and 27 premature infants orally with sodium deoxycholate-extracted O111 : K58(B4) antigen. The haemagglutinin titre seldom rose above 1 : 20. KÖDITZ *et al.* [4] fed 30 infants with streptomycin-dependent living organisms which had been shown to induce immunity in mice; they did not report on the development of an immune reaction. As our antigen differed considerably from the vaccines used by the above authors, the experiments were to be performed on an entirely new basis.

In this paper we present observations on 62 infants aged 1 to 2 months as to reactivity and immune response to Boivin antigens found effective in mouse experiments.

Materials and methods

Strains. Biochemically and antigenically typical *E. coli* O111 : K58(B4) 1872/68, O55 : K59(B5) 1926/68 and O86 : K61(B7) Tb/68 strains were used. These organisms were isolated in our routine laboratory from severe cases of infantile diarrhoea.

Cultivation, preparation of antigens and determination of immunogenicity were described in a previous paper [1]. Differences in the methods due to the nature of the present experiments were as follows.

Preparation of tablets for oral immunization of infants. In order to simplify vaccination, the antigens were compressed into tablet form. Antigens prepared from strains O111 : K58(B4), O55 : K59(B5) and O86 : K61(B7) representing the most frequent *E. coli* serogroups in Hungary, were used in amounts to give haemagglutination inhibition identical with the standard. One tablet contained 0.5 mg of each antigen, thus the total amount of antigen was 1.5 mg per tablet. The tablets disintegrated in water easily.

For checking the antigen content, 2 tablets were suspended in 1 ml saline (1 mg per ml for each antigen). After centrifugation the antigen concentration in the supernatant was determined parallel with the original antigen preparation by the use of haemagglutination inhibition. The antigen was recovered in 90—95%. The loss was probably due to an adsorption of the antigen to the deposit. Sterility testing on blood agar showed that one tablet contained at most one or two micrococci.

Immunization and collection of specimens. Several different immunization schedules were applied as indicated in the text and the Tables. Revaccination* was always performed with one tablet (0.5 mg of each antigen). The tablets were dissolved in tea and given before the morning meal. Their administration presented no difficulty. Immunization was performed at 5 day intervals, except in one experiment, where vaccination was carried out daily. Revaccination was carried out always at 7 day intervals.

For the examination of antigenicity blood samples and, for the determination of copro-antibodies, faecal specimens were obtained before and 5 days after immunization. The duration of immunity was estimated by examining specimens collected several weeks after the last dose.

Passive mouse protection test. Serum dilutions were injected intraperitoneally to mice. Challenge was immediately performed, likewise intraperitoneally with 5 to 50 LD_{50} of bacteria cultured on agar slant and standardized by the use of the NIH-Human opacity standard. Determination of the LD_{50} was carried out in control animals infected in the same manner.

Determination of copro-antibodies. The faecal specimens were stored in the deep freezer until use. An estimated amount (10—15 g) of the specimen was suspended in 15 ml cool distilled water and centrifuged at 10 000 *g*. Extracts originating from infants belonging to the same test group were united, passed through Seitz filter and freeze-dried. The dry material was rehydrated in 6 ml saline and used for passive immunization and haemagglutination.

* Revaccination means that small antigen doses were given at intervals for maintaining the immunity induced by priming.

Results

Reactivity of the vaccine. Few data have been available as to oral immunization of infants with coli vaccine. MOCHMANN *et al.* [2] and OCKLITZ *et al.* [3] applied a freeze-dried filtrate of the deoxycholate extract of 10^{10} cells in 5 subsequent doses; the vaccine showed no reactivity. KÖDITZ *et al.* [4] used $10^9 - 10^{11}$ living cells of a streptomycin-dependent strain and observed mild diarrhoea among the infants vaccinated with 10^{11} organisms. Neither of these vaccines can be compared with our antigen preparation. We have had experience in children with an oral dysentery vaccine of similar toxicity [5], but not in infants and, therefore, we studied first the effect of low graded doses. A cautious administration was essential, as the patients consisted of undernourished dystrophic infants without a previous history of enteric infection. Temperature, weight and the presence or absence of diarrhoea were considered. A total of 20 infants aged 1 to 8 months (mainly under 6 months) were studied for the antigen's reactivity. The results are shown in Table I.

In different weight groups of infants the antigen dose was cautiously increased. Doses ranging from 0.5 to 2.4 mg per kg (1 to 6 tablets) were applied.

After having been convinced of the innocuity of even the largest dose studies of the vaccine's immunogenicity were commenced. The results suggested that the highest doses could safely be administered. This finding was confirmed later when 8 tablets (12 mg) were given in one dose.

Immunogenic effect. Priming was performed in 7 dystrophic infants 1 to 9 months of age; 4 of them were 1 to 4 months of age and weighed 3500 g

Table I

Reactivity of the vaccine

Age, months	Weight, g	Average weight, g	No. of infants vaccinated	Antigen dose			Reactivity
				No. of tablets	mg	mg/kg	
1.2—4	2700—3400	3200	1	1	1.5	0.5	Nil
			3	2	3	0.9	Nil
1.5—6	3500—4400	3800	1	1	1.5	0.4	Nil
			5	2	3	0.8	Nil
			2	6	9	2.4	Nil
2—8	4500—5400	5300	2	2	3	0.6	Nil
			2	4	6	1.14	Nil
5—6	5500—5800	5600	1	2	3	0.5	Nil
			2	4	6	1.0	Nil
			1	6	9	1.6	Nil

on the average. This group received 2×3 tablets; the interval between the two doses was 5 days. The remaining 4- to 9-month-old infants weighed 4800 g on the average and were fed with 2×5 tablets (Table II).

Table II

Protective value of sera of infants immunized orally

Chick embryo test; challenging strain, O111 : K58(B4); infective dose, 75 LD_{50}

Infants	Age, months	Weight, g	No. of tablets	Protective value of sera (ml), ED_{50}		Rise in titre as compared to pre-immunization value (RP)
				before immunization	after immunization	
1	1	3600	2×3	0.05	0.009	5.5
2	3	3300		0.05	0.005	10.0
3	4	3700		0.05	0.0028	17.8
4	4.5	3600		0.05	0.016	3.1
5	4	4600	2×5	0.05	0.009	5.5
6	9	5400		0.05	0.0028	17.8
7	9	4400		0.05	0.005	10.0

Table II presents the result of passive chick embryo protection test against O111 : K58(B4). Passive mouse protection test carried out with the usual technique [6] failed to give reliable results. It is seen that two doses of either 3 or 5 tablets produced a seroconversion, the protective antibody level against the challenging organism increased 3 to 17 times. In immunogenic effect there was no difference between the two kinds of dosage.

Table III presents protective values against three different *E. coli* strains in the sera of the same infants pooled at equal proportions. Irrespective of the dose, the rise in protective titre was 10- to 31-fold against all challenging organisms. It is thus evident that the tablets exerted a polyvalent immunizing effect.

Subsequently, the protective value of the serum pool of 11 immunized infants aged 1 to 9 months was examined against strain O111 : K58(B4). Six infants received 2×3 tablets, the remaining 5 infants 2×5 tablets. In accordance with previous findings, the protective titre showed an 11-fold increase. The immune response of an adult given 5×5 tablets was similar in degree to that observed in the infants.

Effect of revaccination. Mouse experiments [1] have indicated that the effect of priming is not only maintained but even increased on the subsequent administration of small antigen doses. (In view of the booster effect of small doses, the term revaccination seems more correct than maintenance of immunity.) The experiments were performed in two groups of 5 infants each. One

Table III

Protective value of serum pools of infants

Chick embryo test; challenging strains, O111 : K58(B4), O55 : K59(B5) and O86 : K61(B7), infective dose, O111 = 5, O55 = 7.5, O86 = 75 LD_{50}

No. of tablets	Blood samples collected	Immunity against strains					
		O111 : K58(B4)		O55 : K59(B5)		O86 : K61(B7)	
		ED_{50}/ml	RP	ED_{50}/ml	RP	ED_{50}/ml	RP
2×3	Before immunization	0.05	1	0.05	1	0.05	1
	5 days after immunization	0.0016	31	0.005	10	0.0016	31
2×5	Before immunization	0.05	1	0.05	1	0.05	1
	5 days after immunization	0.005	10	0.005	10	0.0028	17

group received 2×3 tablets, the other 3×3 tablets at 5 day intervals. Each member of the first group was revaccinated after priming with 1 tablet at weekly intervals. The other group received no revaccination. The time of observation lasted 70 days in the revaccinated and 60 days in the control group. The results of passive chick embryo protection test against O111 : K58(B4) are summarized in Table IV.

Table IV

Protective value of serum pools of infants after revaccination

Chick embryo test; challenging strains, O111 : K58(B4), O55 : K59(B5) and O86 : K61(B7); infective dose 50 LD_{50}

Group	No. of tablets	Blood samples collected	Relative value of immunity (RP) against strain		
			O111 : K58(B4)	O55 : K59(B5)	O86 : K51(B7)
Revaccinated	2×3	Before priming	1	1	1
		5 days after priming	5	6	6
		5 days after the 9th revaccination* (70 days after priming)	20	100	60
Not revaccinated	3×3	Before priming	1	1	1
		5 days after priming	10	6	10
		60 days after priming	1	1	1

* Revaccination: 1 tablet weekly.

After priming the rise in protective antibodies was similar in both groups. After 9 revaccinations (70 days after priming) the protective antibody levels highly increased in the first group: the difference as compared to values after priming was significant (about 10-fold for all test strains, $P < 0.01$). After a

similar period (60 days) antibodies in the not revaccinated group decreased to the value observed before priming. This finding, in view of humoral factors, indicates the ceasing of immunity.

Correlation between dose and immunity and duration of immunity. The question arose whether the obvious effectiveness of revaccination is or is not attributable to an insufficient priming. The answer is presented in Table V, which shows data for 3 groups of 5 infants each. The first group received 2×3 tablets at 5 day intervals. The second group was given 3×8 tablets at 5 day intervals (these infants were examined for protective antibodies after the second dose). The third group was immunized on 8 consecutive days with daily doses of 3 tablets, thus the second and third groups received the same total dose of 12 mg. The sera were examined with the chick embryo test against O111 : K58(B4).

Infants receiving 2 doses of 3 tablets showed a 5-fold rise in protective antibodies, similarly to those immunized earlier with the same dose (Table IV). After 8 revaccinations this level increased 10-fold. The difference between the two results is significant ($0.02 > P > 0.01$).

Infants given 8 tablets twice at a 5 day interval showed an increased antibody response. Under the effect of 2×8 tablets the protective titre rose 18-fold. The antibody level developing after the third dose was 3 times higher than that observed after the first two doses (1 : 55). Both values differed significantly from the effect of 2×3 tablets ($P \sim 0.05$), but the difference in immune response between the second and third doses of 8 tablets was not significant (1 : 18 *vs.* 1 : 55; $0.5 < P > 0.3$). Neither was significant ($0.20 < P > 0.10$) the 2-fold rise (1 : 100) after 5 revaccinations. The effect of daily doses of 3 tablets given on consecutive days was less favourable than that of immunization with the same dose at 5 day intervals (1 : 31 *vs.* 1 : 55). Two revaccinations did not increase the values significantly ($0.5 > P > 0.3$). Similarly, there was no significant difference between this result and that found in the former group (3×8 tablets) revaccinated 5 times ($0.80 > P > 0.70$).

In a subsequent experiment, in the group immunized with 3×8 tablets and revaccinated 5 times the protective titre was examined 30 and 51 days after the last revaccination dose. The decrease had became significant in 30 days and after 51 days the titre decreased nearly to the values obtained before immunization.

In a further group it was shown that the protective titre developing under the effect of 2×8 tablets given at 5 day intervals decreased considerably after 24 days.

Accordingly, priming with a total dose of 16 to 24 tablets fed at 5 day intervals or distributed in daily doses resulted in an immunity which was not further increased by revaccinations; without revaccination, however, the protective titre decreased rapidly.

Table V

Immunogenic effect of different immunization schedules

Chick embryo test; challenging strain, O111 : K58(B4); infective dose 75 LD_{50}

Groups immunized	No. of tablets	Blood samples collected	Protective value of sera	
			ED_{50}/ml	Rise in titre (RP)
5 day intervals, on 2 occasions	2×3	Before priming	0.05	1
		5 days after priming	0.01	5
		5 days after the 8th revaccination (70 days after priming)	0.009	55
5 day intervals, on 2 and 3 occasions	2—3×8	Before priming	0.05	1
		5 days after the 2nd priming dose	0.0028	18
		5 days after the 3rd priming dose	0.0009	55
		5 days after the 5th revaccination (40 days after priming)	0.0005	100
On 8 consecutive days	8×3	Before priming	0.05	1
		5 days after the 8th priming dose	0.0016	31
		5 days after the 2nd revaccination (14 days after priming)	0.0009	55

Passive haemagglutination. Haemagglutinins incongruent with the protective antibody levels were detected in low titres. Thus, in our opinion, haemagglutinins are unreliable and poor indicators of immunity.

Copro-antibodies. Our mouse experiments demonstrated that oral vaccination with coli antigens gave rise to copro-antibodies [1]. It was, accordingly, advisable to examine the infants for the presence of these factors. The chick embryo test could not be used, as even 0.01 or 0.001 ml amounts of faecal extracts killed the embryos. The modified passive mouse protection test as described under "Materials and methods" was suitable to study the problem.

Table VI compares mouse protective serum titres with copro-antibodies in groups of infants shown in Table IV. Blood and faecal specimens were taken at the same time. It is clear that the protective value of serum antibodies was considerably lower in degree with the mouse test as compared to the chick embryo test. Nevertheless, the rise in protective titre was clearly demonstrated also in mice especially after revaccinations.

The appearance after priming of protective antibodies in faecal extracts is evident. The titre rose further after 9 revaccinations and approached the level of the protective serum antibodies demonstrated with chick embryo test (Table IV). As the aim of these experiments was to obtain qualitative data, the amount of faecal extracts was not standardized. Yet, it may be suggested that the rise in titre was due to an intensive production of copro-antibodies which may be considered as the humoral principle of protection against infan-

Table VI

Protective value of serum and copro-antibodies

Passive mouse protection test; challenging strain, O111 : K58(B4); infective dose, 25 LD_{50}

Samples	No. of tablets	Samples collected	Protective value	
			ED_{50}/ml	RP
Serum pool	2 × 3	Before priming	0.1	1
		5 days after priming	0.05	2
		5 days after the 9th revaccination (70 days after priming)	0.017	5
Faecal extract pool	2 × 3	Before priming	0.1	1
		5 days after priming	0.031	3
		5 days after the 9th revaccination (60 days after priming)	0.005	20

tile *E. coli* infections. Copro-antibodies, accordingly, are formed in infants corresponding to the rules of the development of artificial immunity.

Two observations should be considered for a correct evaluation of copro-antibodies as the principle of immunity. On the one hand, the high protective levels in infants revaccinated 9 times decreased in 50 days after the last dose to values found before vaccination; on the other, copro-antibody titres in the group treated with 2 × 8 tablets at 5 day intervals decreased to pre-immunization values 24 days after the second dose of vaccine. Accordingly, revaccination is essential for the maintenance of high level copro-antibodies.

Discussion

The fact that the incidence of infantile *E. coli* gastroenteritis has remained at the same level year by year is attributable mainly to the multiple resistance of the causative agent [7, 8]. In recent years more than half of about 2000 infants involved have become infected in communities; from 1962 onward the number of fatal cases varied between 16 and 40 [9]. In view of the primary role of infant communities in the spread of infection, vaccination seems to be the very means to solve the problem.

There are no sufficient data in the literature concerning the immunity following *E. coli* enteritis. Haemagglutination may be regarded as the simplest method: in accordance with the findings of MERÉTEY and BACKHAUSZ [11] and of several other authors, we could regularly demonstrate haemagglutinins in the serum of infants and adults [10]. However, KÖDLITZ *et al.* [12] reported variable haemagglutinin titres in infants recovered from *E. coli* enteritis and RALOVICH *et al.* [13] described similar findings. As already mentioned, there was no

correlation between haemagglutinin and protective antibody titres. Evaluation of the haemagglutination test in this respect is difficult since, in addition to the general opinion that there is no strict correlation between circulating antibodies and immunity [12, 14], the fact that older age groups show a decreasing susceptibility of non-immune character to *E. coli* infection [15] should be considered. This particular epidemiological-clinical relation presents special conditions for active immunization; the infants should be vaccinated as early as possible and a high degree of immunity should be reached. On the other hand, the immunity need not last longer than 6 or 12 months.

Our studies were performed after favourable experiments in mice immunized with Boivin antigen [1]. Studies on vaccination against dysentery revealed that, according to the mouse test, Boivin extracts were 3 to 4 times more effective than the corpuscular vaccine [6]. Tablets of *Shigella* Boivin antigens induced marked seroconversion in adults and children and immunity could be maintained as long as desired by oral administration of small doses [16].

Several data are available for the areactivity of Boivin antigens given orally. *Salmonella* and *Shigella* antigens are ingested without symptoms by mice at 50, by adults at 100 mg amounts [16, 17]. In immunization against dysentery no reaction was observed after feeding 8 mg Boivin antigen [5]. There are no data regarding the toxicity of *E. coli* Boivin antigens in infants. As to other antigens, Ocklitz *et al.* [2, 3] used deoxycholate-treated dried antigens representing 10^{10} cells for the immunization of new-born infants. Köditz *et al.* [4] showed that the highest limit of tolerability of a living streptomycin-dependent strain was 10^{11} cells. Our antigen essentially differs from these preparations and, accordingly, before starting the experiments we had to check its areactivity.

Our vaccine contained antigens for the three most frequently occurring *E. coli* serogroups: O111 : K58(B4), O55 : K56(B5) and O86 : K61(B7). For facilitating administration, the antigens were compressed in tablets. One tablet contained 0.5 mg of each of the three antigens, thus, similarly to our oral dysentery vaccine [16], the total antigen content was 1.5 mg. The reactivity of the vaccine was studied after checking the antigen content by haemagglutination inhibiting test and after conducting the sterility test. The antigen tablets were given to 20 mainly dystrophic infants 1 to 6 months of age. The dose was gradually increased from 1.5 to 9 mg (0.5—2.4 mg per kg). After obtaining evidence of the complete absence of toxicity, the vaccine was given also in single 12 mg doses without any untoward effect. Recent experiments have indicated that the above doses were not harmful for premature infants, that is, the antigen is sufficiently safe under different conditions for resorption. Areactivity of the vaccine was a constant property, since the LD_{50} in mice weighing 15—18 g showed a narrow range of difference (100 μg on the average) [18].

The immunogenic effect of the vaccine was to be studied by detecting conversion in the protectivity of paired sera and by demonstrating the presence of protective copro-antibodies. First, an attempt was made to use passive mouse protection test which had been proved suitable for similar studies on immunity to typhoid [19] and dysentery [6, 16]. The effect of *E. coli* vaccines could not properly be evaluated by this method. Later, a slight modification of the mouse test (see Materials and methods) was successfully used especially in the determination of copro-antibodies. The intravenous chick embryo method applied already for endotoxin assay [20] was, accordingly, substituted for the mouse test.

The chick embryo test indicated that 2×3 or 3×3 tablets given to 7 dystrophic infants aged 1 to 7 months increased the protective value 10 to 31 times against all strains incorporated in the vaccine. This finding has proved the polyvalent effect of the vaccine. It should be noted that the protective titre of infants' sera was similar in degree to titres of adults immunized with 5×5 tablets. It may thus be concluded that the immune response of infants is not inferior to that of adults. It seems also that — as indicated by the difference between antibody levels detectable by the mouse test and by the sensitive chick embryo test — immunity developing after *E. coli* vaccination is superficial, similarly to the immunity induced against dysentery [21], and differs only quantitatively from the immunity to enteric infections. Accordingly, the slighter degree of immunity is not associated with the age of the immunized individuals. This assumption has been confirmed by ERDŐS [22] who showed with other antigens that the immune response is not less definite in infants than in adults.

As to the duration of immunity and the effect of weekly administered small antigen doses, 60 to 70 days after immunization with $2-3\times3$ tablets the protective value of sera decreased to pre-immunization levels. When prolonged revaccination was carried out during the same period with 1 tablet weekly the protective titre increased significantly. This agrees well with the increase in the serum antibodies of revaccinated mice [1] or of infants revaccinated orally against dysentery [16].

For studying the effect of high doses, the infants were given 8 tablets 2 or 3 times at 5 day intervals or doses of 3 tablets daily for 8 days, then both groups were revaccinated 2 to 5 times. The above amount of antigen (24 tablets in 3 doses with intervals or in 8 consecutive days) gave statistically homogeneous response and revaccinations resulted in no further rise in titre. In contrast, the priming effect of $2\times3-5$ tablets was significantly less definite, although immunity could be increased by revaccination to a level obtained by high priming doses. This finding is in agreement with RAETTIG's observation showing that the dose of antigen and the protective effect are strictly correlated [23]. Although in the absence of a sufficient priming the prolonged ad-

ministration of small antigen doses may also produce a satisfactory antibody level, rapid immunization should be the guiding principle. Maximum immune response was observed on the 10th day after the administration of 2×8 tablets (4 mg for each antigen). Mouse experiments indicate that the distribution of this amount into consecutive daily doses is even more favourable [1]. The question remains to be studied. Weekly revaccination, however, must not be omitted even after optimum priming.

Haemagglutination does not reflect the protective value of sera. RAETTIG [23] failed to demonstrate antibodies in orally immunized mice. MOCHMANN *et al.* [2], in contrast, showed antibodies by FAGREUS's haemadsorption method after oral immunization of mice. Both the above papers agree in that oral immunization stimulates the antibody producing capacity, as in immunized mice high amounts of haemagglutinin were detected after intraperitoneal infection. In our conception the significance of serum agglutinins should not be overestimated and from the laboratory methods only the protection test should be relied upon.

In view of the above discussed findings it is justified to raise the question whether experiments with serum antibodies reflect the state of immunity, whether protective serum antibodies really contribute to rendering the infants insusceptible to *E. coli* enteritis. The importance of the question is obvious in view of the finding that mouse-protective antibodies were difficult to demonstrate and more delicate differences between the effect of various dosages were demonstrated only by the sensitive chick embryo test. The final answer to the question has to be obtained from epidemiological studies. The chick embryo test demonstrated protective antibodies which differ from mouse-protective antibodies only quantitatively. Thus, an acceptable indicator of immunity has been elaborated which allows the determination of the most suitable immunization schedule for epidemiological investigations.

In view of the actual immunological value of vaccination, observations on copro-antibodies are even more relevant. It is known that patients suffering from, or persons immunized against, cholera [24—29] and dysentery [30—33] excrete antibodies with the intestinal mucus and the faeces. These factors can be demonstrated by agglutination and exert a protective effect against the corresponding organism. KARAKAŚEVIĆ [34] showed the presence of copro-agglutinins in the intestinal content of infants with *E. coli* gastroenteritis. We detected protective antibodies in the intestinal extract of mice immunized orally with shigellae [35] and assumed that these factors were produced in the immune apparatus of the intestinal wall and, since part of the circulating antibodies derive from this very site, the serum antibodies were good indicators of the presence of copro-antibodies. As shown previously, the intestinal extract of mice immunized with O111 : K58(B4) antigen also contained copro-antibodies [1].

The presence and rise on vaccination of protective antibodies in faecal extracts of immunized infants may be considered a direct proof of immunity. It is known from KÉTYI's experiments that *E. coli* O101 : K30 in suckling mice causes an enteric infection which is located to the intestinal wall and can be regarded as an analogy of infantile gastroenteritis [36]. Immune serum administered orally exerted a more effective protection than did parenteral immunization [15]. In infantile gastroenteritis, intestinal antibodies obviously serve as a defence against the infective agent entering from the intestine and attacking the intestinal wall. FRETER and GANGAROSA [29] regarded copro-antibodies as protective principles against enteric infections. The studies of BURROWS [24, 25] have confirmed this assumption. These primary factors of immunity, like the serum antibodies, disappear after immunization. They were completely absent as soon as 24 days after feeding the optimum dose. It is, however, important that as an effect of revaccination they remained at the desired level and even increased significantly when small priming doses (2×3 tablets) were given. The need for revaccination is clearly shown by these data.

The favourable laboratory findings should, of course, be followed by large-scale immunization studies in infants' communities, this being the only means for demonstrating the effectiveness of the vaccine and the duration of immunity.

The practical applicability of oral immunization is suggested by the fact that all the infants in the present study were undernourished, dystrophic, without a history of enteritis, whose immune apparatus, owing to their general condition, was far from functioning normally. In our opinion, immunization of healthy, well-developed infants would yield better results than those presented in this paper. Immunization of every infant in the population in order to eradicate the disease may be regarded as the final goal of such studies.

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TYPE DISTRIBUTION OF STREPTOCOCCUS PYOGENES IN THE YEARS 1964—65 AND 1968—69

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Summary. As the international surveys of streptococcal infections in 1964—65 and 1968—69 were identical in aim and conditions, their results are comparable.

(i) The surveys in Hungary showed that in 1964—65, 395 *Streptococcus pyogenes* strains fell into 27 types; in 1968—69, 427 strains belonged to 22 types (cultures with complex antigens were regarded as separate types).

(ii) Eight out of the 27 types isolated in the first survey were not met with in 1968—69. During the second survey 4 new types were identified.

(iii) By agglutination and precipitation approximately 99% of the strains were typable in the two surveys.

(iv) Complexes *5, 11, 12, 27, 44* and *3, 13,* B_{3264} and type 12 occurred frequently in both periods. The incidence of other frequent types showed a remarkable change in that the number of type 3 strains decreased to half and type 28 disappeared. In contrast, the incidence of complexes *4, 24, 26, 28, 29, 46, 48* and *8, 25,* Imp_{19} and of type 19 increased in the second survey.

(v) There was no definite association between different types and various streptococcal infections. In the first survey, type 3, in the second, type 19 were frequent in scarlet fever.

(vi) No significant difference was revealed between types originating from urban and rural areas.

(vii) Tetracycline sensitivity of the strains was similar in both surveys.

(viii) International collaboration on the basis of identical principles offers a ground for elaborating the specific prevention of streptococcal infections.

On the request of the International Subcommittee on Streptococci and Pneumococci we have taken part in two surveys* of the type distribution of *Streptococcus pyogenes* in different countries.

Results in Hungary of the first survey have been described [1]; the collected data from 11 participating countries were reported by Parker [2]. The aim of the examinations was the same in both surveys, being a comparison of type distribution, improvement of typing technique and organization of a close cooperation between streptococcus laboratories. In view of immunization experiments with streptococcal M protein [3] the data were to be used in choosing the components of the vaccine.

As conditions for the investigation were almost identical in the two surveys, the type distribution of streptococci in the two periods has been compared.

* The first survey lasted from June 1, 1964, to June 1, 1965, under the direction of Professor R. E. O. Williams and Dr. M. T. Parker (Cross-Infection Reference Laboratory, London); the second from April 1, 1968, to April 1, 1969, under the direction of Professor W. Köhler (Streptococcus Reference Laboratory, Jena).

Table I

Type distribution of Str. pyogenes strains

Type	1964–65					1968–69				
	No. of strains determined by antigens			Total		No. of strains determined by antigens			Total	
	M	T	M + T	No.	per cent	M	T	M + T	No.	per cent
1	—	9	12	21	5.3	—	8	2	10	2.3
2	—	4	—	4	1.0	—	4	3	7	1.6
3M (3R)	1	—	38	39	9.9	—	—	19	19	4.5
3R	—	—	2	2	0.5	—	—	8	8	1.9
3, 13, B_{3264}	—	67	—	67	16.9	—	45	—	45	10.5
4, 24, 26 . . .	—	15	—	15	3.8	—	45	—	45	10.5
5	7	—	7	14	3.5	—	—	9	9	2.1
5, 11, 12, 27, 44	—	74	—	74	18.7	—	42	—	42	9.8
6	2	2	16	20	5.0	1	2	5	8	1.9
8	—	—	2	2	0.5	—	—	8	8	1.9
8, 25, Imp_{19}	—	17	—	17	4.3	—	59	—	59	13.8
9	—	3	—	3	0.8	—	21	—	21	4.9
11	—	—	27	27	6.8	—	—	18	18	4.2
12	—	—	27	27	6.8	1	—	42	43	10.1
13	—	—	—	—	—	—	—	12	12	2.9
14	2	9	6	17	4.3	—	—	—	—	—

14, 49	—	—	—	—	—	3	—	—	3	0.7
15	3	—	3	6	1.5	—	2	—	2	0.5
15, 17, 19, 23, 30, 47	—	2	—	2	0.5	—	—	—	—	—
17	—	—	1	1	0.3	—	—	—	—	—
18	—	1	—	1	0.3	—	—	3	3	0.7
19	15	—	—	15	3.8	31	—	2	33	7.7
22	—	6	1	7	1.8	—	16	—	16	3.8
24	1	—	—	1	0.3	—	—	—	—	—
25	—	—	—	—	—	—	—	10	10	2.3
26	—	—	—	—	—	—	—	2	2	0.5
28R	—	—	27	27	6.8	—	—	—	—	—
29	5	—	1	6	1.5	—	—	—	—	—
36	2	—	—	2	0.5	—	—	—	—	—
46	—	—	1	1	0.3	—	—	—	—	—
48	—	—	1	1	0.3	—	—	—	—	—
Total typable	38 (9.6%)	209 (52.9%)	145 (36.7%)	392	99.2	35 (8.2%)	245 (57.4%)	143 (33.5%)	423	99.1
Untypable				3	0.8				4	0.9
Total				395	100.0				427	100.0

Materials and methods

Strains. Laboratories collaborating in the survey were so chosen as to represent all areas of Hungary. The number of laboratories taking part was 10 (11 in the first survey), *i.e.* the public health laboratories in the counties Baranya, Békés, Borsod-Abaúj-Zemplén, Hajdú-Bihar, Pest, Szabolcs-Szatmár, Vas and Veszprém and in the cities of Budapest and Szeged. Streptococcal strains sent to our laboratory were checked only by serological grouping instead of the bacitracin + grouping method used in the previous survey. The laboratories were asked to send strains from (a) scarlet fever, (b) tonsillitis, (c) other infections, (d) healthy carriers. In the first survey, strains of healthy carriers were not investigated. The strains were sent on Loeffler or blood agar slants in both surveys.

Serological methods were also identical (agglutination with trypsinized suspension and M precipitation with Lancefield extract). The same set of sera was used. Identification of types 1, 2, 6, 9, 18 and 22 was accepted on the basis of not only M-precipitation but also of T-agglutination. The strain examined was regarded to belong to one of the complexes (*3, 13,* B_{3264}; *5, 11, 12, 27, 44*; *4, 24, 26, 28, 29, 46, 48*; *15, 17, 19, 23, 30, 47* or *8, 25,* Imp_{19}) if it agglutinated in one or more type sera for members of the corresponding complex but failed to react in anti-M sera.

Penicillin and tetracycline sensitivity of the cultures was determined by the disk method in both surveys.

Reporting of the results was performed, as in the previous survey, on special forms. The code number of the regional laboratory, site of isolation, clinical diagnosis and serological results were reported. In both surveys, approximately 30 strains were typed monthly. Checking of the results by sending 5% of the strains to a streptococcus laboratory situated in a foreign country was omitted in the second survey.

Results

Type distribution of streptococci isolated during the two surveys is presented in Table I.

In the first survey, 395 *Str. pyogenes* strains fell into 27 different types. In the course of the second survey, 427 strains were examined and classified into 22 different types. (Complexes were regarded as separate types.) Eight types, viz. *15, 17, 19* . . . , 17, 24, 28R, 29, 36, 46 and 48 encountered in the first survey were not met with in the second, which, in contrast, showed the presence of four new types: 11, 13, 25 and 26.

Typing by T-agglutination and by M-precipitation showed no difference between the two surveys as in 1964—65, 99.2, while in 1968—69, 99.1% of the strains were typable [4]. The incidence of cultures defined only by precipitation decreased from 46.3% to 41.7%, while only by T-agglutination in the first survey 89.5%, in the second 90.9% of the strains could be identified. In the second survey the symbol *14, 49* was used for the designation of strains not precipitating in serum 14M [6, 7], instead of KÖHLER's term *14, 35, 49* [5].

If types occurring at a frequency higher than 5% are arbitrarily regarded as prevalent streptococci, in both surveys complexes *5, 11, 12,* . . . , *3, 13,* B_{3264} and type 12 fell into this category (Table II). As compared to the first survey, in the second the incidence of type 3 decreased considerably and type 28 disappeared, while complexes *4, 24* . . . and *8, 25,* Imp_{19} and type 19 had become prevalent organisms.

Table III presents the association between the source of streptococcal strains and types. In 1964—65, strains from scarlet fever showed the lowest incidence (25.6%); tonsillitis and other infections were represented by an almost identical number of strains (37.9 and 36.5%, respectively). In 1968—69, the difference between the disease categories was less marked (28.6, 21.3 and 27.4%, respectively); the new category, healthy carriers, yielded 22.7% of the strains.

Like in the first survey, no definite association was demonstrated in the present investigations between types and the clinical form of the disease. In scarlet fever, types 3 (24.8%) and 19 (11.9%) were the most frequent in the first survey. In 1968—69, type 19 was the commonest (18.0%) and complex *4, 24, 26* . . . was next in order (12.3%). In the second survey, tonsillitis was frequently caused by complexes *8, 25,* Imp_{19} (20.8%) and *5, 11, 12* . . . (15.4%),

Table II

Distribution of prevalent types

1964—65			1968—69		
Type	Number	per cent	Type	Number	per cent
5, 11, 12, 27, 44	74	19.0	*3, 13,* B_{3264}	45	10.5
3, 13, B_{3264}	67	17.0	*4, 24, 26, . . .*	45	10.5
3	41	10.0	*5, 11, 12, 27, 44*	42	9.8
12	27	7.0	*8, 25,* Imp_{19}	59	13.8
28R	27	7.0	12	43	10.1
			19	33	7.7
Prevalent types, total	236	60.0	Prevalent types, total	267	62.4
Other types	159	40.0	Other types	160	37.6
Total	395	100.0	Total	427	100.0

while in the first period by complexes *3, 13,* B_{3264} (20.0%) and *5, 11, 12* . . . (17.2%). In other streptococcal infections examined in 1964—65, complexes *3, 15,* B_{3264} and *5, 11, 12* . . . were prevalent, but in 1968—69, complexes *8, 25,* Imp_{19} and *3, 13,* B_{3264} were the commonest agents. In healthy persons, complexes *3, 13,* B_{3264}, *5, 11, 12* . . . and *8, 25,* Imp_{19} and type 12 occurred at the same frequency; complex *4, 24, 26* . . . was also prevalent.

Table III

Distribution of Str. pyogenes types

Type	1964–65							
	Scarlet fever		Tonsillitis*		Other streptococcal infections		Total	
	No.	per cent	No.	per cent	No.	per cent	No.	per cent
1	7	6.9	6	4.0	8	5.6	21	5.3
2	—	—	2	1.3	2	1.4	4	1.0
3M (3R)	25	24.8	10	6.7	6	4.1	41	10.4
3R	—	—	—	—	—	—	—	—
3, 13, B_{3264}	6	5.9	30	20.0	31	21.5	67	16.9
4, 24, 26 . . .	3	3.0	10	6.7	2	1.4	15	3.8
5	1	1.0	4	2.7	9	6.2	14	3.5
5, 11, 12, 27, 44	9	8.9	26	17.2	39	27.1	74	18.7
6	4	4.0	8	5.3	8	5.6	20	5.0
8	—	—	1	0.6	1	0.7	2	0.5
8, 25, Imp_{19}	5	4.9	5	3.3	7	4.8	17	4.3
9	—	—	2	1.3	1	0.7	3	0.8
11	—	—	—	—	—	—	—	—
12	8	7.9	14	9.3	5	3.5	27	6.8
13	—	—	—	—	—	—	—	—
14	5	4.9	4	2.7	8	5.6	17	4.3
14, 49	—	—	—	—	—	—	—	—
15	—	—	4	2.7	2	1.4	6	1.5
15, 17, 19, 23, 30, 47	—	—	2	1.3	—	—	2	0.5
17	1	1.0	—	—	—	—	1	0.3
18	—	—	—	—	1	1.4	1	0.3
19	12	11.9	3	2.0	—	—	15	3.8
22	6	4.9	—	—	1	0.7	7	1.8
24	—	—	—	—	1	0.7	1	0.3
25	—	—	—	—	—	—	—	—
26	—	—	—	—	—	—	—	—
28R	3	3.0	15	10.0	9	6.2	27	6.8
29	4	4.0	1	0.7	1	0.7	6	1.5
36	1	1.0	—	—	1	0.7	2	0.5
46	—	—	1	0.7	—	—	1	0.3
48	—	—	1	0.7	—	—	1	0.3
Total typable	99	98.0	149	99.3	144	100.0	392	99.2
Untypable	2	2.0	1	0.7	—	—	3	0.8
Total	101	100.0	150	100.0	144	100.0	395	100.0

* Including sore throat

according to disease groups

1968–69									
Scarlet fever		Tonsillitis*		Other streptococcal infections		Healthy carriers		Total	
No.	per cent	No.	per cent	No.	per cent	No.	per cent	No.	per cent
2	1.6	4	4.4	1	0.9	3	3.1	10	2.3
3	2.4	1	1.1	—	—	3	3.1	7	1.6
11	9.0	3	3.3	2	1.7	3	3.1	19	4.5
3	2.4	1	1.1	1	0.9	3	3.1	8	1.9
9	7.3	7	7.7	17	14.5	12	12.4	45	10.5
15	12.3	5	5.5	15	12.8	10	10.4	45	10.5
2	1.6	3	3.3	2	1.7	2	2.1	9	2.1
6	4.9	14	15.4	11	9.4	11	11.3	42	9.8
—	—	5	5.5	3	2.5	—	—	8	1.9
1	0.8	1	1.1	5	4.3	1	1.0	8	1.9
9	7.3	19	20.8	20	17.1	11	11.3	59	13.8
4	3.2	4	4.4	4	3.4	9	9.3	21	4.9
6	4.9	2	2.2	6	5.1	4	4.1	18	4.2
12	9.8	8	8.8	12	10.2	11	11.3	43	10.1
4	3.2	1	1.1	4	3.4	3	3.1	12	2.9
—	—	—	—	—	—	—	—	—	—
1	0.8	—	—	—	—	2	2.1	3	0.7
1	0.8	—	—	1	0.9	—	—	2	0.5
—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—
—	—	2	2.2	—	—	1	1.0	3	0.7
21	18.0	3	3.3	5	4.3	4	4.1	33	7.7
8	6.5	3	3.3	2	1.7	3	3.1	16	3.8
—	—	—	—	—	—	—	—	—	—
3	2.4	1	1.1	5	4.3	1	1.0	10	2.3
3	2.4	1	1.1	5	4.3	1	1.0	10	2.3
1	0.8	1	1.1	—	—	—	—	2	0.5
—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—
122	100.0	88	96.7	116	99.1	97	100.0	423	99.1
—	—	3	3.3	1	0.9	—	—	4	0.9
122	100.0	91	100.0	117	100.0	97	100.0	427	100.0

Table IV demonstrates the distribution of prevalent types according to the residence of the examined persons. It is seen that in both surveys these types occurred in rural as well as in urban population at a frequency of more than 5%, except type 12 which in 1964—65 was shown in rural areas only in 2.8%.

Sensitivity of the strains to penicillin and tetracycline was tested in both periods. As shown in Table V, all strains were sensitive to penicillin. In the incidence of tetracycline resistance, which shows an increasing tendency in many countries, no change was demonstrated.

Table IV

Percentage distribution of prevalent types in urban and rural areas

1964—65			1968—69		
Type	Urban areas	Rural areas	Type	Urban areas	Rural areas
5, 11, 12, 27, 44	17.5	20.6	12	14.6	6.9
3, 13, B_{3264}	14.2	21.3	*4, 24, 26 . . .*	12.4	6.9
3	11.0	9.3	*8, 25,* Imp_{19}	12.4	17.3
12	9.4	2.8	*5, 11, 12, 27, 44*	9.6	10.2
28	6.1	8.6	19	6.3	10.2

Table V

Sensitivity to penicillin and tetracycline of Str. pyogenes strains isolated during the two surveys

Survey	Penicillin			Tetracycline		
	Sensitive	Moderately sensitive	Resistant	Sensitive	Moderately sensitive	Resistant
1964—65	395 (100.0%)	—	—	346 (87.6%)	28 (7.1%)	21 (5.3%)
1968—69	427 (100.0%)	—	—	394 (92.3%)	10 (2.3%)	23 (5.4%)

Discussion

The desired number of strains could not be collected by every laboratory in every month. However, the laboratories represented fairly heterologous areas to obtain a picture characteristic of the distribution of streptococcal

types in the whole country. In order to avoid false data, the laboratories were asked not to send strains associated with outbreaks.

In analysing the type distribution in the two surveys a slight qualitative difference was evident: 8 types occurring in 1964—65 were not isolated in 1968—69, and the second survey yielded 4 new types. It should be noted that 5 out of the afore-mentioned 8 types can be regarded as separate units only by M antigen analysis but not by T-agglutination as they possess a common T antigen (types 24, 29, 28, 46, 48 and the corresponding complex as well as type 17 and complex *15, 17, 19, 23, 30, 47*). The change in the incidence of these types was not necessarily due to a disappearance of some types (this was certain only for type 36) but may have been due to a different degree of detectability of the M antigen. T antigen relationships different from the usual were not observed in either of the surveys.

The alteration in the incidence of prevalent strains was not significant as regards complexes *5, 11, 12* . . . and *3, 13,* B_{3264} and type 12. In the second as compared to the first survey, type 3 occurred less frequently while complex *4, 24, 26* . . . and type 19 isolated earlier in a low percentage had become prevalent. Complex *8, 25,* Imp_{19} classified in 1968—69 as a prevalent type was encountered in the 1958—62 material [8] more frequently than the latter types.

In 1964—65, scarlet fever was caused in 24.8% by type 3, but in the second survey type 19 was the most frequently associated with this disease. That types 3 and 19 may be regarded as regular causative agents of scarlet fever was indicated by the fact that these types occurred at lower frequencies in sore throat and tonsillitis. Investigations into the toxin-producing capacity of streptococcal strains and into the antitoxic and antibacterial immunity of the population might be useful for elucidating this problem. As to an eventual association between the type of the agent and infections other than scarlet fever (including tonsillitis and all purulent conditions caused by streptococci), the present examinations, similarly to the result of the previous survey, failed to give an answer. According to data collected in the second survey, types prevalent in pathological conditions comprised the most frequent types also in healthy adults.

No significant difference has been demonstrated between urban and rural areas as regards the distribution of streptococcal types. This may reflect the fact that differences between towns and villages are disappearing.

The constant sensitivity of *Str. pyogenes* to penicillin has been confirmed in both surveys. There was no difference between the two periods as to the incidence of tetracycline resistant streptococci. Similar data were reported by ROBERTSON [9] who found that the incidence of tetracycline resistant streptococci in Southwest Essex remained practically at the same level between 1963 and 1967. In ROBERTSON's material tetracycline resistant strains occurred more frequently than among our culture. Penicillin is still regarded as the best

antibiotic against streptococcal infections, but the possibility of an increase in the number of tetracycline resistant strains cannot be excluded. After having closed the collection of streptococci for the second survey, one of our laboratories (Veszprém) reported an outbreak of nephritis caused by penicillin sensitive but tetracycline resistant strains.

Surveys carried out in the manner described may be considered adequate for collecting data concerning changes occurring in the type distribution of streptococci. If the surveys will be conducted at identical periods, under the same conditions and with the same technique in many countries, a reliable basis will be created for assessing the epidemiological situation in whole continents and for offering an effective help in the elaboration of the specific prevention of streptococcal diseases.

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ENHANCED INTERFERON PRODUCTION IN VITRO BY LEUCOCYTES FROM CHILDREN WITH INFECTIOUS MONONUCLEOSIS

By

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Summary. Interferon production *in vitro* by leucocytes from 10 children with infectious mononucleosis was studied. The production tended to be more intensive than in control cultures of the same cell density, prepared in the same manner from leucocytes of healthy children. The possible causes of the difference are discussed.

Since GRESSER's discovery [1] that human leucocytes produce interferon *in vitro* numerous authors have investigated the interferon production by pathological leucocytes. We have examined leucocytes from patients suffering from chronic or lymphoid leukaemia [2], polycythaemia [3] and lupus erythematosus [4].

Ever since it had been shown that infectious mononucleosis (IM) is a benign lymphoproliferative disease which may have in the Epstein—Barr virus an aetiological agent common with that of the malignant Burkitt lymphoma [5, 6], the interest of oncovirologists has been focussed on IM. This interest has been increased by the recent discovery that the Epstein—Barr virus can cause a malignant transformation of human leucocytes [7, 8].

DAMESHEK [9] considers IM a self-limited form of leukaemia, in which progression is stopped and reversed by the organism's protective mechanisms. It has already been demonstrated [10] that continuously cultured leucocytes from IM patients, like cultured normal leucocytes, are capable of spontaneous interferon production. In the present work, interferon production *in vitro* by leucocytes from IM patients and by normal leucocytes has been studied.

Materials and methods

Leucocytes were purified according to the method of STRANDER and CANTELL [11]. The purified leucocytes, 10^7/ml, were incubated in a rolling drum revolving at 18 r.p.m. As medium Parker's 199 enriched with 10% calf serum was used.

Interferon production was induced by Sendai virus. Interferon was assayed in a continuous line of human amnion cells, using vesicular stomatitis virus as indicator. The techniques used were described in detail previously [2].

Results

Table I shows the interferon production by leucocytes from 10 IM patients and from 10 healthy controls. The control subjects corresponded in sex and age to the patients in each case. From every subject two blood samples were obtained; from the patients the first sample was taken during the acute phase of the illness and the second one after convalescence.

As regards the acute-phase samples, the IM leucocytes produced four or more times more interferon than the control ones in 5 cases (Nos 4, 5, 6, 8 and 10); in two cases (Nos 7 and 9) the quotient was 3 and 2, respectively; in two cases (Nos 1 and 2) the titres were equal, and there was a single case (No. 3) where the control titre exceeded the titre produced by the patient's leucocytes.

Table I

Interferon production in vitro by leucocytes from children with infectious mononucleosis and by healthy control leucocytes

Case No.	Initials	Age (years)	Sex	Blood sample	Interferon titre produced by	
					patient's leucocytes	control leucocytes
1	B. J.	6	♂	1st	128	128
				2nd	1024	256
2	P. J.	9	♂	1st	16	16
				2nd	48	64
3	M. I.	7	♀	1st	32	128
				2nd	16	32
4	B. F.	6	♂	1st	1024	128
				2nd	64	64
5	M. T.	10	♂	1st	512	32
				2nd	256	256
6	Cs. K.	12	♀	1st	512	32
				2nd	128	256
7	M. E.	3	♀	1st	96	32
				2nd	256	128
8	T. J.	13	♀	1st	1024	256
				2nd	256	256
9	T. A.	1 1/2	♂	1st	256	128
				2nd	128	64
10	K. Z.	2 1/2	♂	1st	1024	256
				2nd	1024	1024

There was hardly any difference in interferon production between the convalescent-phase leucocytes and the corresponding controls.

Table II shows the geometric means (g.m.) for the acute-phase and convalescent-phase leucocytes and for the corresponding controls. The quotient g.m. acute-phase/g.m. control was 2.9, whereas the quotient g.m. convalescent-phase/g.m. control was 1.1. The significance of the first difference was at the 0.05 level.

Table II

Geometric means for the interferon titres in Table I

Phase of illness	Geometric means for titres		A/B
	patients A	healthy children B	
Acute	232	78	2.9
Convalescence	164	147	1.1

Discussion

The increased interferon production shown by the leucocytes from the majority of the IM patients might be attributed to an elevated percentage of interferon-producing leucocytes and/or to an increased yield by individual leucocytes. HOOVER *et al.* [12] have shown that in the initial phase of IM, RNA synthesis in the atypical lymphoid cells is very intensive. It is with this intensive RNA synthesis that the enhanced interferon production might be in correlation. The variations in interferon production by the leucocytes from individual IM patients may depend upon the percentage of mononuclears showing an elevated RNA synthesis and consequent protein accumulation. This percentage also shows a great individual variation [13].

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PYOCINE TYPING OF PSEUDOMONAS AERUGINOSA: ASSOCIATION BETWEEN ANTIGENIC STRUCTURE AND PYOCINE TYPE

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Summary. Pyocine typing of 1043 *Pseudomonas aeruginosa* strains isolated from a variety of sources has been carried out by using the schema of Govan and Gillies. The majority of the cultures belonged to types 1 (28.7%), 3 (20.1%), 5 (5.5%) and 10 (14.8%). Unclassifiable and untypable strains occurred in 7.6 and 9.2%, respectively. Among type 1 strains, subtypes 1b, 1c, 1d, 1f and 1h predominated.

A total of 543 strains falling according to Lányi's antigenic schema into 53 serotypes and 16 further, partially defined serological units were subdivided into 165 combined seropyocine types. An interesting association has been revealed between O antigens and pyocine types. The practical applicability of combined typing in epidemiological investigations is discussed.

Pseudomonas aeruginosa is widely distributed in nature and is responsible for various pathological conditions. Its importance as a nosocomial infecting agent has been increasing since the wide-spread use of antibiotics. In addition to pathological materials it is frequently encountered in air and on fomites especially in ventilators and their accessory parts. The organism may be present in antiseptics, ointments and drinking water and is a common inhabitant of sewage.

In epidemiological studies on *Ps. aeruginosa* infections, bacteriocine (pyocine) production by the strains can reliably be utilized. In the present work we examined the distribution of *Ps. aeruginosa* strains isolated from a wide variety of sources according to pyocine types and studied the association between antigenic structure and pyocine production.

Materials and methods

Strains. A total of 1043 *Ps. aeruginosa* strains isolated in different parts of Hungary were examined. Of the strains, 543 had been subjected to full antigenic and biochemical analysis [12, 13]. Ninety-eight strains were isolated from the Szeged municipal water supply invaded by *Ps. aeruginosa* as a sole polluting agent [11] and, finally, 402 strains were collected from pathological materials sent to the Nógrád County Public Health Station. According to materials, the 1043 strains were distributed as follows: faeces 422, non-faecal human material 403, water and sewage 218.

Indicator strains together with pyocine-producing control strains P_1 and P_{16} were obtained from Dr. R. R. GILLIES. Eight indicator strains (Nos 1—8) were used for primary typing. Subdivision of type 1 strains was performed with 5 indicator strains of the supplementary set [6].

Culture media. Blood agar base prepared as described in the Standard Methods of Hungarian Public Health Laboratories [14] was supplemented with 5—7% horse or ox blood. Comparative examinatiou of horse and ox blood agar by the use of various pyocine types including control strains P_1 and P_{16} indicated that both kinds of blood were suitable. The indicator strains were precultured in broth.

Pyocine typing was carried out essentially in the same manner as described by GILLIES and GOVAN [4] and GOVAN and GILLIES [6]. The strain under examination was seeded diametrically across a blood agar plate with a loop 5 mm in diameter and grown at 32°C for 14—18 hours. Then the growth was removed with a glass slide and a piece of cotton wool immersed in chloroform was placed in the lid of the Petri dish. After standing for 1 hour the cotton wool was removed and the chloroform vapour was eliminated from the plate by opening the lid for a few minutes. The indicator strains were streaked at right-angles to the line of the original inoculum. The results were read after 20 hours incubation at 37°C according to the schema of GOVAN and GILLIES [6].

Serological analysis was performed as described previously [12, 13].

Results

A total of 1043 *Ps. aeruginosa* strains were examined for pyocine production. Thirty-two out of the 37 pyocine types described by GOVAN and GILLIES were encountered (Table I). Similarly to the results of the above authors, pyocine type 1 was the most frequent (28.7%). Further common types were 3 (20.1%), 10 (14.8%) and 5 (5.5%). Pyocine-producing strains showing a pattern different from those included in the GOVAN—GILLIES schema occurred in 7.6%. Strains not acting on the indicator strains were encountered in 9.2%. Examination with the supplementary set of indicator strains showed that within type 1, subtypes 1c, 1d and 1b predominated.

It was studied whether the serotypes of LÁNYI's *Ps. aeruginosa* antigenic schema can be subdivided into further units by pyocine typing. In these investigations 543 strains isolated from a wide variety of sources and identified according to biochemical and serological characters [13] were used. The results are presented in Table II.

The 543 strains fell according to O and H antigens into 53 serotypes and into 16 other, not fully defined serological units. These serological units were subdivided into 165 combined sero-pyocine types. The majority of serotypes contained two or more different pyocine types. Serotypes frequently encountered in pathological materials as 1 : 1, 3a, 3b : 1, 3a, 3d : 1, 3c : 1, 3d, 3f : 1, 4a, 4d : 2a, 2b, 2f, 5a, 5d : 1 were each classified into 5 or more different sero-pyocine types. Other common serotypes as 4a, 4b : 2a, 2b, 4a, 4c : 2a, 2b, 2f and 7a, 7b : 2a, 2c contained two or three sero-pyocine types.

There was a definite association between O antigen groups and pyocine types. The frequency of pyocine types 5 and 29 within group 1 was remarkable. In group O3, encountered frequently in pathological materials, pyocine types 1c and 1d predominated. The overwhelming majority of O4 strains, which

Table I

Distribution of 1043 Ps. aeruginosa strains according to pyocine type

Pyocine type*	No.	per cent	Pyocine type*	No.	per cent
1a	9	0.8	14	2	0.2
1b	43	4.1	15	3	0.3
1c	124	11.9	17	12	1.1
1d	70	6.7	18	1	0.1
1e	2	0.2	19	2	0.2
1f	20	1.9	22	8	0.8
1g	6	0.6	23	1	0.1
1h	16	1.5	24	2	0.2
1uc	11	1.0	25	2	0.2
2	12	1.1	27	3	0.3
3	210	20.1	28	6	0.6
4	2	0.2	29	18	1.7
5	58	5.5	30	5	0.6
6	10	1.0	31	7	0.7
7	1	0.1	32	1	0.1
8	7	0.7	33	4	0.4
9	4	0.4	34	2	0.2
10	155	14.8	35	5	0.5
11	16	1.5	37	6	0.6
12	1	0.1	uc	79	7.6
13	1	0.1	ut	96	9.2

* According to the schema of GOVAN and GILLIES [6].
uc = unclassifiable strains (not producing pyocine detectable with the standard indicator strains).
ut = untypable strains (pyocine-producing cultures giving patterns other than those included in the schema).

represent the commonest serogroup in faeces, water and sewage, fell into pyocine type 3. Pyocine 10 was characteristic of O5, O7 and O11 strains; in groups O7 and O11 type 1b, a pyocine type almost entirely absent from other groups, was frequently demonstrated. It is interesting to note that between the last-named two groups there is a minor serological relationship [12]. No definite association was revealed between pyocine types and serological subgroups or serotypes.

Table III presents the distribution of pyocine types according to serogroups. The data can be interpreted similarly to the above ones. It is seen that untypable and unclassifiable strains belonged mainly to serogroups O1, O4 and O6.

Table II

Subdivision of Ps. aeruginosa serotypes into pyocine types

Serogroup	O antigen	H antigen	Frequent pyocine types, number of strains									Other pyocine types*	Not determined types, No. of strains		Total No. of strains
			1b	1c	1d	1f	1h	3	5	10	29		uc	ut	
1	1	1	—	—	—	—	—	—	12	—	12	17 (2, 6, 9, 13, 15, 31, 35, 37)	12	9	62
	1	2a, 2b	—	—	—	—	—	—	1	—	—	—	—	—	1
	1	2a, 2c	—	—	—	—	—	—	1	—	—	—	—	—	1
2	2	1	—	1	—	1	—	—	—	8	—	1 (1a)	—	—	11
	2	2a, 2b	—	5	—	—	—	1	—	1	—	3 (1e, 1g, 11)	—	—	10
	2	2a, 2c	—	1	—	—	—	—	—	—	—	—	—	—	1
	2	ND	—	—	—	—	—	—	—	—	—	1 (11)	—	—	1
3	3a, . . .	1	—	1	4	—	—	—	—	1	—	—	—	—	6
	3a, 3b	1	—	13	9	—	—	4	—	1	—	3 (luc, 6, 17)	—	—	30
	3a, 3b	ND	—	1	1	—	—	—	—	—	—	—	—	—	2
	3c	1	—	6	15	—	—	—	—	1	—	4 (1a, 6, 8)	—	—	26
	3a, 3d	1	—	21	5	—	—	1	—	1	—	2 (luc)	—	—	30
	3a, 3d	2a, 2b, 2f	—	1	—	—	—	—	—	—	—	—	—	—	1
	3a, 3d	ND	—	—	1	—	—	—	—	—	—	—	—	—	1
	3a, 3d, 3e	1	—	5	6	—	—	—	—	—	—	—	—	—	11
	3a, 3d, 3e	2a, 2c	1	—	—	—	—	—	—	—	—	—	—	—	1
	3d, 3f	1	—	15	1	—	—	3	—	1	—	4 (1a, 7)	—	—	24
	3d, 3f	2a, 2b, 2f	—	1	—	—	—	—	—	—	—	—	—	—	1
4	4a, . . .	1	—	—	—	—	—	1	—	—	—	—	—	—	1
	4a, . . .	2a, . . .	—	—	—	—	—	1	—	—	—	—	—	2	3

	4a, . . .	2a, 2b	—	—	—	—	—	13	—	—	—	1 (1a)	—	1	15
	4a, . . .	2a, 2c, (2f)	2	—	—	—	—	1	—	—	—	1 (33)	—	—	4
	4a, . . .	ND	—	—	—	—	—	—	—	—	—	1 (2)	—	—	1
	4a, 4b	2a, 2b	—	—	—	—	—	12	—	—	1	1 (27)	—	3	17
	4a, 4b	2a, 2d, 2e, 2f	—	—	—	—	—	—	—	—	—	—	—	1	1
	4a, 4c	—	—	—	—	—	—	1	—	—	—	—	—	—	1
	4a, 4c	1	—	—	—	—	—	1	—	—	—	—	—	—	1
	4a, 4c	2a, 2b, 2f	—	—	—	—	—	11	5	—	—	—	—	—	16
	4a, 4c	2a, 2c	—	—	—	—	—	3	—	—	—	—	—	—	3
	4a, 4c	2a, 2c, 2f	—	—	—	—	—	—	—	—	—	—	—	1	1
	4a, 4d	2a, 2b, 2f	—	—	—	—	—	5	2	—	—	6 (2, 14, 17, 28, 31)	5	6	24
	4a, 4d	2a, 2c, 2f	—	—	—	—	—	—	—	—	—	2 (17, 27)	—	—	2
5	5a, 5b, 5c	1	—	—	—	—	—	—	—	5	—	—	1	—	6
	5a, 5b, 5c	2a, 2d	—	—	—	—	—	—	—	1	—	—	—	—	1
	5a, 5b, 5d	1	2	—	—	—	—	—	—	3	—	—	—	—	5
	5a, 5b, 5d	2a, 2d	—	—	—	—	—	—	—	8	—	—	—	—	8
	5a, 5d	1	—	7	1	1	—	—	—	17	—	2 (18, 34)	—	1	29
	5a, 5d	2a, 2c	—	2	—	—	—	—	—	—	—	—	—	—	2
	5a, 5d	2a, 2d	—	—	—	—	—	—	—	1	—	—	—	—	1
6	6	1	—	—	—	—	—	1	1	—	—	1 (31)	—	9	12
	6	2a, 2b	—	—	—	—	—	—	1	—	—	5 (9, 11, 31)	—	12	18
	6	2a, 2c	—	—	—	—	—	—	1	—	—	6 (22)	4	—	11
7	7a, 7b	1	1	—	—	—	1	—	—	2	—	—	—	—	4
	7a, 7b	2a, 2c	10	—	—	—	2	—	—	30	—	—	—	—	42
	7a, 7b	2a, 2d	—	—	—	—	—	—	—	4	—	—	—	—	4
	7a, 7c	1	—	—	—	—	—	—	—	1	—	—	—	—	1
	7a, 7c	2a, 2b	1	—	—	—	—	—	—	—	—	—	—	—	1

Table II (continued)

Serogroup	O antigen	H antigen	Frequent pyocine types, number of strains									Other pyocine types*	Not determined types, No. of strains		Total No. of strains
			1b	1c	1d	1f	1h	3	5	10	29		uc	ut	
	7a, 7c	2a, 2c	3	—	—	—	—	—	—	—	—	—	—	—	3
8	8	1	—	—	3	—	—	—	—	—	—	—	—	—	3
9	9	2a, 2d	—	—	—	—	—	—	—	2	—	—	—	—	2
	9	2a, (2d), 2e, 2f	—	—	—	—	—	1	—	—	—	—	—	—	1
10	10a	1	—	—	—	1	—	—	—	—	—	—	—	—	1
	10a	2a, 2b, 2f	—	—	—	8	—	—	—	—	—	2 (luc)	—	2	12
	10a	2a, 2c	—	—	—	6	—	—	—	—	—	4 (luc)	—	—	10
	10a	2a, 2c, (2f)	—	—	—	—	—	1	—	—	—	—	—	—	1
	10a, 10b	1	—	1	—	—	1	—	—	—	—	2 (19)	—	—	4
	10a, 10b	2a, . . .	—	1	—	—	—	—	—	—	—	—	—	—	1
	10a, 10b	2a, 2c	—	—	—	—	—	—	—	—	—	1 (31)	—	—	1
11	11	1	3	—	—	—	5	—	—	5	—	2 (1a)	—	—	15
	11	2a, 2c	2	—	—	—	—	—	—	3	—	—	—	—	5
	11	2a, 2c, 2f	3	—	—	—	—	—	—	8	—	—	—	—	11
	11	ND	1	—	—	—	—	—	—	—	—	—	—	—	1
12	12	1	2	—	—	—	—	—	—	3	—	—	—	—	5
	12	2a, 2c	—	—	—	—	—	—	—	—	—	—	1	—	1
13	13	1	—	—	—	—	—	—	—	1	—	—	—	—	1
	ND	2a, 2b	—	—	—	—	—	1	—	2	—	—	—	—	3
	ND	2a, 2c	3	—	—	—	—	—	—	1	—	—	—	—	4
	ND	2a, 2c, 2f	—	—	—	—	—	1	—	—	—	—	—	—	1
	ND	ND	—	—	1	—	—	—	—	1	—	—	—	—	2
Total			34	82	47	17	9	63	24	112	13	72	23	47	543

* Figures before the brackets indicate the number of strains; bracketed figures stand for unfrequent pyocine types; uc = unclassifiable strains; ut = untypable strains; ND = antigen not specified in LÁNYI's schema.

Table III

Distribution of Ps. aeruginosa pyocine types according to serogroups

Pyocine type	Serogroup														Total No. of strains
	1	2	3	4	5	6	7	8	9	10	11	12	13	ND	
1a	—	1	4	1	—	—	—	—	—	—	2	—	—	—	8
1b	—	—	1	2	2	—	15	—	—	—	9	2	—	3	34
1c	—	7	64	—	9	—	—	—	—	2	—	—	—	—	82
1d	—	—	42	—	1	—	—	3	—	—	—	—	—	1	47
1e	—	1	—	—	—	—	—	—	—	—	—	—	—	—	1
1f	—	1	—	—	1	—	—	—	—	15	—	—	—	—	17
1g	—	1	—	—	—	—	—	—	—	2	—	—	—	—	3
1h	—	—	—	—	—	—	3	—	—	1	5	—	—	—	9
1uc	—	—	3	—	—	—	—	—	—	6	—	—	—	—	9
2	1	—	—	2	—	—	—	—	—	—	—	—	—	—	3
3	—	1	8	49	—	1	—	—	1	1	—	—	—	2	63
5	14	—	—	7	—	3	—	—	—	—	—	—	—	—	24
6	1	—	3	—	—	—	—	—	—	—	—	—	—	—	4
7	—	—	1	—	—	—	—	—	—	—	—	—	—	—	1
8	—	—	1	—	—	—	—	—	—	—	—	—	—	—	1
9	3	—	—	—	—	1	—	—	—	—	—	—	—	—	4
10	—	9	5	—	35	—	37	—	2	—	16	3	1	4	111
11	—	2	—	—	—	2	—	—	—	—	—	—	—	—	4
13	1	—	—	—	—	—	—	—	—	—	—	—	—	—	1
14	—	—	—	1	—	—	—	—	—	—	—	—	—	—	1
15	2	—	—	—	—	—	—	—	—	—	—	—	—	—	2
17	—	—	1	3	—	—	—	—	—	—	—	—	—	—	4
18	—	—	—	—	1	—	—	—	—	—	—	—	—	—	1
22	—	—	—	—	—	6	—	—	—	—	—	—	—	—	6
27	—	—	—	2	—	—	—	—	—	—	—	—	—	—	2
28	—	—	—	1	—	—	—	—	—	—	—	—	—	—	1
29	12	—	—	1	—	—	—	—	—	—	—	—	—	—	13
31	1	—	—	1	—	3	—	—	—	1	—	—	—	—	6
33	—	—	—	1	—	—	—	—	—	—	—	—	—	—	1
34	—	—	—	—	1	—	—	—	—	—	—	—	—	—	1
35	4	—	—	—	—	—	—	—	—	—	—	—	—	—	4
37	4	—	—	—	—	—	—	—	—	—	—	—	—	—	4
uc	12	—	—	5	1	4	—	—	—	—	—	1	—	—	24
ut	9	—	—	14	1	21	—	—	—	2	—	—	—	—	47
Total	64	23	133	90	52	41	55	3	3	30	32	6	1	10	543

uc = unclassifiable strains.
ut = untypable strains.
ND = antigen not specified in LÁNYI's schema.

No association was demonstrated between the biochemical characters and pyocine types of the 543 strains. Similar conclusions have been drawn as to colony morphology, pyocyanin and fluorescent pigment production. Different colony types of *Ps. aeruginosa* isolated from the same material were always identical in pyocine type.

In addition to the above 543 strains, 98 cultures isolated by LANTOS *et al.* [11] in studies of a *Ps. aeruginosa* mass pollution of the Szeged municipal water system were also examined. Table IV shows that group 4a, 4c strains responsible for the invasion belonged, with one exception, to pyocine type 3. Other *Ps. aeruginosa* strains isolated from the Szeged water system but not associated with the mass pollution belonged to different subgroups and different pyocine types.

Table IV

O antigens and pyocine types of 98 Ps. aeruginosa strains isolated from the Szeged municipal water supply

O antigen	Pyocine type	Number of strains
1*	uc	1
3a, 3b*	ld	1
4a, 4c	3	93
4a, 4c	uc	1
5a, 5b, 5d*	10	1
ND*	3	1
Total		98

* Strains belonging to these serogroups were isolated from peripheral sampling sites and were not associated with the invasion of the drinking water system by *Ps. aeruginosa* O4a, 4c.

Stability of pyocine types was confirmed by the fact that repeated examinations of the same patient yielded identical pyocine types and the original type remained unchanged after several subcultures.

Discussion

For the epidemiological marking of *Ps. aeruginosa* strains, phage typing, pyocine typing and serological determination of the cultures may be used. The strains are frequently lysogenic [1]. The practical utilization of various lysotyping schemes [7, 16—18] is rendered difficult by the instability of phage patterns [21] and by the existence of a large number of types [19].

Bacteriocine (pyocine, aeruginocine) produced by *Ps. aeruginosa* was described first by JACOB [10]. HOLLOWAY [8] recommended pyocine typing for the subdivision of the species. The two widely used methods [2, 4] are based on the detection of pyocine production by the isolated strains and their technique resembles the method elaborated for the colicine typing of *Shigella sonnei*. Classification of *Ps. aeruginosa* was attempted by determining the pyocine sensitivity of the strains [3, 15]. For the serological characterization of *Ps. aeruginosa* many schemes have been devised. The most extensive of these is that described by LÁNYI [12, 13].

Pyocine typing of Hungarian *Ps. aeruginosa* strains gave results similar to the findings of GILLIES and GOVAN for cultures isolated in the United Kingdom. In both countries, pyocine type 1 was the most frequent, indicating the importance of classifying this unit into subtypes.

Combined serological-pyocine typing offers a reliable and delicate characterization of *Ps. aeruginosa* strains. The method allowed the classification of 543 strains originating from a variety of sources into not less than 165 types. Serotypes frequently met with in pathological material could be divided into a number of different pyocine types. It should be noted that although the number of distinguishable sero-pyocine types was great, within each frequent serotype the majority of strains belonged to one or two pyocine types. This finding means that the applicability of the method, similarly as in the case of typing by H antigens [13], is somewhat limited. We failed to demonstrate the uniform distribution of pyocine types as described by WAHBA [20]. The disagreement was due probably to the fact that WAHBA's method differentiated a considerably smaller number of types either by serological examination or on the basis of pyocine production.

Our experiments revealed an important association between O antigens and pyocine types of *Ps. aeruginosa*. In this respect it is interesting to note that according to HOMMA and SUZUKI [9] the protein part of endotoxin isolated from the cell wall of *Ps. aeruginosa* is related to, or identical with, pyocine. K colicine in *Escherichia coli* K 235 is also associated with the O antigenic structure of the organism [5].

The uniformity of pyocine types of *Ps. aeruginosa* strains associated with the invasion of the Szeged water system has confirmed other findings concerning the stability of pyocine production.

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ATYPICAL MYCOBACTERIA ISOLATED FROM DIAGNOSTIC MATERIAL

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Summary. A total of 32 strains of acid-fast bacteria was isolated from routine tuberculous material. On the basis of morphological, cultural, biochemical, pathogenic and resistance characteristics the strains were: 2 *Mycobacterium tuberculosis* (human type), 1 *M. avium*, 8 *M. aquae* I, 3 *M. aquae* III, 3 *M. smegmatis* and 1 *M. phlei*. The human pathogenicity of niacin positive strains, because of their irregular behaviour, was doubtful. Six strains remained unclassified. Contradictions in the classification of mycobacteria are discussed. Standardization of the methods is regarded as a most important step for proper identification. A classification scheme suitable for small laboratories is recommended.

In recent years an increasing number of papers has dealt with the identification of "atypical" mycobacteria [1—3]. Most authors attempted to elaborate a classification by the use of international type strains [3, 4].

Practically every author has described new biochemical reactions or growth factor tests for improving classification. TSUKAMURA [3] described 98 characters for each examined strain, JUHLIN [4] and RUNYON [5] determined an almost equally high number of properties. Today the number of tests used for the classification of mycobacteria is well over one hundred. Many of them have been forgotten (thioglycollate, Desbordes and neutral red reaction) and some of them are used by few workers. The number of tests in common use is not more than 50. Unfortunately, some reactions such as phage typing [6, 7] and immunological examination [8, 9] have not been generally introduced because of technical and other difficulties. In view of the wide variety in the methods the results are difficult to compare. The media and their ingredients differ in composition and quality and the tests are carried out in different manners. For example, at least 6 different methods are routinely employed for the niacin test. It is generally agreed that the results are reproducible only when the standard procedures are strictly followed, but very few — and mainly unsuccessful — attempts have been made to standardize the identification methods. Even the simplest character for grouping — the time of growth — has not been accepted unanimously.

In the present work, methods giving reproducible results have been chosen so as to allow a proper identification of mycobacteria in small laboratories.

Materials and methods

Strains. A total of 32 atypical mycobacterial strains isolated from routine tuberculous material was used. The specimens were cultured after pretreatment with sodium triphosphate [10]. All cultures other than human and bovine type *M. tuberculosis* were collected during a 6-month period. Subcultures of the strains were suspended by the aid of glass beads 3 mm in diameter to 1 mg bacteria per ml. Aliquots of 0.5 ml of the suspension were used for seeding cultural and resistance test media and also for animal inoculation. Biochemical properties were examined by seeding parallel test media from Loewenstein—Jensen culture.

Media. Modified Loewenstein—Jensen medium was prepared from the standard concentrate obtained from the National Institute for Tuberculosis "Korányi", Budapest. *PNF medium:* to Loewenstein—Jensen medium 0.05% analytical grade p-nitrophenol was added. *Meat infusion agar:* dehydrated medium, Human, Budapest. *TTC medium:* Loewenstein—Jensen medium supplemented with analytical grade triphenyl tetrazolium chloride (500 μg/ml). *Picronitric acid agar:* to Sauton agar 0.2% picronitric acid was added. *Salicyl medium:* sodium salicylate (1 mg/ml) was dissolved in Ogawa medium.

Biochemical tests. Niacin: KONO's method as modified by TARSHIS [11]. *Catalase:* MIDDLEBROOK's method [12]. *Peroxidase:* according to VISCHER *et al.* [13]. *Lipase:* according to TISON *et al.* [14]. *CFA:* ferric ammonium citrate utilization as described by SZABÓ [15]. *Nitrate and nitrite reduction:* according to TACQUET *et al.* [16]. *Tween hydrolysis:* WAYNE's method [17]. *Amidase tests* with 10 substrates were performed as described by BOENICKE [18].

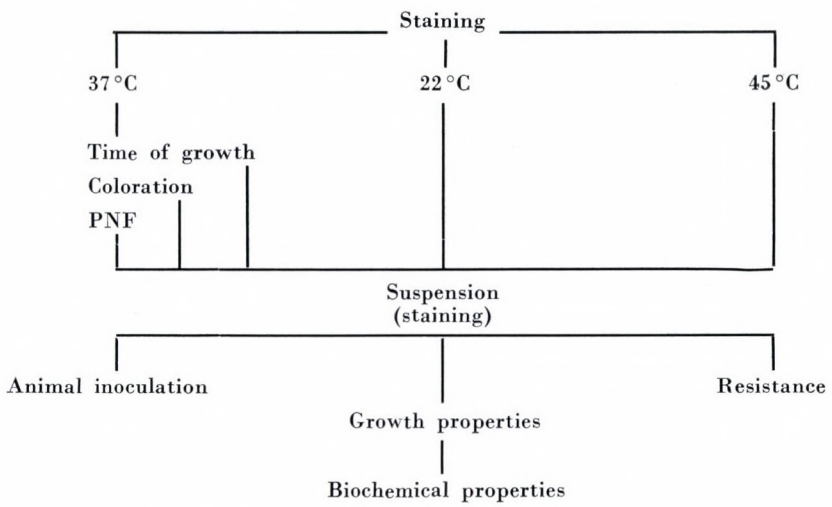
Resistance to antibiotics. The method of CANETTI *et al.* [19] was used at the following concentrations: INH, 20 μg/ml; streptomycin, 8 μg/ml; PAS, 10 μg/ml; ethionamide, 80 μg/ml; cycloserin, 80 μg/ml; viomycin, 80 μg/ml; TB-I, 4 μg/ml; rifampicin (AMP), 40 μg/ml; pyrazin amide, 400 μg/ml; ethambutol, 3 μg/ml, kanamycin, 80 μg/ml.

Animal experiments. The bacterial suspension (1 mg/ml) was inoculated at 0.5 ml aliquots in the inguinal region to guinea pigs and at 0.1 ml aliquots intraperitoneally and subcutaneously to mice. Readings were performed 6 weeks after the injection. The results were considered positive if the tested agent was recovered from macroscopic changes in the spleen, liver, lung and lymph nodes. Animals dying 48 hours after the infection were examined by autopsy.

Identification of isolates was performed as shown in Table I. Medium PNF was used for selecting *Nocardia* [20].

Table I

Scheme for the identification of mycobacteria



Results

The isolates were classified into two groups on the basis of the time of growth. A total of 14 rapidly growing strains was isolated; they began to multiply after 2—3 days and showed optimum growth within 6 days (Tables II, III and IV).

Table II

Cultural characters and animal pathogenicity of rapidly growing isolates

Designation	Time of growth, days	Growth at		Colour	Photo-induction	Mouse	Guinea-pig	Meat infusion agar	Picro-nitric acid	TTC
		20°	45°							
3	3	+	+	white	—	—	—	+	—	+
11	3	+	—	white	—	—	—	+	+	+
12	3	+	—	white	—	—	—	+	+	+
14	3	+	—	white	—	—	—	+	+	+
17	2	+	+	white	—	+	—	+	+	+
18	6	+	—	orange	—	—	—	—	+	+
19	6	+	—	white	—	+	—	+	+	+
20	2	+	—	white	—	—	—	—	+	+
21	3	+	—	yellow	—	—	—	—	—	+
25	3	+	—	orange	—	+	—	+	—	+
26	2	+	+	orange	—	—	—	+	+	+
27	6	+	—	white	—	—	—	+	+	+
28	6	+	—	white	—	—	—	+	+	+
29	3	+	—	orange	—	—	—	+	—	+

None of the rapidly growing isolates were photochromogenic and pathogenic to the guinea pig. All grew at room temperature and showed reducing colonies on TTC medium. As expected, they were catalase positive, niacin negative and PAS resistant. On the basis of amidase tests isolates 11, 12 and 14 were identified as *M. smegmatis*. These cultures were uniform in behaviour and differed only in peroxidase production and kanamycin resistance. There were more considerable differences between isolates 17, 19, 27 and 29 identified as *M. aquae* group II strains (time of growth, colour of culture, etc. as seen in Table II).

None of the strains attacked nitrate and nitrite, and peroxidase positivity was unfrequent. In antibiotic sensitivity the strains varied considerably. In addition to PAS resistance, a frequent incidence of INH, streptomycin, TB-I and pyrazinamide insusceptibility was noted.

Table III

Biochemical characters

Designation	NO_3	NO_2	CFA	Catalase	Peroxidase B	Lipase	Niacin	Tween hydrolysis
5	+	--	−	+	+	++	−	+
11	+	--	+	+	+	+++	−	+
12	+	−	+	+	−	+++	−	+
14	+	−	+	+	+	+++	−	+
17	−	+	−	+	−	−	−	−
18	−	+	−	+	−	++	−	−
19	−	+	−	+	−	−	−	(±)
20	−	+	−	+	−	−	−	(±)
21	−	+	−	+	−	+++	−	++
25	−	+	−	+	−	+++	−	++
26	−	−	−	+	−	+++	−	−
27	−	+	−	+	−	−	−	(±)
28	−	+	−	+	−	−	−	(±)
29	−	+	−	+	−	+++	−	−

− negative, ± weakly positive, + positive, ++, +++ strongly positive

Table IV

Antibiotic resistance of rapidly growing isolates

Designation	INH	Streptomycin	PAS	Ethionamide	Cycloserin	Viomycin	TB-I	AMP	PZA	EMB	Kanamycin
5	S	S	R	S	R	S	R	R	R	R	S
11	R	R	R	R	R	R	R	R	R	R	R
12	R	R	R	R	R	R	R	R	R	R	S
14	R	R	R	R	R	R	R	R	R	R	R
17	R	R	R	S	S	S	S	S	S	S	S
18	S	R	R	R	S	S	S	S	S	S	S
19	R	R	R	S	S	R	R	S	R	S	S
20	R	R	R	S	S	S	R	S	R	S	S
21	R	R	R	R	S	S	R	S	R	S	S
25	R	R	R	R	S	S	R	S	R	S	S
26	R	S	R	R	S	S	R	R	R	S	S
27	R	R	R	S	R	S	R	S	R	S	R
28	R	R	R	S	R	S	R	S	R	S	R
29	S	S	R	S	S	S	R	S	R	S	S

S = sensitive, R = resistant.

of rapidly growing isolates

Amidase									
1	2	3	4	5	6	7	8	9	10
−	−	−	−	±	−	±	−	−	−
++	−	+	−	±	+	±	+	±	−
±	±	+	−	++	+	±	+	±	±
++	−	+	−	±	±	−	+	±	±
−	−	−	−	−	−	−	−	−	−
−	−	−	++	−	−	−	−	−	−
−	−	−	−	−	−	−	+	−	−
−	−	−	−	+	+	−	−	−	−
−	−	++	−	±	±	−	±	−	−
−	−	++	−	−	±	−	+	−	−
+	+	−	−	++	−	±	++	−	±
−	−	−	−	−	−	−	−	−	−
±	−	−	−	−	−	−	±	−	−
−	−	−	−	−	−	−	−	−	−

Table V

Cultural characters and animal pathogenicity of slowly growing isolates

Designation	Time of growth, days	Growth at		Colour	Photo-induction	Mouse	Guinea pig	Meat infusion agar	Picro-nitric acid	TTC
		20°	45°							
1	17	−	−	white	−	+	−	−	−	−
2	17	−	−	white	−	+	+	−	−	−
3	13	+	−	yellow	−	−	−	+	+	+
4	17	−	−	yellow	−	−	−	−	−	+
6	17	−	−	white	−	−	−	−	+	+
7	17	+	−	white	−	+	−	−	+	+
8	13	+	−	white	−	−	−	−	+	+
9	17	−	−	orange	−	−	−	−	−	−
10	13	+	−	white	−	+	−	−	+	+
13	13	+	−	yellow	−	−	−	−	−	+
15	13	+	−	white	−	−	−	−	+	+
16	13	+	−	orange	−	−	−	−	−	+
22	8	+	−	white	−	−	+	−	−	−
23	8	+	−	white	−	−	−	−	−	−
24	8	+	−	white	−	+	−	−	+	+
30	8	+	−	white	−	−	−	+	−	−
31	8	+	−	white	−	+	+	+	−	−
32	8	+	−	white	−	+	+	+	−	−

Table VI

Biochemical characters

Designation	NO_3	NO_2	CFA	Catalase	Peroxidase B	Lipase	Niacin	Tween hydr.
1	+	−	−	−	±	−	+	−
2	+	−	−	+	+	+++	+	−
3	−	−	−	+	+	+	−	−
4	+	−	−	−	+	+++	−	−
6	−	+	−	+	−	−	−	+
7	−	+	−	+	−	−	−	+
8	−	+	−	+	−	−	−	+++
9	−	−	−	+	+	−	+	−
10	−	+	−	+	−	+	−	−
13	−	+	−	+	−	+++	−	+
15	−	−	−	+	−	−	−	++
16	−	+	−	+	−	+++	−	+
22	+	−	−	−	+	++	+	−
23	+	−	−	−	+	++	+	−
24	−	+	−	+	−	+	−	+
30	+	−	−	+	−	+++	+	−
31	+	−	−	−	+	+++	+	+
32	+	−	−	+	+	+++	+	++

− negative, ± weakly positive, + positive, ++, +++ strongly positive.

Isolates showing visible multiplication after 6 days were classified as slowly growing organisms (Tables V, VI and VII). None of the 18 cultures were photochromogenic or grew at 45 °C. Culturing, pathogenicity and biochemical tests showed that isolates 1 and 2 were human pathogens. On the basis of characters determined in this study the classification of the majority of slowly growing cultures was difficult.

With the exception of strains 1 and 2, niacin positivity was not in agreement with the systematic position of the strains determined by other characters. In the literature only human type *M. tuberculosis* and *M. niacinogenes* are known to produce niacin. Thus strains 9, 22, 23, 30, 31 and 32 were atypical in biochemical behaviour. Slowly growing strains were usually more sensitive to antibiotics than rapidly growing ones. There was no significant relation between antibiotic resistance and other characters.

of slowly growing isolates

Amidase									
1	2	3	4	5	6	7	8	9	10
−	−	+	−	+	−	−	−	−	−
−	−	±	−	+	−	−	−	−	−
++	−	++	−	±	+	−	±	−	−
−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−
−	−	++	−	+	+	−	−	−	−
−	−	−	−	−	−	−	±	−	−
−	−	++	−	−	−	−	±	−	−
−	−	−	−	−	−	−	−	−	−
−	−	−	−	+	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−
±	−	−	−	+	−	−	−	−	−
−	−	−	−	+	−	−	−	−	−
−	−	−	−	+	−	±	±	−	−

Discussion

Cultural and morphological behaviour were the first characters examined in the present work. Only strains forming R or S colonies on Loewenstein—Jensen medium, different in colour from *M. tuberculosis*, showing uniform turbidity in liquid Sula medium and lacking cord formation were selected.

M. tuberculosis strains with atypical morphology are unfrequently encountered. The incidence of two such isolates in our material indicates that their possible occurrence should always be considered in routine work.

In recent years a frequent incidence of pathogenic atypical mycobacteria has been noted [5, 21]. It is difficult to prove the pathogenic role of such isolates. Usually, only organisms cultured on repeated examinations or from surgical material are accepted as pathogens. The present work was not aimed at proving the pathogenicity of the strains. The fact that most of the atypical mycobacteria were isolated from chronic lesions of incurable patients seems to confirm XALABARDER's selection theory [22]. The selective action of laboratory pre-treatment of the material and the effect of drugs cannot, of course,

Table VII

Antibiotic resistance of slowly growing isolates

Designation	INH	Strepto-mycin	PAS	Ethion-amide	Cyclo-serin	Vio-mycin	TB-I	AMP	PZA	EMB	Kana-mycin
1	S	S	S	S	S	S	S	S	R	S	S
2	S	S	S	S	S	S	S	S	R	S	S
3	S	S	S	S	S	S	S	S	S	S	S
4	S	R	S	S	S	S	S	S	R	S	S
6	R	R	R	S	R	S	R	S	R	S	R
7	R	R	R	S	S	S	R	S	R	S	S
8	R	R	R	S	S	R	R	S	R	S	S
9	S	S	S	S	S	S	S	S	R	S	S
10	R	R	R	S	S	S	R	S	R	S	S
13	S	S	R	S	S	S	R	S	R	S	S
15	R	R	R	R	R	S	R	S	R	S	R
16	R	R	R	S	S	S	R	S	R	S	S
22	S	R	R	R	S	S	R	S	R	S	S
23	S	S	R	S	S	S	S	S	S	S	S
24	R	R	R	S	S	R	R	S	R	S	S
30	R	S	S	S	S	S	S	S	R	S	S
31	S	S	R	S	S	S	S	S	S	S	S
32	S	S	S	S	S	S	S	S	R	S	S

S = sensitive, R = resistant.

be excluded. Some of the isolates may have been contaminants; this is indicated by the relative frequency of *M. aquae.*

In the identification of mycobacteria, great importance should be attributed to the standardization of the methods. It is recommended that the tests should be started by seeding with logarithmic phase cultures grown on an appropriate standard medium and suspended to contain 1 mg bacteria per ml by the use of glass beads. All growth tests, biochemical reactions and animal experiments should be started from the same culture simultaneously. The use of a standard medium is important because the organism grown in liquid medium may exert an activity different from that of a solid medium culture. As mycobacteria frequently produce aggregates, care should be taken to use homogeneous cultures.

There are many disagreements between the authors as regards biochemical tests. For example, PENSO [23] regards *M. minetti* catalase negative, while according to other workers [24] it is catalase positive.

In the present work tests not giving variable results for members of one mycobacterium group were regarded as having a primary importance in classification. Differences in the intensity of the same reaction (+, ++ and +++) cannot be accepted as a firm taxonomic basis. The widely used scheme of taxonomic analysis [25] has several disadvantages. In our opinion only strains showing identical behaviour in certain basic characters (time of growth, optimal temperature, coloration and animal pathogenicity) should be classified into the same group. In evaluating animal pathogenicity it should always be considered whether the patient had or had not received antibiotic treatment.

Comparison of *M. smegmatis* strains 11, 12 and 14 indicated that a similarity of 90.3% existed between these isolates. The values for other mycobacteria classifiable on the basis of amidase tests in the same group was about 50%. In the present work final classification was based on BOENICKE's amidase tests. At present this method supplemented with certain other tests seems to be the only means of obtaining a precise diagnosis.

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ANTI-COMPLEMENTARY SUBSTANCES PRODUCED BY KB CELL CULTURES INFECTED AND NOT INFECTED WITH VIRUS

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Summary. Anti-complementary substances from nutrient media of KB cell cultures, infected and not infected with virus have been studied. One fraction of the anti-complementary substance produced by non-infected KB cells was found to have several properties in common with $C\bar{1}$. Infection of the cell culture with type 1 poliovirus induced a decrease, whereas infection with type 3 adenovirus an increase, in the anti-complementary activity of the tissue culture fluid. The increase in activity related to adenovirus infection was due not so much to the increment of the originally produced substance, as to the formation of a new substance which differed from the former in several properties.

The anti-complementary (AC) effect of viral complement fixing antigens often interferes with the results of serological tests [1]. In experiments on the preparation of adenovirus complement fixing (CF) tumour antigen, GILDEN *et al.* [2] could use exclusively the 10th—30th passages of KB cell cultures, as in the higher passages the high AC activity of the fluid medium interfered with the evaluation of the CF tests. VON ZEIPEL demonstrated in the fluid media of non-infected HeLa and KB cell cultures a thermolabile substance with AC effect, which was later identified as an enzyme of esterase activity decomposing N-acetyl-L-tyrosine-ethyl-ester [3, 4]. In view of this characteristic substrate specificity it seemed worthwhile to examine whether the substance responsible for the AC activity of the fluid media of KB cell cultures was identical with the activated first component of human complement, the C1 esterase (further on referred to as $C\bar{1}$, according to the WHO nomenclature). After these experiments had been brought to an end, VON ZEIPEL's recent paper [4a] was published stating that the substrate specificity of one fraction separated from the AC substance of the fluid medium of HeLa cell cultures was identical with that of $C\bar{1}$.

In a further series of experiments, KB cell cultures were infected with SABIN's attenuated poliovirus type 1 and adenovirus type 3 virus strains to examine the influence of these viruses on AC substance production by KB cells.

Materials and methods

Cell culture. KB cells were grown in a nutrient medium consisting of 16% inactivated human serum, 3% lactalbumin hydrolysate, 19% Parker's 199 solution and 62% Hanks' solution. The medium was exchanged two days after passage (growth medium) and on the

sixth day the tube cultures were washed 3 times with Parker's 199 solution to remove human serum. Subsequently, part of the cultures was infected with virus and as maintenance medium Parker's 199 was added to both infected and non-infected cultures. After 5, 24 and 48 hours the fluid media of the infected and non-infected cultures were pooled (15 tubes per series), centrifuged at 2500 r.p.m. for 10 minutes and, after addition of Sephadex G-50, concentrated to about one third of the original volume. In other experiments, the cultures were subjected to alternate freezing and thawing three times prior to the withdrawal of the tissue culture fluids, to release cellular AC activity. Subsequently the material was centrifuged and the supernatant was concentrated by freeze-drying to about one sixth of its original volume. In every case, the concentrated tissue culture fluid was dialysed overnight against veronal buffer pH 7.4.

Virus strains. SABIN's attenuated type 1 poliovirus strain LSc2ab and type 3 adenovirus prototype strain from the collection of the National Institute of Public Health were used throughout.

Sera. Pooled sera from healthy subjects and one serum from a patient with hereditary angioneurotic oedema (HANO serum), stored at −20 °C, were used.

Reagents. The titres of the four "classical" complement components (C1, C2, C3, C4) were determined in human serum using R-reagents, by the method described earlier [5]. The R-reagents were prepared from guinea-pig serum by standard methods [6].

Haemolytic system. A mixture of equal volumes of 2% sheep erythrocyte suspension and 1 : 2000 diluted haemolysin solution.

Diluent. Veronal buffer solution (VBS) of pH 7.4, containing 0.001 *M* Ca^{++} and 0.0005 *M* Mg^{++}.

Determination of C4 inactivating effect. The C4 inactivating effect of the fluid media of KB cell cultures was estimated by LAURELL and SIBOO's method [7], using inactivated human serum. To 0.9 ml concentrated tissue culture fluid 0.1 ml of 1 : 15 or 1 : 30 diluted inactivated human serum was added and the mixture was incubated at 37 °C for 60 minutes. To the control tubes, 0.9 ml VBS or Parker's 199 was added. After incubation, the titre of C4 was determined in each tube and expressed in 50% haemolytic units (C4H50). The difference between the C4 titres of the control and KB tissue culture fluid containing tubes corresponded to the number of C4H50 bound by the latter.

Gel filtration. Six ml concentrated KB tissue culture fluid was applied to columns 310 mm long and 20 mm wide (Sephadex G-100 coarse and G-200 coarse, Pharmacia, Uppsala). The material was eluted in 100—120 ml VBS, at pH 7.4. Flow rate was 12 ml/hour. A total of 28—32 fractions were collected, of 4 ml volume each. The C4 inactivating activity of the fractions was determined, and their extinctions were measured in a Unicam spectrophotometer at 280 mμ.

Mathematical-statistical evaluation. Significance of differences was determined by Student's *t*-test.

Results

Examination of AC substance produced by non-infected KB cell cultures. Concentrated tissue culture media of 24-hour and 48-hour non-infected cell cultures were tested for AC activity against human complement components by the above method. Table I shows that the 24-hour tissue culture fluid caused a decrease of the C4 and C2 titres by 21 and 19%, respectively, while the titres of C1 and C3 remained unaffected. With the 48-hour tissue culture fluid the same effect was more pronounced.

The fact that of the four components only C4 and C2 were inactivated by the test material was in support of its identity with $C\bar{1}$. Further experiments were performed to substantiate this hypothesis, in which AC activity was measured by the decrease of the titre of C4 in human serum.

1. Since unlike most AC substances (antigen-antibody complex, aggregated gammaglobulin), the $C\bar{1}$ (activated C1 component) exerts an effect against C4 also in inactivated serum which does not contain C1, it was examined

Table I

Effect of fluid phase from KB cell culture on titres of complement components in active human serum

(0.9 ml tissue culture fluid + 0.1 ml serum dilution; incubation at 37 °C for 60 min.)

Complement components	Original activity CH_{50}	Activity bound by KB tissue culture fluid			
		after 24 hours		after 48 hours	
		CH_{50}	per cent	CH_{50}	per cent
C1	15.7	0.0	0	0.0	0
C4	15.9	3.3	21	5.7	35
C2	15.9	3.1	19	3.8	24
C3	15.4	0.0	0	0.0	0

whether the 48-hour KB tissue culture fluid would reduce the titre of C4 in inactivated human serum. A decrease of the C4 titre took place in fact also in the inactivated serum, as shown in Table II.

Table II

Effect of fluid phase from KB cell culture on C4 titre of human serum inactivated and not inactivated with heat

Serum	Original activity CH_{50}	Activity bound by tissue culture fluid			
		after 24 hours		after 48 hours	
		CH_{50}	per cent	CH_{50}	per cent
Serum inactivated at 56 °C for 30 min	8.3	0.9	11	2.2	27
Control serum	12.6	1.4	11	1.8	14

In further experiments, the AC-effects of KB tissue culture fluids were examined in inactivated human sera.

2. In view of the fact that the activity of $C\bar{1}$ is lost at 56 °C for 30 minutes, the C4 inactivating effect of the KB tissue culture fluid was examined after this kind of heat treatment. No C4 inactivator was demonstrable in the heat treated tissue culture fluid.

3. The next experiment was performed to clarify whether the C1 esterase inhibitor, present in all normal human sera, would suppress the activity of the C4 inactivator in the KB tissue culture fluid. From the human serum C4 was previously removed by precipitating it twice with 40% $(NH_4)SO_4$, and the fluid phase was used in the experiments. (According to literary data [8], the full amount of the C1 esterase inhibitor remains in the supernatant.) As a control, a similarly treated serum sample from a patient with hereditary angioneurotic oedema was used, as such sera are known to contain no C1 esterase inhibitor [9].

The supernatants of normal sera and of the HANO serum were mixed with tissue culture fluid at a ratio of 1 : 5, incubated for 60 minutes at 37 °C and titrated for C4 inactivator in the usual way. Incubation with the supernatant of the normal human serum significantly reduced the C4-inactivating effect of the KB tissue culture fluid, whereas the HANO serum induced no inhibition (Table III).

Table III

Inhibition by C1-esterase inhibitor of the C4-inactivator action of fluid phase from KB cell culture

	Original activity CH_{50}	Bound activity CH_{50}
Tissue culture fluid incubated with VBS (control)	12.18	3.01**
Tissue culture fluid incubated with supernatant* of normal human serum	12.18	1.16**
Tissue culture fluid incubated with supernatant* of HANO-serum	12.18	2.98

* Supernatant of serum precipitated with 40% $(NH_4)_2SO_4$, incubated at 37 °C for 60 minutes.

** Significant difference ($P < 0.01$).

To check whether the AC substance examined was actually synthetized by the KB cells, AC activity of the fluid media of cell cultures treated and not treated by triple freezing and thawing were compared. The difference of the two values corresponded to the quantity of AC substance present in the disintegrated cells at a given time. The C4-inactivator synthesizing capacity of the KB cells was shown by a significant increase in the total activity of cells and the tissue culture fluid between 24 and 48 hours. Cellular activity tended to decrease during the same period (Table IV).

Table IV

C4-inactivating action of fluid phase and cells of KB cell culture

	Bound activity of	
	24-hour cell culture CH_{50}	48-hour cell culture CH_{50}
Tissue culture fluid	5.69**	8.69**
Tissue culture fluid + cells*	7.71**	9.49**
Cells	2.02	0.80

* Disintegrated by alternate freezing and thawing 3 times.

** The difference between the corresponding 24-hour and 48-hour values is statistically significant ($P < 0.05$).

Effect of viral infection on C4-inactivator production by KB cell cultures. KB cell cultures were infected with 10^2 CPD_{50} type 1 poliovirus or 10^4 CPD_{50} type 3 adenovirus to obtain a full cytopathic (CP) effect in 48 hours. Five hours after poliovirus infection the C4 inactivator activity of the tissue culture fluid showed no change, but 24- and 48-hour tissue culture fluids had significantly less C4-inactivating effect than the control tissue culture fluids. In contrast, in the case of adenovirus infection, 24-hour and 48-hour tissue culture fluids showed a significantly higher activity than did the controls (Table V).

Table V

Effect of type 1 poliovirus or type 3 adenovirus infection on C4-inactivator production by KB cell culture

	Activity (CH_{50}) bound by tissue culture fluid		
	5 hours	24 hours	48 hours
	after infection		
Cell culture infected with poliovirus	1.54	2.19*	3.35*
Cell culture infected with adenovirus	N. T.	5.05*	8.25*
Non-infected cell culture (control)	1.38	3.60	4.68

* Difference from control values is statistically significant ($P < 0.05$).
N.T. = not tested.

To find an explanation for the apparent stimulation of C4-inactivating activity by adenovirus, it was supposed that during the reproduction of adenovirus, in addition to the AC substance produced by the non-infected cells another substance was also formed, the C4-inactivating action of which had been added to that of the former substance. This hypothesis was based on the experience that inactivation of the 48-hour tissue culture fluid from adenovirus-infected cell cultures for 30 minutes at 56 °C effected a decrease, but not a lack, of the C4-inactivating effect (Table VI). This suggested the responsibility of an unidentified thermoresistant C4-inactivator substance for the residual activity of the infected cultures.

Table VI

Effect of heat inactivation on C4-inactivator activity of fluid phase from KB cell cultures infected and not infected with type 3 adenovirus

Tissue culture fluid	Bound activity (CH_{50})	
	before	after
	inactivation at 56 °C for 30 minutes	
From 48-hour non-infected culture (control)	2.80	0.00
From 48-hour culture infected with adenovirus	4.95	1.85

To obtain more information on the problem, an attempt was made to isolate the supposed two types of substances by gel filtration. For this purpose, tissue culture fluids from cell cultures disintegrated by 3 times alternate freezing and thawing were used after concentration by freeze-drying. Since for technical reasons no infective tissue culture fluid could be used in the experiments, 48-hour fluids from non-infected cells and inactivated (56 °C, 30 minutes) 48-hour fluids from adenovirus-infected KB cells were run simultaneously on a Sephadex G-100 column 310 mm long by 20 mm wide. C4 inactivator activity was determined in all but the first fractions (Fig. 1). Extinctions

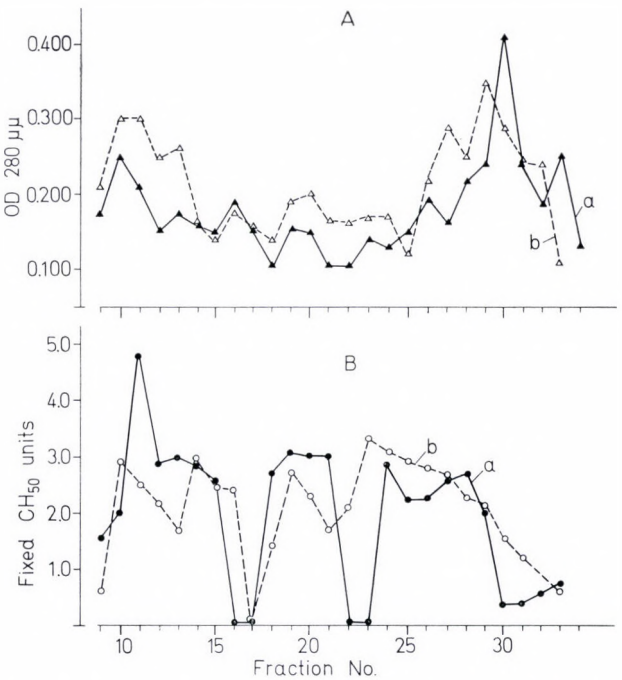

Fig. 1. Extinction values (A) and C4-inactivator activities (B) of fractions obtained by filtration through Sephadex G-100 from the fluid phases of KB cell cultures infected and not infected with type 3 adenovirus. A = fluid phase from non-infected control cultures, B = fluid phase from cultures infected with type 3 adenovirus

measured at 280 mμ were plotted in parallel. The extinction values for fractions Nos 28—34 were disregarded, as they contained colouring matter. The curves representing the activities of the fractions obtained from the two columns were of a similar type, suggesting that, in contrast with our original hypothesis, the AC substance was non-uniform not only in fluids from infected cultures, but also in those from non-infected cultures. A notable difference between the

two types of curve was found in two points: (1) on the control curve, activity values were highest for fractions Nos 11—13, whereas the corresponding fractions of the infected tissue culture fluid showed a low activity. It was supposed that the control fractions Nos 11—13 contained the $C\bar{1}$-like substance (molecular weight of Cl = 1 200 000) which was absent from the corresponding fractions of the infected tissue culture fluid owing to destruction by inactivation. (2) While the control fractions Nos 22—23 showed no activity, the corresponding fractions of the other series showed maximum activity. This implies that fractions Nos 22—23 of the tissue culture fluid from infected KB cells contained a C4-inactivator substance of much lower molecular weight than that of C1 esterase, which was not present in the non-infected cultures and perhaps corresponded to the originally sought thermoresistant substance. Gel filtration on Sephadex G-100 and G-200 columns was repeated in two additional instances, and the above results were consistently reproduced.

To verify the above findings, further experiments were performed with the most active fractions pooled for series Nos 11—13 and Nos 22—23, concentrated by freezing and drying. First, the C4-inactivator activity of the fractions was tested for inhibition by the C1-esterase inhibitor of normal human serum (Table VII). In this experiment, too, HANO-serum was used as a control. As expected, a significant inhibitory action was found with fractions Nos 11—13. In contrast, the C1-esterase inhibitor had no influence on the C4-inactivator action of fractions Nos 22—23.

Table VII

Inhibition by C1-esterase inhibitor of C4-inactivator action of Sephadex fractions

Sephadex G-100 fraction tested	Bound activity CH_{50}		
	Control (incubated in VBS)	Sample incubated in supernatant** of normal human serum	Sample incubated in supernatant** of HANO-serum
Control fractions Nos 11—13*	2.82***	1.68***	2.98
Fractions Nos 22—23* from adenovirus-infected material	2.01	2.15	2.05

* Designations of the fractions apply to the experiment presented graphically in Fig. 1.
** Supernatant of serum precipitated with 40% $(NH_4)_2SO_4$, incubated at 37 °C for 60 minutes.
*** Statistically significant difference ($P < 0.05$).

Finally, dose-response curves were constructed for the C4-inactivator action of the mentioned pooled fractions and of the whole control tissue culture fluid (Fig. 2). A linear relationship was obtained for fractions 22—23 in which the response (number of bound C4H50) was directly related with the dose

(volume added to the system). The dose-response relationship was non-linear with the whole control tissue culture fluid and fractions 11—13 as well, but a linear transformation of the curves was possible by plotting the logarithms of the dose on axis X. This implied a linear relationship between the number of inactivated C4 molecules and the logarithm of the dose of test material.

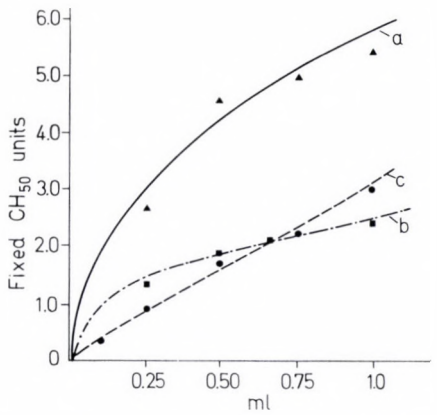

Fig. 2. Dose-response curves of C4-inactivator activity of Sephadex G-100 fractions of fluid phases from KB cell cultures infected and not infected with type 3 adenovirus and of fluid phase from the control culture. a = fluid phase from non-infected control culture, b = fractions Nos 11—13 obtained from fluid phase of control culture by filtration through Sephadex G-100, c = fractions Nos 22—23 obtained from fluid phase of adenovirus-infected culture by filtration through Sephadex G-100. Designations of the fractions apply to the experiment presented graphically in Fig. 1

Discussion

Experiments with fluid medium from non-infected KB cell cultures have shown that the AC substance produced by the KB cells was not uniform and the fraction responsible for most of the AC activity was identical with $C\bar{1}$, the first activated component of human serum. Essentially the same conclusions were drawn by VON ZEIPEL [4a] from enzymological studies in which a comparison of the substrate specificity of purified AC substance from HeLa tissue culture medium and $C\bar{1}$ showed the esterolytic properties of the two substances to be similar.

Component C1, as an inactive proesterase, is present in the serum in a macromolecular (19 S) form and is transformed to active C1 esterase under the influence of antigen-antibody complexes or other factors such as plasmin. Of the three subcomponents of C1, $C1_s$ carries the esterase activity. C4 and C2 being natural substrates of $C\bar{1}$, their molecules are activated by the enzyme,

thus becoming capable of binding to surfaces of antigen-antibody complexes, *e.g.* sensitized sheep erythrocytes. This circumstance provides for the binding of further complement components and finally for the lysis of the red cells. If, however, $C\bar{1}$ acts on C4 and C2 in the absence of an antigen-antibody complex (in the fluid phase), these components are rapidly inactivated and lose their haemolytic capacity.

The $C\bar{1}$-like substance produced by KB cell cultures differs from the commonly known AC substances (antigen-antibody complexes, aggregated gammaglobulin, plasmin) by its ability to act on C4 also in the absence of the C1 component, *viz.* in inactivated serum; the C4-inactivating action of the substance is analogous with the $C\bar{1}-C4$ reaction taking place in the fluid phase. Also, the thermosensitivity of the substance in question corresponds to that of $C\bar{1}$, being completely inactivated by exposure at 56 °C for 30 min. Of the four classical complement components, only C4 and C2 are affected by the KB tissue culture medium. As demonstrated by LEPOW *et al.* [10], exclusively these complement components were inactivated by purified $C\bar{1}$ in human serum. The same authors also showed the non-linearity of the relationship between the quantity and effect (number of bound C4H50) of $C\bar{1}$. The dose-response curve constructed for KB tissue culture fluid and the activity of the fraction supposed by us to contain $C\bar{1}$ disclose no linear relation, either. The hypothesis advanced to explain these phenomena is greatly supported by the finding that the C4-inactivating action of the substance produced by KB cell cultures was inhibited by normal human serum, but not inhibited by the HANO-serum which differed from the normal serum exclusively in the absence of the C1 esterase inhibitor [9].

A final substantiation of the theory remains to be obtained by demonstrating the antigenic identity of the two substances. Further experiments are scheduled to be carried out to clarify the precise nature of the antigenic relationship between C1 and the AC substance produced by the KB cell culture.

In the present experiments the $C\bar{1}$-like substance produced by normal KB cell cultures did not pass through Sephadex G-200, which implied its molecular weight to be above 200 000. This virtually contradicts VON ZEIPEL's finding who reported on a molecular weight of 115 000 for the AC substance produced by HeLa cell cultures [4]. Since there seems to be no reason to suppose that the AC substances produced by KB and HeLa cells were different, the explanation offered for the dissimilarity is that in the absence of Ca^{++}, C1 is split into its subcomponents $C1_q$, $C1_r$ and $C1_s$ [11]. The esterase activity remains localized to the $C1_s$ subcomponent, the molecular weight of which is 100 000 [12]. If gel filtration is performed with a Ca^{++}-free buffer — as in the experiments of VON ZEIPEL — the esterase will appear in the 100 000 molecular weight fractions. Since in the present experiments a Ca^{++} containing buffer solution was used throughout, this may have been the cause of detecting

the esterase activity corresponding to $C\bar{1}$ in the first exclusion peak, *viz.* with the whole C1 molecule in the molecular weight range well above 1 000 000.

The synthesis of the whole C1 molecule was studied by COLTEN *et al.* [13] in various human organs isolated post mortem, with the result that the bulk of this complement component was produced in the colon, the minor part in the ileum; there was no indication of its synthesis in other organs. An eventual isolated synthesis of the subcomponent $C1_s$ has not been reported.

Further experiments in this laboratory have shown that infection with either type 1 poliovirus or type 3 adenovirus was influencing the production of C4 inactivator by the KB cells. On infection with poliovirus, synthesis of the inactivator decreased significantly after 24 and 48 hours, while on infection with adenovirus it increased significantly in the same time interval. Thus, the significant change of inactivator production coincided with the development of the cytopathic effect of both viruses. This is in good correlation with literary data on other enzymes, stating that the increase or decrease of enzyme activity of cultured cells could be demonstrated exclusively when the cytopathic effect had already developed [14—16].

Infection of KB cells with type 3 adenovirus resulted in the release of a substance identical in action with the original enzyme but differing from it in certain other properties. The molecular weight of that substance was lower than that of C1 esterase, it was not destroyed by heat inactivation (56 °C for 30 minutes), and its activity was not inhibited by the C1-esterase inhibitor in human serum.

The available experimental data permit no conclusion as to whether the infection of the KB cells by adenovirus had initiated the synthesis of a new enzyme in these cells or simply effected the structural alteration of an enzyme regularly produced by the cells.

Further studies are in progress on the properties of the AC substance initiated in KB cells by adenovirus infection.

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BOOKS RECEIVED

REGAMEY, R. H., DE BARBIERI, A., HENNESSEN, W., IKIC, D., PERKINS, F. T. (eds): *International Symposium on Brucellosis. Standardization and Control of Vaccines and Reagents.* Proceedings of the 24th Symposium Organized by the Permanent Section of Microbiological Standardization at the Bourse du Travail, Tunis. Symposia Series in Immunological Standardization. Vol. 12. S. Karger, Basel—München—New York 1970. XII + 412 pages, 49 Figures, 145 Tables. Price: SFr/DM 60.—, US $ 14.40, 120 s.

CHARLES, H. P., KNIGHT, B. C. J. G. (eds): *Organization and Control in Prokaryotic and Eukaryotic Cells.* 28th Symposium of the Society for General Microbiology held at Imperial College, London, April 1970. Cambridge University Press, Cambridge 1970. XI + 457 pages. Price: £ 5.

WOLSTENHOLME, G. E. W., O'CONNOR, M. (eds): *Mutation as Cellular Process.* A Ciba Foundation Symposium. J. and A. Churchill Ltd., London 1969. XI + 244 pages. Price: 60 s.

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VAN HEMERT, P. A., VAN RAMSHORST, J. D., REGAMEY, R. H. (eds): *International Symposium on Pertussis.* Proceedings of the 25th Symposium Organized by the Permanent Section of Microbiological Standardization. Bilthoven 1969. S. Karger, Basel—München—New York 1970. XII + 294 pages, 59 Figures, 119 Tables. Price: SFr/DM 60.—, US $ 14.40, 120 s.

INDEX

VIIth INTERNATIONAL CONGRESS OF CHEMOTHERAPY PRAGUE, CZECHOSLOVAKIA, AUGUST 1971

The International Society of Chemotherapy has entrusted the Chemotherapeutical section of the J. E. Purkyně Society for Internal Medicine with the organization of the VIIth International Congress of Chemotherapy which will be held from August 23—28, 1971 in Prague. The preliminary programme is divided as follows:

A—1 Pharmacodynamics and Pharmacokinetics of Antibiotics and Antibacterial Chemotherapeutics —
theoretical basis of pharmacodynamics and pharmacokinetics and its application, effect of various factors on the kinetics of antibiotics.

A—2 The Role of the Lymphatic System in the Absorption and Distribution of Antibiotics —
the role of the lymphatic system in the absorption and distribution of antibiotics, cytostatics and chemotherapeutics, changes in kinetics in pathological states.

A—3 Chemotherapy in Immunosuppression and Transplantation —
present state of clinical research, prevention and therapy of bacterial, viral and mycotical complications in immunosuppression and transplantation.

A—4 Chemotherapy of Mycotic Disease —
new antimycotic agents; chemotherapy of cryptococcosis, histoplasmosis, North American blastomycosis, candidiasis, sporotrichosis, coccidioidomycosis, chromoblastomycosis, opportunistic mycotic infections and adiaspiromycosis.

A—5 Chemotherapy of Viral Infections —
results from testing of antiviral substances in vivo and in vitro; mechanism of action; chemotherapy of viral diseases in man.

A—6 Chemotherapy of Parasitic Infections —
new antiparasitic drugs; chemotherapy of protozoal infections and helminthoses.

A—7 Adverse Reaction to Chemotherapeutics —
their occurrence, pathogenesis and prevention, diagnostics.

A—8 Chromosomal and Extrachromosomal Resistance of Microorganisms to Chemotherapeutic Agents —
ribosomal resistance to antibiotics affecting proteosynthesis; structure and function of nucleic acids in relation ro resistance; impermeability to antibiotics; molecular biology of microbial resistance; origin, function and structure of R-factors, R-plasmids and satellite DNA.

A—9 Surveillance of Drug Resistance —
methodology of comparative studies; survey of the resistance of pathogens; problems of multiresistance; attempts to affect resistance; criteria for long-term testing of defensive mechanism affected by long-term administration of antibiotics.

A—10 Laboratory Control of Chemotherapy —
sensitivity of pathogenic and potentially pathogenic microorganisms to new chemotherapeutics; chemotherapy in laboratory practice and its correlation with clinical results.

A—11 Mode of Antibiotic Action —
molecular basis of antibiotic action; binding mechanism of antibiotics to DNA, and RNA; relation between chemical structure and biological activities, etc.

A—12 Experimental Chemotherapy —
tests and screening of chemotherapeutics in vivo; pharmacokinetics in animals and comparison with man.

A—13 New Antibacterial Antibiotics —
production, chemistry and biological activities of new antibacterial antibiotics.

A—14 Clinical Evaluation of Antibacterial Chemotherapeutics —
experience with new antibiotics; present position of established antibacterials in patients with infectious diseases or complications.

A—14.1 In Medicine
A—14.2 In Surgery
A—14.3 In Urology
A—14.4 In Dermatovenerology

B—1 New Antineoplastic Chemotherapeutics —
the chemistry, biological effects, mechanism of action, metabolism, chemico-biological correlations, resistance development, combined therapy, new biochemical and therapeutic aspects.

B—1.1 Alkylating Agents
B—1.2 Antimetabolites of Pyrimidine and Purine Type
B—1.3 Antitumor Antibiotics
B—1.4 Other Compounds

B—2 Screening Methods in Evaluation of Cancerostatics —
antineoplastic effect of cancerostatics on experimental neoplasms; study of histological and cytological effects; biochemical and metabolic changes of malignant cells; metastatic inhibition as a screening method. Tissue cultures in screening. Microbiological screening.

B—3 Pharmacology of Antineoplastic Agents —
action on malignant cells and tissues in vitro and in vivo. Teratogenic effect. Toxicological properties and possibilities of prediction of cytogenetic action and toxicity. Preliminary testing of cytostatics, their pharmacokinetics and metabolism. Adverse reactions in man.

B—4 Evaluation of Tumor Cell Sensitivity to Cytostatics in vitro —
the determination of human tumor sensitivity to cytostatic therapy. The effects of cytostatics on the degree of incorporation of labelled compounds in tumor cells.

B—5 The Kinetics of Tumor Cells and Chemotherapy —
sensitivity of malignant cells in different cell cycle phases. Experimental chemotherapy of synchronized cell populations. Proliferative activity, doubling time of tumors and drug sensitivity. Importance of non-dividing tumor cells in cancer chemotherapy.

B—6 Clinical Chemotherapy of Cancer as a Part of General Management —
chemotherapy in combination with other methods of therapy.

B—7 Chemotherapy of Advanced Cancer —
criteria for selection of cytostatics and their combinations in the resistant tumorous diseases.

B—8 Clinical Evaluation of Antitumor Agents —
clinical experience with new antitumor agents. Present clinical position of established cancerostatics.

C—1 Radioprotectives —
the experimental and clinical research into different structural types of radioprotectives.

Everyone interested in the participation at the Congress should apply for information to the Secretariat of the 7th Congress of Chemotherapy, Sokolská 31, Praha 2, phone number 29 72 71.

The official Congress language is English without simultaneous translation.

Summaries of papers not exceeding 200 words, typed in double spacing in English, should be sent to the Congress Secretariat not later than April 1, 1971. The time limit for presenting a paper must not exceed 10 minutes including the projection of slides.

The commercial exhibition 'Chemotherapia' will take place in the Congress building.

Post Congress Symposia:

'Biosynthesis of antibiotics and related compounds' will be held in Warsaw, Poland, August 30 and 31, 1971.

'Infectious Antibiotic Resistance' will take place at Smolenic, Czechoslovakia, August 30—September 2, 1971.

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T. Trencséni and B. Keleti

Clinical Aspects and Epidemiology of Haemorrhagic Fever with Renal Syndrome

The merit of this monograph lies in the fact that the authors give a detailed analysis of the clinical and epidemiological aspects of haemorrhagic fever with renal syndrome as well as its aetiology and pathology based on the nearly 130 cases they have encountered since 1953 in Hungary. All pertaining observations in the literature are also reported. Moreover, the epidemiological criteria of the epidemic and sporadic cases which have occurred in Hungary are discussed. Based on the knowledge of general epidemiology recent data on haemorrhagic fevers concomitant with renal syndrome are examined with special regard to unsolved problems. The appendix provides information on various virus induced haemorrhagic fevers. Another valuable feature of this monograph is that there are 310 reference works in the Bibliography and another 100 in the Appendix.

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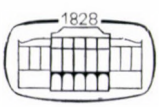

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ACTA MICROBIOLOGICA

ТОМ XVII.

РЕЗЮМЕ

GROWTH KINETICS OF YEASTS

E. K. NOVÁK, T. DEÁK.

КИНЕТИКА РОСТА ДРОЖЖЕЙ

Е. К. НОВАК, Т. ДЕАК

Авторы изучали изменения кинетических параметров роста, таких как постоянная степени размножения (k), lag-фаза (L), конечная плотность (Д) и показатель размножения (M), в отношении ***Candida beverwijkii, C. claussenii, C. krusei, C. utilis, Procandida albicans, Pc. tropicalis, Saccharomyces carlsbergensis S. cerevisiae***, выращиваемых в аэробных условиях в глюкозе, галактозе, сахарозе, мальтозе и раффинозе. За исключением мальтозы, во всех субстратах наблюдалась тесная взаимосвязь между величинами k и M. Оценка результатов проводится с точки зрения углеводного обмена у дрожжей. Обсуждается связь между параметрами роста в различных субстратах и первыми фазами углеводного обмена (транспортирование сахара, вне и внутриклеточный распад олигосахаридов).

GROWTH FACTOR REQUIREMENT OF SHIGELLA FLEXNERI AND SHIGELLA SONNEI

A. VERTÉNYI, I. KÉTYI, B. KOCSIS

НЕОБХОДИМОСТЬ В ФАКТОРЕ РОСТА У SHIGELLA FLEXNERI И SHIGELLA SONNEI

А. ВЕРТЕНЬИ, И. КЕТЬИ, Б. КОЧИШ

Необходимость в факторе роста у 271 штамма ***Sh. flexneri*** и ***100 Sh. sonnei*** была изучена на минимальной среде Falkow-a. Все штаммы ***Sh. sonnei*** и большинство штаммов ***Sh. flexneri*** оказались прототрофными культурами; было найдено всего лишь 11 ауксотрофных штаммов ***Sh. flexneri*** (2,9%).

PROPERTIES OF THREONINE DEAMINASE IN MYCOBACTERIUM PELLEGRINO

A. SZENTIRMAI, I. HORVÁTH

СВОЙСТВА ТРЕОНИН-ДЕАМИНАЗЫ У MYCOBACTERIUM PELLEGRINO

А. СЕНТИРМАИ, И. ХОРВАТ

Изолейцин подавляет активность треонин-деаминазы, выделенной из ***Mycobacterium pellegrino.*** Степень подавления зависит от концентрации субстрата. Валин оказывает активизирующий эффект и частично прекращает подавление, проявляемое изолейцином. В свежих препаратах энзима кривая субстратного насыщения соответствует кинетике по **Michaelis—Menten.** Хранение при 0° С сопровождается повышением активности и понижением термостабильности и чувствительности к изолейцину. Устойчивость энзима может быть повышена не только пиридоксал-фосфатом и изолейцином, но также валином и тиамин-пирофосфатом.

VIRAL GROWTH INHIBITION BY A BIGUANIDINE DERIVATIVE IN TISSUE CULTURE

L. GERGELY, L. VÁCZI, GY. HADHÁZI, F. D. TÓTH

ПОДАВЛЕНИЕ ВИРУСНОГО РОСТА ПРОИЗВОДНЫМ БИГУАНИДИНА В ТКАНЕВОЙ КУЛЬТУРЕ

Л. ГЕРГЕЙ, Л. ВАЦИ, ДЬ. ХАДХАЗИ, Ф. Д. ТОТ

Авторы изучали токсический и вирусоподавляющий эффект производного бигуанидина в тканевой культуре. Упомянутое соединение заметно и довольно селективно подавляло репликацию некоторых штаммов миксовирусов и парамиксовирусов во вращающихся культурах фрагментов хориоаллантоисной мембраны.

SEROLOGICAL PROPERTIES OF PSEUDOMONAS AERUGINOSA II. TYPE-SPECIFIC THERMOLABILE (FLAGELLAR) ANTIGENS

B. LÁNYI

СЕРОЛОГИЧЕСКИЕ СВОЙСТВА PSEUDOMONAS AERUGINOSA II. ТИПО-СПЕЦИФИЧЕСКИЕ ТЕРМОЛАБИЛЬНЫЕ (ЖГУТИКОВЫЕ) АНТИГЕНЫ

Б. ЛАНЬИ

В специфической Н-антисыворотке, истощенной от О-агглютининов, клетки ***Ps. aeruginosa***, растущие на среде, способствующей развитию жгутиков, вначале становились неподвижными, затем, склеиваясь, образовывали характерные хлопья. Иммуногенность, агглютинабельность и агглютининсвязывающая способность Н-антигенов после формалинизации оставались неизменными, но исчезали после температурного воздействия 75° С и выше и обработки соляной кислотой. Эталон прекращал агглютинабельность и понижал иммуногенность и агглютининсвязывающую способность. Автор пришел к заключению, что термолабильные антигены ***Ps. aeruginosa*** связаны со жгутиками.

Серологическое типирование показало, что из выделенного 541 штамма ***Ps. aeruginosa*** 228 могли быть характеризованы серологически униформным антигеном Н1 и 246 — комплексным антигеном Н2. Последние культуры были подразделены на частичные антигены 2a, 2b, 2c, 2d, 2e и 2f. Один штамм оказался неподвижным, и 6 штаммов содержали неопределяемые Н-антигены.

На основе различных комбинаций О-антигенов, описанных в предыдущей работе, и Н-антигенов для практических целей была создана антигенная схема ***Ps. aeruginosa***, содержащая 53 серотипа.

ISOLATION AND CHARACTERIZATION OF SOME NEWLY ISOLATED B. CEREUS PHAGES

J. FÖLDES, V. GAÁL, J. MOLNÁR

ВЫДЕЛЕНИЕ И ХАРАКТЕРИСТИКА НЕКОТОРЫХ ВЫДЕЛЕННЫХ ФАГОВ B. CEREUS

Й. ФЁЛДЕШ, В. ГААЛ, Й. МОЛНАР

Излагаются выделение и свойства фагов, выделенных из двух групп ***B. cereus***, отличающихся по структуре клеточной стенки. Выделенные фаги образовывали чистые мишень-подобные и мутные бляжки. Мутный (дикий) тип в 5—10% содержал чистые и мишень-подобные бляшковые мутанты. Изучение спектра чувствительного хозяина показало, что часть выделенных фагов оказалась типоспецифической, а остальная часть обладала групповой специфичностью. Некоторые фаги имели поливалентную природу, будучи специфичными как в отношении ***B. anthracis***, так и в отношении ***B. cereus***. Была отмечена редкая антигенная разница между фагами двух групп ***B. cereus***. Выделенные фаги ***B. cereus*** были изучены также с точки зрения burst size, латентного периода и чувствительности к температуре, pH и ультрафиолетовому облучению.

THE EFFECT OF BORDETELLA PERTUSSIS VACCINE AND ADRENAL HORMONES ON 5-HYDROXYTRYPTAMINE LEVEL IN RAT TISSUES

B. CSABA, L. MUSZBEK

ЭФФЕКТ ВАКЦИНЫ BORDETELLA PERTUSSIS И АДРЕНАЛОВЫХ ГОРМОНОВ НА УРОВЕНЬ 5-ГИДРОКСИТРИПТАМИНА В ТКАНЯХ КРЫСЫ

Б. ЧАБА, Л. МУСБЕК

Авторы изучали эффект вакцины ***Bordetella pertussis*** (БПВ), адреналэктомии и лечения кортизоном на уровень 5-гидрокситриптамина (5—ГТ) в тканях крысы. Введение БПВ обусловливало повышение уровня 5—ГТ в пилорической области желудка и 12-перстной кишке и повышение содержания 5-гидроксииндолуксусной кислоты в моче. После адреналэктомии уровень 5—ГТ повышался во всех тканях за исключением крови и легких. Лечение кортизоном понижало содержание 5—ГТ во всех тканях кроме пилорической области желудка и 12-перстной кишки. В пилорической области желудка кортизон повышал количество 5—ГТ. Предполагается, что БПВ влияет на уровень 5—ГТ в тканях непрямым путем, посредством адреналовых гормонов.

IMMUNOFLUORESCENT STUDIES ON THE REACTIVITY OF „EARLY" AND „LATE" HERPES-IMMUNE RABBIT SERA WITH VIRUS-INDUCED ANTIGENIC FORMATIONS OF HEp-2 CELLS

L. GÉDER, L. VÁCZI, F. LEHEL, É. GÖNCZÖL, E. JENEY

ИММУНОФЛУОРЕСЦЕНТНОЕ ИССЛЕДОВАНИЕ РЕАКТИВНОСТИ «РАННЕЙ» И «ПОЗДНЕЙ» ГЕРПЕС-ИММУННОЙ КРОЛИЧЬЕЙ СЫВОРОТКИ С ВИРУС-ИНДУЦИРОВАННЫМИ АНТИГЕННЫМИ ФОРМАЦИЯМИ КЛЕТОК НЕр-2

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Была изучена по ходу иммунизации реактивность сыворотки кроликов, зараженных вирусом герпеса, с антигенными формациями клеток НЕр—2, инфицированных вирусом **Herpes simplex.** Пробы сыворотки, взятые через неделю от начала иммунизации, окрашивали диффузные цитоплазматические, околоядерные и некоторые внутриядерные мелкозернистые элементы в клетках НЕр—2, зараженных вирусом **Herpes simplex.** Иммунная сыворотка, взятая 2 неделями позже, в дополнение к диффузной цитоплазматической и околоядерной флуоресценции выявила только большие включение-подобные тельца и несколько больших гранул в ядрышках тех же самых препаратов. В то же самое время было отмечено значительное повышение титра вируснейтрализующих антител.

«Ранняя» и «поздняя» иммунная сыворотка реагировала с околоядерными антигенными формациями клеток, обработанных цитозинарабинозидом.

«Ранние» антитела соответствовали **IgM** иммуноглобулинам.

THE EFFECT OF TEMPERATURE ON THE DEVELOPMENT OF IMMUNOFLUORESCENT ELEMENTS IN HERPES SIMPLEX VIRUS INFECTED BS-C-1 CELLS

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ЭФФЕКТ ТЕМПЕРАТУРЫ НА РАЗВИТИЕ ИММУНОФЛУОРЕСЦЕНТНЫХ ЭЛЕМЕНТОВ В КЛЕТКАХ BS-C-1, ИНФИЦИРОВАННЫХ ВИРУСОМ HERPES SIMPLEX

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Инкубация клеток BS—C—I, инокулированных вирусом **Herpes simplex**, при температуре 40° С не влияла на развитие «ранних» антигенных формаций, но задерживала появление больших внутриклеточных включение-подобных телец, которые являются

поздними продуктами инфекции. Вырабатывались как «ранние», так и «поздние» вирусспецифические комплементсвязывающие антигены, причём первые в большем количестве. Титр внутриклеточного инфективного вируса был заметно редуцирован.

Инкубация при комнатной температуре обуславливала латентную вирусную инфекцию клеток с аккумуляцией «ранних» антигенных формаций, полностью блокирующих синтез «поздних» антигенных элементов и инфективного вируса.

COMPARATIVE STUDY ON SENSITIVITY TO TOXOPLASMA GONDII OF HUMAN PRIMARY AMNIOTIC CELL CULTURE AND OF MICE

R. CSÓKA, G. KULCSÁR

СРАВНИТЕЛЬНОЕ ИЗУЧЕНИЕ ЧУВСТВИТЕЛЬНОСТИ МЫШЕЙ И ПЕРВИЧНОЙ КЛЕТОЧНОЙ КУЛЬТУРЫ АМНИОНА ЧЕЛОВЕКА К TOXOPLASMA GONDII

Р. ЧОКА, Г. КУЛЧАР

Авторы изучали чувствительность белых (швейцарских) мышей и первичной клеточной культуры амниона человека к штамму RH *Toxoplasma gondii*. При количественной и качественной оценке чувствительность к культивированным клеткам оказалась одинаковой или выше таковой в отношении мышей.

Данные подтверждают то наблюдение, что количество переживающих токсоплазм значительно понижалось после 24 часов хранения.

INDUCTION OF MEMBRANE-BOUND PENICILLINASE SYNTHESIS IN B. CEREUS 569

V. CSÁNYI

ИНДУКЦИЯ СИНТЕЗА ПЕНИЦИЛЛИНАЗЫ, СВЯЗАННОЙ С ОБОЛОЧКОЙ, У B. CEREUS 569

В. ЧАНЬИ

Была изучена индукция пенициллиназы, связанной с оболочкой, и экзопенициллиназы с применением в качестве индукторов пенициллина и высоких концентраций фосфата. Как пенициллин, так и фосфат индуцировали синтез пенициллиназы, связанной с оболочкой, но пропорция связанного энзима в общей продукции энзима оказалась значительно ниже в присутствии высоких концентраций электролита. Обсуждается возможное происхождение энзима, связанного с оболочкой.

THE EFFECT OF LYSOZYME AND METHICILLIN ON THE GROWTH OF METHICILLIN RESISTANT AND SENSITIVE STAPHYLOCOCCUS AUREUS STRAINS

F. ROZGONYI, I. RÉDAI

ЭФФЕКТ ЛИЗОЗИМА И МЕТИЦИЛЛИНА НА РОСТ МЕТИЦИЛЛИН-УСТОЙЧИВЫХ И ЧУВСТВИТЕЛЬНЫХ ШТАММОВ STAPHYLOCOCCUS AUREUS

Ф. РОЗГОНЬИ, И. РЕДАИ

В присутствии лизозима устойчивость к метициллину у естественно метициллин-устойчивых стафилококков слегка повышалась. В тех же самых условиях штаммы, чувствительные к антибиотику или располагающие адаптированной устойчивостью к метициллину, проявляли повышенную чувствительность.

В присутствии метициллина + лизозима lag-фаза естественно устойчивых стафилококковых культур понижалась примерно наполовину, тогда как чувствительные или располагающие адаптированной устойчивостью штаммы в 4—7 раз повышали свои значения по сравнению с таковыми в присутствии только одного метициллина. Когда добавлялись сублетальные концентрации метициллина + лизозима в логарифмической фазе, число переживающих бактерий в случае естественно устойчивых культур понижалась на 1 значение и на 5—6 — в отношении чувствительных или располагающих адаптированной устойчивостью штаммов.

PROPERTIES OF ACETOHYDROXY ACID SYNTHETASE IN STREPTOMYCES RIMOSUS

A SZENTIRMAI, I. HORVÁTH, J. ZSADÁNYI

СВОЙСТВА УКСУСНОГИДРОКСИКИСЛОЙ СИНТЕТАЗЫ В STREPTOMYCES RIMOSUS

А. СЕНТИРМАИ, И. ХОРВАТ, Й. ЖАДАНЬИ

Уксусногидроксикислая синтетаза в ***Streptomyces rimosus*** требует присутствия тиамин-пирофосфата и флавинаденин динуклеотида. Тот факт, что хотя валин является ингибитором при pH 7,4 и 28° C, он всё-таки оказывает значительный активирующий эффект при высоких значениях pH и температуры, может быть объяснен его заметным стабилизирующим действием на энзим.

Были изучены пропорция α-уксусномолочной кислоты и синтез α-уксусно-α-гидроксибутириновой кислоты путём определения действия концентраций соответствующих субстратов (пирувиновой и α-кетобутириновой кислот). По сравнению с энзимом в других микроорганизмах, родство α-кетобутириновой кислоты с уксусногидроксикислой синтетазой ***S. rimosus*** было более тесным и поэтому синтез прекурзора изолейцина в последней был выше. Это явление может быть объяснено депрессивным действием α-кетобутириновой кислоты.

SUCROSE AND RAFFINOSE BREAKDOWN BY ESCHERICHIA COLI

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РАЗЛОЖЕНИЕ САХАРОЗЫ И РАФФИНОЗЫ ESCHERICHIA COLI

М. АРР, Т. ПЕРЕНЬИ, Е. К. НОВАК

Проверка ассимиляции сахарозы и раффинозы 56 лабораторными и свежевыделенными штаммами ***E. coli*** выявила, что большинство культур или вовсе не утилизирует этих сахаров, или утилизирует оба из них. Культуры, ассимилирующие только сахарозу, составляли 1/5, только раффинозу — 1/20 всех исследованных штаммов. Опыты с одним представителем каждой из четырех групп показали, что за разложение как раффинозы, так и сахарозы ответственен адаптивный энзим («бактериальная инвертаза»). Энзим мог быть индуцирован обоими сахарами. Естественно сахарозо-отрицательный штамм в соответствующей среде мог быть адаптирован к низким степеням разлагающей активности сахарозы. Этот сахарозо-отрицательный и раффинозо-положительный штамм действовал на раффинозу в основном мелибиазой. Штамм, ассимилирующий сахарозу, после адаптации к сахарозе в выборочном опыте вырабатывал только α-глюкозидазу. Штаммы, неутилизирующие сахарозу и раффинозу, могли быть также индуцированы для выработки соответствующих энзимов, обладающих, однако, низкой активностью.

В отличие от других микроорганизмов инвертаза и мелибиаза располагаются внутриклеточно в бактерии. Подобно разложению лактозы, что является основным диагностическим признаком ***Escherichia***, могло быть индуцировано разложение сахарозы и раффинозы, в противоположность разложению конструктивной трехалозы и мальтозы.

ACTION OF CATION TRANSFER ATP-ASE INHIBITORS ON EFFICIENCY OF INFECTION WITH POLIOVIRUS

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ДЕЙСТВИЕ ИНГИБИТОРОВ КАТИОН-TRANSFER АТФ-азы НА ЭФФЕКТИВНОСТЬ ИНФЕКЦИИ ВИРУСОМ ПОЛИОМИЕЛИТА

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В системе, состоящей из полиовируса, адсорбированного на взвешенных клетках почки обезьяны, редукция содержания ионов K^+ до или ниже 0,91 mEq/1, по крайней мере, в течение часа вызывала 4-кратное повышение эффективности инфекции, измеряемой выходом вируса в конце цикла. Эффект прекращался, если концентрация K^+ возвращалась к норме в конце первых 30 минут цикла. Отсутствие ионов Mg^{++} в течение первого часа цикла вызывало 70% редукцию конечного выхода. Принимая во внимание известный эффект ионов K^+ и Mg^{++} на мембранную АТФ-азу, полученные результаты являются дальнейшим непрямым доказательством возможной взаимосвязи между конформацией АТФ-азы и степенью необратимой адсорбции вириона.

AUXOTROPHIC MUTATION ASSOCIATED WITH LOW STREPTOMYCIN RESISTANCE AND SLOW GROWTH IN BACILLUS SUBTILIS

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АУКСОТРОФНАЯ МУТАЦИЯ BACILLUS SUBTILIS, СВЯЗАННАЯ С НИЗКОЙ РЕЗИСТЕНТНОСТЬЮ К СТРЕПТОМИЦИНУ И МЕДЛЕННЫМ РОСТОМ

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Чашки с питательным агаром, содержащим 100—200 μг стрептомицина /мл, были массивно заражены ***Bacillus subtilis*** (штамм 168 ind$^-$). Через несколько дней после инкубации при 37° С выросли колонии небольших и средних размеров в пропорции 10^{-6}. Приблизительно 1/4 часть изолятов при переносе на кровяной агар давала точечные колонии. Изоляты, производящие колонии нормальных размеров, на кровяном агаре проявляли некоторые новые требования в питании, позволяющие классифицировать мутанты в 5 групп. Большинство мутантов хорошо размножалось на комплетной среде, такой как дрожжевой пептоновый агар, но не росло в определенных условиях питания. Приблизительно 1/10 мутантов показывала недостатки в синтезе тетрапорфирина (протогем-а). Было также найдено несколько изолятов, зависимых от ароматических аминокислот. Хотя изоляты были получены в высоких стрептомициновых концентрациях, они показывали низкую толеранцию к этому антибиотику.

Другой чертой мутантов был медленный рост. Ауксотрофия, медленный рост и относительно повышенная стрептомициновая толерантность, очевидно, являлись результатом мутации в единственном локусе, тогда как реверсия ауксотрофии в прототрофию восстановила их чувствительность и нормальный рост. Можно сделать вывод, что прямое действие стрептомицина на хромосомальную репликацию могло быть включено в генез этих своеобразных мутантов.

FAST SEDIMENTING FRACTIONS CONTAINING DNA FROM STREPTOMYCES GRISEUS

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БЫСТРОЕ ОСАЖДЕНИЕ ФРАКЦИЙ, СОДЕРЖАЩИХ ДНК, ПОЛУЧЕННОЙ ИЗ STREPTOMYCES GRISEUS

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Авторы изучали в различных условиях осаждаемость ДНК, полученной из мицелия **Streptomyces griseus**, штамм № 52—I, после дезинтеграции. Значительное число быстрооседающих фракций было получено после переваривания лизозимом в среде, содержащей

полиэтиленгликоль 300, при 6000 g и после механического размельчения в среде ТМК при 25 000—38 000 g (39 и 30% соответственно).

Число быстрооседающих фракций зависело от выбранного штамма *Streptomyces*, возраста культуры и от состава среды, в которой проводились отмывка, размельчение и осаждение.

В тех же условиях, которые оказались оптимальными для *S. griseus*, в отношении *Escherichia coli* и *Bacillus cereus* быстрооседающих фракций получить не удалось.

Очистка фракций, содержащих ДНК, осуществлялась центрифугированием в градиенте плотности, затем был проведен анализ этих фракций на содержание ДНК, РНК, протеина и полисахаридов. Отношение протеина к ДНК в некоторых фракциях, полученных центрифугированием в градиенте плотности, оказалось ниже чем 1:0.

SEROLOGICAL AND CHEMICAL STUDIES OF SH. SONNEI, PSEUDOMONAS SHIGELLOIDES AND C27 STRAINS

K. RAUSS, T. KONTROHR, A. VERTÉNYI, L. SZENDREI

СЕРОЛОГИЧЕСКОЕ И ХИМИЧЕСКОЕ ИЗУЧЕНИЕ ШТАММОВ SH. SONNEI, PSEUDOMONAS SHIGELLOIDES И С 27

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Биохимическое поведение штаммов *Ps. shigelloides* Bader-a и C 27 Ferguson-a было характерно роду *Aeromonas*.

Серологически формы S и мутанты R обоих штаммов были одинаковыми друг с другом, а также с I и II фазой *Sh. sonnei*.

Идентичность в антигенной структуре *Sh. sonnei* и *Ps. shigelloides* была также продемонстрирована опытом защиты на мышах.

LPS, полученный из обоих *Aeromonades*, содержал составные сахара, качественно идентичные с *LPS Sh. sonnei* в отношении S или R формы, но *Aeromonades*, кроме того, располагали серологически инактивным компонентом галактуроновой кислоты, а LPS штамма C27 содержал небольшое количество галактозамина.

Sh. sonnei и исследованные два штамма *Aeromonas* представляли собой различные хемотипы, но даже штаммы *Ps. shigelloides* и C27 не принадлежали к тому же хемотипу.

ISOLATION OF HFR DERIVATIVE BY THE USE OF SHIGELLA FLEXNERI 4b-MODIFIED F FACTOR

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ВЫДЕЛЕНИЕ HFR-ПРОИЗВОДНОГО ПУТЕМ ИСПОЛЬЗОВАНИЯ 4b-МОДИФИЦИРОВАННОГО ФАКТОРА F SHIGELLA FLEXNERI

И. КЕТЬИ

4b-модифицированный фактор F *Sh. flexneri* был получен передачей **hsp gene Shigella flexneri** на F^+ *Escherichia coli K-12*. Передача этого фактора другому мутанту **Shigella**-хозяина сопровождалась получением F^+-культуры, обеспечивающей выход устойчивого **Hfr**-производного.

Место F-интеграции 4b Hfr-производного **Sh. flexneri** находится между маркерами **Mtl** и **Ilv** — при проектировании на хромосомную карту *E. coli* K—12 — и появляется приблизительно на 72 минуте. Порядок передачи следовал в направлении часовой стрелки: **O—ilv—met—thr—trp.**

IMMUNOLOGICAL AND BIOCHEMICAL ACTIVITY OF ANTI-RIBOSOME IMMUNE SERUM

K. MERÉTEY, J. HOLLAND, V. VÁRTERÉSZ

ИММУНОЛОГИЧЕСКАЯ И БИОХИМИЧЕСКАЯ АКТИВНОСТЬ АНТИРИБОСОМНОЙ ИММУННОЙ СЫВОРОТКИ

К. МЕРЕТЕИ, Й. ХОЛЛАНД, В. ВАРТЕРЕС

Производство иммунной сыворотки против обработанной $MgCl_2$ рибосомной фракции печени морской свинки проводилось в кроликах и крысах. Присутствие антирибосомных антител выявлялось преципитацией в жидкости и геле, а также рибосома-латекс-агглютинацией. Загрязнение контролировалось иммунодиффузным исследованием иммунной сыворотки, полученной против рибосомных и печеночных гомогенатов. Нормальная сыворотка также имела некоторую преципитирующую активность. Преципитины содержались во фракции IgM. Выделенная антирибосомная IgG-фракция в условиях ***in vitro*** подавляла функциональную активность рибосом инкорпорировать аминокислоты.

GENETIC MAPPING OF RHIZOBIOPHAGE 16—3

L. OROSZ, T. SIK

СОЗДАНИЕ ГЕНЕТИЧЕСКОЙ КАРТЫ РИЗОБИОФАГА 16—3

Л. ОРОС, Т. ШИК

Частичная генетическая карта фага 16—3 ***Rhizobium*** была создана путем комплементации и перекрещиванием двух или трех точек. Были выделены и использованы термочувствительные (**ts**), термо-индуцируемые (**ti**), отличающиеся по морфологии бляшек (№ 2; С), спектру хозяина (**h**) и измененным фаговым антигенам (**Ant**) мутанты. Маппированные гены располагались в следующем порядке: № **2-C-ts2-ts6-ts4-h-ts5-Ant.**

ANAEROBIC TRANSFORMATION OF STEROIDS BY MYCOBACTERIUM PHLEI

K. ALBRECHT, E. TÖMÖRKÉNY, A. SZABÓ

ТРАНСФОРМАЦИЯ СТЕРОИДОВ С АНДРОСТАНОВОЙ СТРУКТУРОЙ ПОСРЕДСТВОМ MYCOBACTERIUM PHLEI В АНАЭРОБНЫХ УСЛОВИЯХ

К. АЛБРЕХТ, Э. ТЁМЁРКЕНЬ, А. САБО

В анаэробных условиях ***Micobacterium phlei*** превращала андрост-4-ен-3,17-дион, 17β-гидрокси андрост-4-ен-3-он и его 17β-ацилаты, 3β-гидроксиандрост-5-ен-17-он и 17α-метил-17β-гидроксиандрост-4-ен-3-он в насыщенные химические соединения 5α и 5β. По ходу дальнейшей трансформации за счёт редукции 3- и в некоторых случаях 17-оксогрупп возникают насыщенные гидрокси-производные. В анаэробных условиях также можно было наблюдать образование стероидэстерных формаций.

TRANSFORMATION OF 4,5-EPOXY STEROIDS WITH MYCOBACTERIUM PHLEI II. TRANSFORMATION UNDER ANAEROBIC CONDITIONS

E. TÖMÖRKÉNY, K. ALBRECHT, L. ILA

ТРАНСФОРМАЦИЯ 4,5-ЭПОКСИСТЕРОИДОВ ПОСРЕДСТВОМ MYCOBACTERIUM PHLEI II. ТРАНСФОРМАЦИЯ В АНАЭРОБНЫХ УСЛОВИЯХ

Э. ТЁМЁРКЕНЬ, К. АЛБРЕХТ, Л. ИЛА

В анаэробных условиях 3-оксо (3-гидрокси)-4,5-эпоксистероиды с андростановой структурой могут быть превращены в насыщенные производные 5α—H и 5β—H через δ4—3-оксо-интермедиат. Устранение эпоксидного кольца, повидимому, происходит вначале через производное 3-гидрокси-4,5-эпокси, затем через производное 3,5-дигидрокси.

APPLICATION OF GEL ADSORPTION TO CHARACTERIZE STRAINS OF THE FAMILY ENTEROBACTERIACEAE

E. SZÖLLŐSY

ПРИМЕНЕНИЕ ГЕЛЕВОЙ АДСОРБЦИИ ДЛЯ ХАРАКТЕРИСТИКИ ШТАММОВ СЕМЕЙСТВА ENTEROBACTERIACEAE

Э. СЁЛЛЁШИ

Было найдено, что клеточные суспензии штаммов, принадлежащие к семейству **Enterobacteriaceae**, связывались с поверхностью $Al(OH)_3$-геля с различной интенсивностью. Так как адсорбция задерживалась фосфатными ионами, была сделана попытка характеризовать степень адсорбции молярностью фосфатного буфера. Принимая за штаммовую характеристику нумерическое значение молярности такого буфера, в котором 50% клеток связывались с поверхностью геля (AC_{50}), можно было отличить друг от друга бактериальные штаммы, относящиеся к идентичным серологическим и фаговым типам.

На основе значений AC_{50}, 25 штаммов 0-типа **E. coli** показали следующее распределение. Три штамма проявляли очень слабое адсорбционное свойство даже в отсутствии фасфатных ионов. Семь штаммов проявляли AC_{50} в фосфатном буфере с M, равной 0,0035, тогда как четыре штамма — в интервале 0,0025 — 0,005, семь других — 0,005 — 0,01, три — 0,01 — 0,02 и один — 0,04 — 0,08. Штаммы, выделенные от больных, давали распределение, сходное с таковым штаммов 0-типа.

Большинство клеток патогенных штаммов **E. coli** проявляло очень слабую степень адсорбции даже в отсутствии фосфатных ионов. Заслуживает внимания разница значений AC_{50}, обнаруженная при исследовании штаммов **Shigella flexneri**. Штаммы **Shigella flexneri** и **sonnei**, показывающие идентичный серологический и фаговый тип, могли быть отдифференцированы на основе их различных значений AC_{50}. Салмонеллёзные штаммы, за исключением **S. typhi**, были охарактеризованы растянутой кривой адсорбции. Значения AC_{50} для двух штаммов **S. typhi murium** оказались различными.

Клетки патогенной **E. coli dispepsiae**, располагающие слабой степенью адсорбции, могли быть обратно получены из суспензии, приготовленной из бактерий нормальной **E. coli**, сильнее адсорбирующейся на геле, несмотря на большее количество последних.

Обсуждается связь между антигенным и адсорбционным свойствами.

EFFECT OF FACTOR C ON GLUCOSE REPRESSION OF INDUCED β-GALACTOSIDASE SYNTHESIS

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ВЛИЯНИЕ ФАКТОРА С НА РЕПРЕССИЮ ГЛЮКОЗЫ В СИНТЕЗЕ ИНДУЦИРОВАННОЙ β-ГАЛАКТОЗИДАЗЫ

Й. ШЛАММАДИНГЕР, Г. САБО

Фактор С, агент, содержащий макромолекулы гликопротеидного типа и регулирующий цитодифференциацию у ***Streptomyces griseus***, повышает количество 5%-холодо-ТСА-нерастворимой ^{14}C-урациловой фракции, инкорпорированной в присутствии актиномицина Д как в ***Str. griseus***, так и ***Escherichia coli***, и поднимает Tm-точку ДНК.

Изучение глюкозной репрессии индуцированного синтеза β-галактозидазы у ***E. coli*** показало, что при разных концентрациях глюкозы фактор С понижал глюкозо-индуцированную проходящую и катаболитную репрессию.

В свете полученных данных относительно механизма глюкозо-репрессии можно предположить, что фактор С первоначально действует на ДНК.

ELECTRON MICROSCOPIC STUDIES IN EXPERIMENTAL KERATOCONJUNCTIVITIS LISTERIOSA

I. PENETRATION OF LISTERIA MONOCYTOGENES INTO CORNEAL EPITHELIAL CELLS

P. RÁCZ, K. TENNER, K. SZIVESSY

ИЗУЧЕНИЕ В ЭЛЕКТРОННОМ МИКРОСКОПЕ ЭКСПЕРИМЕНТАЛЬНОГО ЛИСТЕРИОЗНОГО КЕРАТОКОНЪЮНКТИВИТА

I. ПРОНИКНОВЕНИЕ LISTERIA MONOCYTOGENES В ЭПИТЕЛИАЛЬНЫЕ КЛЕТКИ РОГОВИЦЫ

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В условиях экспериментального листериозного кератоконъюнктивита авторы изучали на морских свинках электронно-микроскопическим методом проникновение возбудителя в эпителиальные клетки роговицы. Чаще всего в одну эпителиальную клетку проникали 1—2 живых бактерии. Клеточная мембрана перед приближающейся листерией прогибалась. По мере продвижения бактерии внутрь клетки это вдавление превращалось в вакуолю, ограниченную мембраной, с заключённой в ней бактерией. В дальнейшем ограничивающая мембрана распадалась, и листерия оставалась окруженной своеобразным зернистым материалом. Когда таким образом освобожденная листерия вновь приближалась к клеточной мембране, на последней образовывался выступ, судьба которого зависела от того, возникнет ли в межклеточном пространстве по ходу воспаления значительный отёк или нет. В случае отсутствия межклеточного отёка выступающая часть клетки, содержащая бактерию, внедряется в соседнюю клетку, затем окончательно захватывается ею. При значительном межклеточном отёке выступающая часть клетки с заключенной в ней бактерией может отпочковаться в межклеточное пространство.

В более поздние стадии инфекционного процесса, спустя 36—48 часов, увеличивается число клеточных органелл в клетках.

Авторы считают, что понятие факультативного внутриклеточного паразитизма при листериозном кератоконъюнктивите, которое применяется в отношении взаимодействия хозяина и паразита только в случае клеток типа макрофагов, должно быть распространено и на эпителиальные клетки роговицы.

EFFECT OF SORBIC ACID ON THE GROWTH OF SOME SPECIES OF YEAST

T. DEÁK, M. TÜSKE, E. K. NOVÁK

ЭФФЕКТ СОРБИНОВОЙ КИСЛОТЫ НА РАЗМНОЖЕНИЕ РАЗЛИЧНЫХ ВИДОВ ДРОЖЖЕЙ

Т. ДЕАК, М. ТЮШКЕ, Е. К. НОВАК

Авторы изучали эффект сорбиновой кислоты на параметры кинетики роста в аэробных и анаэробных культурах *Candida beverwijkii*, *C. claussenii*, *C. krusei*, *C. pseudotropicalis*, *C. utilis*, *Procandida albicans*, *Pc. tropicalis* и *Saccharomyces carlsbergensis*. Сорбиновая кислота оказывала различный эффект на постоянную скорости размножения, lag-фазу и конечную клеточную плотность; её действие также менялось в зависимости от примененной концентрации, способа инкубации, присутствия редуцированного глютатиона и от исследуемого вида дрожжей. В первую очередь сорбиновая кислота продляла lag-фазу, тогда как степень подавления скорости роста и конечной клеточной плотности была примерно одинаковой. Действие сорбиновой кислоты в анаэробных условиях проявлялось сильнее. Это частью было вызвано тем, что в аэробных культурах отдельных видов концентрация сорбиновой кислоты понизилась, и подавляющий размножение

эффект, таким образом, проявился в меньшей степени. В присутствии редуцированного глютатиона понижение концентрации сорбиновой кислоты или вовсе не происходило, или происходило, но в незначительной степени; этим самым редуцированный глютатион косвенно повышал подавляющий размножение эффект сорбиновой кислоты.

EFFECT OF SORBIC ACID ON THE GROWTH OF YEASTS ON VARIOUS CARBOHYDRATES

T. DEÁK, E. K. NOVÁK

ЭФФЕКТ СОРБИНОВОЙ КИСЛОТЫ НА РОСТ ДРОЖЖЕЙ НА РАЗЛИЧНЫХ УГЛЕВОДАХ

Т. ДЕАК, Е. К. НОВАК

Авторы изучали рост ***Candida beverwijkii***, ***C. claussenii***, ***C. utilis***, ***Procandida albicans***, ***Pr. tropicalis***, ***Saccharomyces carlsbergensis*** и ***S. cerevisiae*** в аэробной жидкой среде, содержащей глюкозу, галактозу, мальтозу, сахарозу и раффинозу, в присутствии 0,75 мг/мл сорбата калия. Исходя из эффекта ингибитора на изучаемые параметры кинетики роста (постоянная скорости роста, lag-фаза, показатель размножения), на основе сравнительного изучения добавленных отдельных субстратов, был сделан вывод, что причина подавления находится на уровне углеводного обмена веществ. Результаты показали, что причину подавления можно искать довольно часто в процессах переноса, которые являются начальными стадиями углеводного обмена веществ. Влияние на активные процессы переноса может играть важную роль в механизме действия сорбиновой кислоты.

STUDIES ON SWINE ENTEROVIRUSES III. OCCURRENCE OF ENTEROVIRUSES IN HUNGARIAN SWINE HERDS AND IN KIDNEY TISSUE OF NORMAL PIGS

T. SZENT-IVÁNYI

ИЗУЧЕНИЕ ЭНТЕРОВИРУСОВ СВИНЕЙ III. ВСТРЕЧАЕМОСТЬ ЭНТЕРОВИРУСОВ СРЕДИ СВИНЕЙ В ВЕНГРИИ И В КУЛЬТУРАХ ПОЧЕК ЗДОРОВЫХ СВИНЕЙ

Т. СЕНТ-ИВАНЬИ

Автор выделил 505 энтеровирусных штаммов из 1585 фекальных проб, взятых от свиней различного возраста. До недельного возраста выделение энтеровирусов обнаружить не удалось, но в дальнейшем пропорция вирусовыделителей постепенно повысилась в среднем до 75,3%, после того как поросята перестали быть сосунками. Затем пропорция понизилась, и выделение вируса среди животных старше одного года было спорадическим. Распространение инфекции среди помётов в больших свинарниках было связано с условиями изоляции помётов от одной матки.

За исключением 16 изолятов, выделенные штаммы принадлежали к одному из 16 серотипов и 2 цитоморфологическим типам. Повторные исследования фекальных проб от отдельных свиней показали, что некоторые энтеровирусные серотипы выделялись один за другим и реже одновременно.

В условиях изолированного содержания свиней в отдельных клетках энтеровирусная инфекция передавалась вертикально от матки новому поколению. Горизонтальное распространение инфекции наблюдалось среди поросят особенно после того, как они переставали быть сосунками, и их помещали в большие группы.

SPECIFIC ORAL PREVENTION OF INFANTILE GASTRO-ENTERITIS E. EXPERIMENTS IN MICE

K. RAUSS, I. KÉTYI, L. SZENDREI, A. VERTÉNYI

СПЕЦИФИЧЕСКАЯ ОРАЛЬНАЯ ПРОФИЛАКТИКА ГАСТРОЭНТЕРИТА НОВОРОЖДЕННЫХ

I. ЭКСПЕРИМЕНТЫ НА МЫШАХ

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В эксперименте на мышах с целью разработки профилактики гастроэнтерита новорожденных было проведено изучение возможности индукции иммунитета против **E. coli dyspepsiae.** Опыты были выполнены с лиофилизированным антигеном **Boivin**, приготовленным из штамма **O111 : K58(B4).** Иммунизация проводилась ***per os*** повышающимися дозами ежедневно или с 3-дневными интервалами.

Иммунный ответ определялся прямым изучением активной защиты и на основе защитного эффекта сыворотки и интестинальных экстрактов иммунизированных животных. Активно проявленная защитность и защитный эффект интестинальных экстрактов (копро-антитела) были изучены на мышах с помощью муциновой техники. Защитное свойство сыворотки определялось на 10-дневных куриных эмбрионах.

Иммунный ответ был продемонстрирован во всех трех изученных группах. Авторам удалось установить взаимосвязь между иммунизирующей дозой, способом и эффектом иммунизации.

Иммунитет, судя по активной и пассивной защите, значительно понизился спустя 31 день после завершения полной вакцинации. Степень иммунитета можно было поддержать или повысить введением небольших доз антигена с 5-дневными интервалами.

В интестинальном экстракте были обнаружены защитные копро-антитела. Эти антитела также исчезали в пределах 31 дня, тогда как их эффект мог быть поддержан или усилен небольшими дозами антигена.

Копро-антитела являются свидетельством развития местного иммунитета и оправдывают изучение иммуногенности вакцины у новорожденных.

Обсуждаются проблемы живой и убитой вакцин.

PROPHYLACTIC USE OF AMANTADINE DURING HONG KONG INFLUENZA EPIDEMIC

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ИЗУЧЕНИЕ ПРОФИЛАКТИЧЕСКОГО ПРИМЕНЕНИЯ АМАНТАДИНА ВО ВРЕМЯ ЭПИДЕМИИ ГРИППА ГОНГ-КОНГ

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В марте 1969 года, когда в Венгрии широко распространилась эпидемия гриппа Гонг-Конг, было проведено применение 1-амантадин-HCl **(Viregyt R°)** и **placebo** в семи военных подразделениях, всего у 2440 и 2133 молодых солдат (соответственно). Лечение было начато сразу, как только накапливающиеся случаи гриппоподобных заболеваний были распознаны как эпидемия, и продолжалось до спада эпидемии. Ежедневная доза представляла собой 200 мг, содержащихся в 2 капсулах.

Средство не оказывало влияния на заболеваемость, но понижало степень ($P < 0{,}01$) и продолжительность ($P < 0{,}001$) лихорадки, сокращало число койкодней ($P < 0{,}001$) и случаи осложнений вообще ($P < 0{,}05$) и особенно случаи, осложняющиеся заболеванием нижних дыхательных путей ($P < 0{,}01$).

Вирусологические и серологические результаты показали, что в 4 подразделениях (возможно и в 5-ом тоже) эпидемия была вызвана таким штаммом, который практически идентичен A_2**(Hong Kong)** 68. В двух подразделениях эпидемии способствовал нетипирующийся агент, отличный от вируса гриппа А и гриппа Б. Только в последних подразделениях был достигнут значительный благоприятный эффект. Средство не понижало пропорцию сероконверсии, тогда как титр антител в РПГА у лиц, первоначально сероотри-

цательных, после эпидемии оказался несколько ниже в группе, в которой применялся амантадин, по сравнению с группой, в которой использовали **placebo.** Разница не достигала предела достоверности (0,05). В опытах ***in vitro*** штаммы **Hong Kong**(1)68 оказались менее чувствительными к амантадину, чем более ранний штамм вируса гриппа A_2.

STUDIES ON THE PERITONEAL EXUDATE OF ANIMALS EXPERIMENTALLY INFECTED WITH TOXOPLASMA GONDII

III. COMPARATIVE STUDY ON MOUSE PERITONEAL EXUDATE INDUCED WITH COMPLETE FREUND ADJUVANT

M. A. SAMIR

ИССЛЕДОВАНИЕ ПЕРИТОНЕАЛЬНОГО ЭКССУДАТА ЖИВОТНЫХ, ЭКСПЕРИМЕНТАЛЬНО ЗАРАЖЕННЫХ TOXOPLASMA GONDII

III. СРАВНИТЕЛЬНОЕ ИЗУЧЕНИЕ НА МЫШАХ ПЕРИТОНЕАЛЬНОГО ЭКССУДАТА, ИНДУЦИРОВАННОГО КОМПЛЕТНЫМ АДЪЮВАНТОМ ФРОЙНДА

М. АХМЕД САМИР

Посредством комплетного адъюванта Фройнда в нескольких мышах была индуцирована перитонеальная жидкость. Внутривенное введение супернатанта этой жидкости после прогревания при 56° С или 65° С в течение 30 минут вызывало развитие синдрома, сопровождающего внутривенное введение СПЭТ (супернатант перитонеального экссудата токсоплазмозных мышей). Летальная доза варьировала между 0,006 и 0,012 мл на 1 грамм веса тела. Внутрибрюшинное или подкожное введение не вызывало очевидного заболевания. Активность прогретого супернатанта могла быть снята гиалуронидазой и редуцирована трипсином. Мыши переносили (толеранция) внутривенное введение 0,05 мл непрогретой или прогретой мышиной сыворотки на 1 грамм веса тела. Синдром, сопровождающий внутривенное введение СПЭТ-а, мог быть также воспроизведен введением 0,01 мл 4% крахмала или насыщенного раствора резины арабика на 1 грамм веса тела. Мышиный СПЭТ, супернатант мышиной перитонеальной жидкости, индуцированной адъювантом Фройнда и мышиной сывороткой, качественно представляется тождественным. На дисковых электрофоретограммах широкое кольцо формировалось в стартовой зоне за счёт других, которые становились более бледными, узкими и исчезали в 3 жидкостях после прогревания при 56° С в течение 30 минут. Обсуждается значение этих наблюдений в свете литературных данных о СПЭТ-е.

EFFECT OF PHYTOHAEMAGGLUTININ AND TUBERCULIN ON MACROMOLECULE SYNTHESIS IN HUMAN LYMPHOCYTE CULTURES

A. DOBOZY, J. HUNYADI, M. SIMON

СТИМУЛИРУЮЩИЙ ЭФФЕКТ ФИТОГЕМАГГЛЮТИНИНА И ТУБЕРКУЛИНА НА МАКРОМОЛЕКУЛЯРНЫЙ СИНТЕЗ В КУЛЬТУРЕ ЧЕЛОВЕЧЕСКИХ ЛЕЙКОЦИТОВ

А. ДОБОЗИ, Й. ХУНЬАДИ, М. ШИМОН

Авторы изучали макромолекулярный синтез, индуцированный фитогемагглютинином и туберкулином в культурах лимфоцитов от Манту-положительных лиц. Фитогемагглютининовая стимуляция спустя 30 минут повышала метиляцию гистона, а через 6 часов индуцировала синтез РНК и белка. Максимальные значения инкорпорации уридина и лейцина были получены на 48 часу культивирования, тогда как синтез ДНК, начинающийся позже, достигал своего пика спустя 72 часа.

Туберкулин проявлял эффект, подобный таковому фитогемагглютинина, но максимум значений был достигнут на 24 часа позже. Лимфоциты культур от больных, чувствительных к различным лекарственным средствам, на стимуляцию специфическим антигеном реагировали подобным же образом. Актиномицин Д в одинаковой степени подавлял инкорпорацию уридина, индуцированную фитогемагглютинином и туберкулином.

ESTIMATION OF ENDOTOXIN-INDUCED INCREASE IN RESISTANCE BY MEANS OF TRYPANOSOMA EQUIPERDUM GROWTH CURVES

G. FÓRIS, K. UJHELYI, M. CSUKÁS

ИЗМЕРЕНИЕ ПОВЫШЕНИЯ РЕЗИСТЕНЦИИ, ВЫЗВАННОЙ ЭНДОТОКСИНОМ, С ПОМОЩЬЮ КРИВОЙ РОСТА TRYPANOSOMA EQUIPERDUM

Г. ФОРИШ, К. УЙХЕЙИ, М. ЧУКАШ

В опытах на крысах, предварительно получивших эндотоксин **Serratia marcescens** и затем внутривенно зараженных **Trypanosoma equiperdum**, логарифмический рост начинался спустя несколько часов после lag-фазы.

Продолжительность lag-фазы находилась в зависимости от промежутка времени между дачей эндотоксина и заражением. При дозе эндотоксина 200 μг/100 г оптимальный интервал от дачи эндотоксина до заражения был 24—72 часа.

Другими факторами, влияющими на продолжительность lag-фазы кривой трипаносомного роста, являлись вес экспериментального животного и число паразитов, используемое при заражении.

После предварительной дачи эндотоксина, параллельно заражая крыс с одинаковым весом тела идентичным количеством паразита, можно было наблюдать линеарную регрессию между продолжительностью lag-фазы кривой роста трипаносомы и примененными дозами эндотоксина. Математический анализ был проведен на основе опытов, в которых крысы весом 100 г спустя 40 часов после дачи эндотоксина заражались **Trypanosoma equiperdum** таким образом, чтобы начальное содержание паразита составляло $2 \cdot 10^7$/мл.

На основе уже предварительно сообщенного нами явления (12,13), в настоящей работе удалось выработать такую методику, с помощью которой представляется возможным количественное сравнение эффекта различных эндотоксинных препаратов повышать резистенцию.

VITAMIN B_{12} PRODUCING FERMENTATIONS OF SEWAGE SLUDGE ORIGIN WITH A MIXED BACTERIUM POPULATION

I. ROLE OF INDIVIDUAL BACTERIUM SPECIES IN VITAMIN B_{12} PRODUCTION

B. JOHAN

ИЗУЧЕНИЕ ФЕРМЕНТАЦИИ В ИЛЕ КАНАЛИЗАЦИОННОГО ПРОИСХОЖДЕНИЯ СО СМЕШАННОЙ ПОПУЛЯЦИЕЙ БАКТЕРИЙ ПРИ ПРОИЗВОДСТВЕ ВИТАМИНА B_{12}

I. РОЛЬ ИНДИВИДУАЛЬНЫХ ВИДОВ БАКТЕРИЙ В ПРОИЗВОДСТВЕ ВИТАМИНА

Б. ЙОХАН

Смешанная популяция бактерий, участвующая в ферментации при производстве витамина B_{12}, инокулированная вместе с 20% канализационного ила бытового происхождения, оставалась неизмененной в течение нескольких лет, когда пополнение канализационного ила было прекращено, и популяция была адаптирована к обедненной илом среде. Из-за полиморфизма индивидуальных бактериальных видов анализ морфологических типов был затруднен. Однако, это могло быть облегчено тем фактом, что имелась возможность центрифугированием сепарировать эти бактерии в три типичные морфологические группы. Результаты попыток определения таксономии этих бактерий будут опубликованы позже. Определение содержания аналогичного витамину B_{12} в трех отделенных морфологических бактериальных группах предоставляло возможность более ясного понимания их специальной роли в получении общего выхода витамина. Представляется реальным влиять на бактериальный спектр ферментации и этим самым на его витаминаналогичное содержание.

INTERFERON PRODUCING CAPACITY OF WHITE BLOOD CELLS FROM PATIENTS WITH POLYGLOBULIA

GY. HADHÁZY, GY. NAGY L. GERGELY, CS. BALÁZS

ИЗУЧЕНИЕ ИНТЕРФЕРОНПРОДУЦИРУЮЩЕЙ СПОСОБНОСТИ ЛЕЙКОЦИТОВ ОТ БОЛЬНЫХ ПОЛИГЛОБУЛИЕЙ

ДЬ. ХАДХАЗИ, ДЬ. НАДЬ, Л. ГЕРГЕЙ, Ч. БАЛАЖ

Авторы провели сравнительное изучение продукции интерферона *in vitro* лейкоцитами от больных полиглобулией различного происхождения и от здоровых лиц. В качестве индуктора использовали вирус парагриппа —1 (Сендай). Интерферон определялся в культуре перевиваемой линии клеток амниона человека с помощью вируса везикулярного стоматита, как индикатора.

Были изучены лейкоциты от 33 больных, страдающих **polycythaemia vera**; от некоторых из них было взято несколько проб. Геометрический титр интерферона был 1:31, приблизительно в 6 раз ниже, чем таковой у контрольных лейкоцитов.

Лейкоциты от больных полиглобулией в отличие от случаев **polycythaemia vera** (8 случаев) в такой же степени вырабатывали интерферон, как и контрольные клетки.

Обсуждается возможное патогенетическое значение измененной способности лейкоцитов вырабатывать интерферон при разных заболеваниях.

SPECIFIC ORAL PREVENTION OF INFANTILE GASTRO-ENTERITIS

II. EXPERIMENTS IN INFANTS

K. RAUSS, I. KÉTYI, E. MATUSOVITS, L. SZENDREI, A. VERTÉNYI

СПЕЦИФИЧЕСКАЯ ОРАЛЬНАЯ ПРОФИЛАКТИКА ГАСТРОЭНТЕРИТА НОВОРОЖДЕННЫХ

II. ЭКСПЕРИМЕНТЫ НА НОВОРОЖДЕННЫХ

К. РАУШШ, И. КЕТЬИ, Е. МАТУШОВИЧ, Л. СЕНДРЕИ, А. ВЕРТЕНЬИ

1. Реактогенность и иммуногенность антигенов **Boivin E. coli**, вызывающих эффективный иммунитет у мышей, были изучены путем введения **per os** 62, в основном, дистрофичным новорожденным в возрасте от 1 до 8 месяцев. В одной таблетке содержалось по 0,5 мг каждого из антигенов: **O11 : K58(B4)**, **O55 : K59(B5)** и **O86 : K61(B7)**.

2. Доза антигена, постепенно доведенная до 12 мг (8 таблеток) на 1 кг веса тела, оказалась безвредной. Более высокие дозы не изучались.

3. Антигенность вакцины оценивалась по пассивной иммунизации 10-дневных куриных эмбрионов сывороткой новорожденных и по пассивной иммунизации мышей копро-антителами.

4. Субоптимальные дозы (от 2×3 до 3×3 таблеток) повышали уровень сывороточных антител примерно в 5 раз. Эффект мог быть значительно усилен ревакцинацией: 1 таблеткой в неделю. Иммунный ответ был поливалентным. Более высокие дозы ($2—3\times8$ или 8×3 табл.) давали более значительный подъем титров (18—55-кратный) по сравнению с таковыми в упомянутой группе новорожденных, и дальнейшая ревакцинация не сопровождалась значительным повышением уровня антител. Определение оптимальной иммунизирующей дозы, а также оптимальных интервалов между вакцинациями требует дополнительного изучения.

5. Оральный антиген вызывал образование копро-антител, параметры которых были подобны таковым сывороточных антител.

6. Как это было показано на примере возникновения сывороточных- и копро-антител, после примовакцинации для поддержки эффективного иммунитета существенным моментом является ревакцинация.

7. Обсуждается роль сывороточных- и копро-антител в иммунитете. Благоприятный иммунный ответ — особенно появление копро-антител — подтверждает необходимость широкого эпидемиологического изучения профилактического эффекта вакцинации.

TYPE DISTRIBUTION OF STREPTOCOCCUS PYOGENES IN THE YEARS 1964—65 AND 1968—69

J. SZITA, M. KÁLI

СРАВНЕНИЕ ТИПОВОГО РАСПРЕДЕЛЕНИЯ STREPTOCOCCUS PYOGENES В 1964—65 И 1968—69 ГОДЫ

Й. СИТА, М. КАЛИ

Так как международный надзор над стрептококковыми инфекциями в 1964—65 и 1968—69 годах был идентичным по целям и условиям, имелась возможность сравнения результатов.

1. Надзор в Венгрии показал, что в 1964—65 годах 395 штаммов **Streptococcus pyogenes** относились к 27 типам, в 1968—69 годах 427 штаммов принадлежали к 22 типам (культуры с комплексными антигенами были оценены как отдельные типы).

2. Восемь из 27 типов, выделенных при первом изучении, не встречались в 1968—69 гг.; в свою очередь, во втором периоде были идентифицированы 4 новых типа.

3. В обоих исследованиях агглютинацией и преципитацией можно было типировать приблизительно 99% штаммов.

4. Комплексы 5, 11, 12, 27, 44 и 3, 13, B_{3264} и 12-ый тип встречались довольно часто в обоих периодах. Встречаемость других фреквентных типов заметно изменилось: у 3 штаммов понизилось до половины, а 28-ой тип исчез. В противоположность этому во втором исследовании встречаемость комплексов 4, 24, 26, 28, 29, 46, 48 и 8, 25, Imp_{19} и типа 19 повысилась.

5. Не было найдено определенной взаимосвязи между разными типами и различными стрептококковыми инфекциями. При первом исследовании 3-ий тип, при втором — 19-ый тип встречались наиболее часто в случаях скарлатины.

6. Не было отмечено значительной разницы между типами, происходящими из промышленных и сельскохозяйственных областей.

7. Чувствительность штаммов к тетрациклину оказалась подобной в обоих исследованиях.

8. Подчеркивается, что международное сотрудничество на основе общих принципов предоставляет возможность для выработки специфической профилактики стрептококковых инфекций.

ENHANCED INTERFERON PRODUCTION IN VITRO BY LEUCOCYTES FROM CHILDREN WITH INFECTIOUS MONONUCLEOSIS

L. GERGELY, F. D. TÓTH, GY. HADHÁZY, B. SZABÓ

СРАВНИТЕЛЬНОЕ ИЗУЧЕНИЕ ИНТЕРФЕРОНПРОДУЦИРУЮЩЕЙ СПОСОБНОСТИ ЛЕЙКОЦИТОВ ЗДОРОВЫХ И БОЛЬНЫХ ИНФЕКЦИОННЫМ МОНОНУКЛЕОЗОМ ДЕТЕЙ

Л. ГЕРГЕЙ, Ф. Д. ТОТ, ДЬ. ХАДХАЗИ, Б. САБО

Авторы изучали ***in vitro*** выработку интерферона лейкоцитами от 10 детей, больных инфекционным мононуклеозом. Выработка интерферона была более интенсивной, чем в контрольных культурах той же самой клеточной плотности, приготовленных тем же самым способом из лейкоцитов здоровых детей. Обсуждаются возможные причины разницы.

PYOCINE TYPING OF PSEUDOMONAS AERUGINOSA: ASSOCIATION BETWEEN ANTIGENIC STRUCTURE AND PYOCINE TYPE

K. CSISZÁR, B. LÁNYI

ПИОЦИНОВОЕ ТИПИРОВАНИЕ PSEUDOMONAS AERUGINOSA. СВЯЗЬ МЕЖДУ АНТИГЕННОЙ СТРУКТУРОЙ И ПИОЦИНОВЫМ ТИПОМ

К. ЧИСАР, Б. ЛАНЬИ

Пиоциновое типирование 1043 штаммов ***Pseudomonas aeruginosa***, выделенных из различных источников, было проведено по схеме ***Govan*** и ***Gillies***. Большая часть культур принадлежала к типам 1 (28,8%), 3 (20,1%), 5 (5,5%) и 10 (14,8%). Неклассифицируемые и нетипируемые штаммы оказались в 7,6 и 9,2% соответственно. Среди штаммов типа 1 доминировали подтипы **1b, 1c, 1d, 1f, и 1h.**

В общей сложности 543 штамма, несоответствующие антигенной схеме Ланьи, включающей 53 серотипа и 16 дальнейших, частично определенных серологических единиц, были подразделены в 165 комбинированных серо-пиоциновых типов. Была обнаружена интересная взаимосвязь между О-антигенами и пиоциновыми типами. Обсуждается возможность практического применения комбинированного типирования в эпидемиологических исследованиях.

ATYPICAL MYCOBACTERIA ISOLATED FROM DIAGNOSTICAL MATERIAL

K. HAJÓS, P. JUHÁSZ

АТИПИЧНАЯ МИКОБАКТЕРИЯ, ВЫДЕЛЕННАЯ ИЗ ДИАГНОСТИЧЕСКОГО МАТЕРИАЛА

К. ХАЙОШ, П. ЮХАС

При исследовании рутинного туберкулёзного материала было выделено 32 штамма кислото-устойчивой бактерии. На основе морфологических, культуральных, биохимических, патогенетических характеристик и резистенции штаммы были следующие: 2 **Mycobacterium tuberculosis** (человеческий тип), 1 **M. avium**, 8 **M. aquae e**, 1 **M. aquae III**, 3 **M. smegmatis** и 1 **M. phlei.** Патогенность для человека 5 ниацин-положительных штаммов — из-за их иррегулярного поведения — была сомнительной. Шесть штаммов остались неклассифицированными. Обсуждается противоречие в классификации микобактерии. Стандартизация методов рассматривается как наиболее важный шаг для соответствующей идентификации. Авторы рекомендуют схему классификации, пригодную для небольших лабораторий.

ANTI-COMPLEMENTARY SUBSTANCES PRODUCED BY KB CELL CULTURES INFECTED AND NOT INFECTED WITH VIRUS

ZS. KÓSA, GY. FÜST

ИЗУЧЕНИЕ АНТИКОМПЛЕМЕНТАРНЫХ СУБСТАНЦИЙ, ВЫРАБАТЫВАЕМЫХ КЛЕТОЧНЫМИ КУЛЬТУРАМИ КБ, ЗАРАЖЕННЫМИ И НЕЗАРАЖЕННЫМИ ВИРУСОМ

Ж. КОША, ДЬ. ФЮШТ

Авторы изучали антикомплементарные субстанции, обнаруженные в питательной среде клеточных культур КБ, зараженных и незараженных вирусом. Было найдено, что одна из фракций антикомплементарной субстанции, вырабатываемой незараженными клетками КБ, имела много общих свойств с С1. Заражение клеточной культуры полиовирусом типа 1 понижало, тогда как заражение 3 типом аденовируса повышало антикомплементарную активность культуральных жидкостей. Повышение активности, связанное с аденовирусным заражением, являлось следствием не увеличения количества первоначально выработанной субстанции, а возникновения нового материала, отличающегося от упомянутой субстанции.

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