ACTA MICROBIOLOGICA

ACADEMIAE SCIENTIARUM HUNGARICAE

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R. MANNINGER, I. NÁSZ, A. PELC, K. RAUSS, J. SZIRMAI,
J. WEISSFEILER

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G. IVÁNOVICS

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AKADÉMIAI KIADÓ, BUDAPEST

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DIE STRAHLENEMPFINDLICHKEIT VON ESCHERICHIA COLI B-KULTUREN

II. DIE WIRKUNG DER BEHANDLUNG VOR, WÄHREND UND NACH DER BESTRAHLUNG AUF DIE STRAHLENEMPFINDLICHKEIT DER ZELLEN

Von

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(Eingegangen am 8. November 1965)

Zusammenfassung. Die Strahlenempfindlichkeit von *E. coli* B-Kulturen der Rtg- bzw ^{60}Co-Strahlung gegenüber wurde vor und nach der Bestrahlung sowie in Abhängigkeit von der Temperatur und Anoxie während der Bestrahlung untersucht.

Die Strahlenempfindlichkeit der vor der Bestrahlung auf Minimal-Nährboden gewachsenen *E. coli*-Kulturen war größer als die der auf komplettem Nährboden gewachsenen 14stündigen Kulturen.

Das Überleben von Zellsuspensionen nach der Bestrahlung wurde durch Züchtung auf dem Minimal-Nährboden gesteigert. Diese günstige Wirkung wurde durch die Komplettierung des Minimal-Nährbodens mit organischen Bestandteilen (Hefe, Pepton, Knochenmark) beeinträchtigt. Zugleich konnte die auf das Überleben ausgeübte ungünstige Wirkung des kompletten Nährbodens mit stoffwechselhemmenden Mitteln (anoxische bzw. mit Chloramphenicol ausgeführte Vorinkubation) verbessert werden.

Temperaturerhöhung während der Bestrahlung führte bei niedriger Dosisleistung (260 r/min) zur Steigerung der Strahlenempfindlichkeit, die bei hoher Dosisleistung (3200 r/min) nicht zutage trat. Von der während der Bestrahlung mittels N_2-Durchströmung herbeigeführten Anoxie wurde die Strahlenempfindlichkeit der Zellsuspensionen um etwa 2,4 DRF herabgesetzt.

Die Strahlenempfindlichkeit der *E. coli* B-Zellen wird von zahlreichen Faktoren beeinflußt. So ändert sich die Strahlenempfindlichkeit auch im Verlauf der Züchtung. Das Maximum der Empfindlichkeit ist am Ende der Lag-Periode bzw. am Anfang der Log-Phase anzutreffen [1]. Durch Mangel an Glukose und Sauerstoff während der Züchtung wird die Strahlenempfindlichkeit der *E. coli* B/r-Zellen herabgesetzt [2, 3, 4].

Die Temperatur und der Sauerstoffdruck während der Bestrahlung wirken sich ebenfalls auf die Strahlenempfindlichkeit der Zellen aus. Stapleton und Edington [5] untersuchten die Empfindlichkeit von *E. coli* B/r den Rtg-Strahlen gegenüber zwischen —196° C und +40° C und stellten fest, daß bis +30° C keine Veränderung in der Strahlenempfindlichkeit eintritt, diese jedoch bei +40° C bereits um 25—50% zunimmt. Wood [6] beobachtete bei haploiden Hefepilzen bis +40° C eine minimale Erhöhung der Empfindlichkeit. Eine stärkere Erhöhung trat erst über +50° C in Erscheinung.

Auf den Zusammenhang der Strahlenempfindlichkeit mit der Sauerstofftension während der Bestrahlung haben Giles und Beatty [7] sowie Read [8] hingewiesen. Bis zu einem gewissen Maximum ändert sich die Strahlenempfindlichkeit stufenförmig mit den ansteigenden Sauerstoffkonzentrationen. Die anwesende O_2 wirkt als dosismultiplizierender Faktor.

Die Größe der von der ionisierenden Strahlung verursachten Schädigung hängt außerdem auch von den Restitutionsgeschehnissen ab. Diese Prozesse können von den Postirradiationsbehandlungen beeinflußt werden, z. B. von erhöhter oder herabgesetzter Inkubationstemperatur, vom Mangel an einigen Nährstoffen, von stoffwechselhemmenden Mitteln [9—18].

LATARJET [19] stellte bei Saccharomyces ellipsoideus nach Anwendung einer an und für sich hochgradige Abtötung herbeiführenden Dosis ein Überleben in höherem Prozentsatz fest, wenn diese 24 Stunden bei +5° C inkubiert und erst danach in die optimale Wachstumstemperatur verbracht wurden. Für *E. coli* B gibt es zwei optimale Überlebenstemperaturen; die eine liegt bei +12° C, die andere über +30° C [20].

Für die Nährbodenwirkungen nach der Bestrahlung ist bezeichnend, daß das Verhältnis der Überlebenden nach der Bestrahlung wächst, wenn wir den untersuchten Zellen suboptimale Wachstumsbedingungen gewährleisten [13, 14].

Im Rahmen unserer Versuche wollten wir unter Berücksichtigung obiger Tatsachen folgendes untersuchen: (1) Wie wirken die Präirradiations-Nährbodenfaktoren, welche die physiologisch-biochemischen Verhältnisse der Kultur beeinflussen, auf die Strahlenempfindlichkeit des Stammes *E. coli* B? (2) Auf welche Weise beeinflussen die Temperatur während der Bestrahlung und die mittels Stickstoffdurchströmung zustandegebrachte Anoxie die Strahlenempfindlichkeit der Zellsuspensionen? (3) Wie wirken sich die Postirradiations-Nährbodenverhältnisse, gewisse Temperaturvariationen und stoffwechselhemmende Mittel auf das Überleben aus?

Material und Methoden

(1) *Präirradiations-Züchtung.* Die mit gleicher Zellzahl abgeimpften, geschüttelten Kulturen von *E. coli* B (Hammersmith Hospital, London) wurden in Bouillon (total) bzw. im (minimale Nährstoffe enthaltenden) ROBERTS C-Nährmedium [21] bei 37° C 14 Stunden gezüchtet, worauf wir nach dreimaligem Waschen mit physiol. NaCl-Lösung eine Suspension mit der Zellzahl 10^6/ml herstellten.

(2) *Bestrahlung.* Von obiger Suspension wurde 1 ml in das Bestrahlungsgefäß eingemessen. Die totale Anoxie während der Bestrahlung wurde mindestens 5 min. vor Beginn der Bestrahlung, die partielle Anoxie hingegen durch die zugleich mit der Bestrahlung eingeleitete Stickstoffdurchströmung zustande gebracht. Nach der Bestrahlung und Verdünnung wurde das Substrat auf Nähragar ausgebreitet und nach 24stündiger Inkubation bei 37°C die Anzahl der aus überlebenden Zellen gewachsenen Kolonien festgestellt. Die Abweichung der Zahl der in Paralleluntersuchungen auf Agarplatten gewachsenen Kolonien überstieg nicht ±20% des Durchschnitts.

Jedes einzelne in dieser Arbeit mitgeteilte Ergebnis bildet den Mittelwert von jeweils 6 nach 3 parallelen Bestrahlungen (insgesamt also 18) untersuchten Agarplattenbefunden.

Die für die Versuche erforderliche Bestrahlung mit niedriger Dosisleistung (260 r/min) erfolgte mit der teletherapeutischen ^{60}Co-Apparatur Gravicert, die Bestrahlung mit hoher Dosisleistung (3200 r/min) unter Anwendung der tiefbestrahlenden Rtg-Apparatur Siemens-Stabilivolt. Die Strahlendosen wurden mit der Ionisationskammer und mit dem FRICKEschen chemischen Dosimeter [22] gemessen.

(3) *Postirradiationsbehandlungen.* I. Veränderung der Nährbodenverhältnisse: Als kompletten Nährboden verwendeten wir Nähragar, als Minimal-Nährboden den 2% Agar enthaltenden ROBERTSschen synthetischen C-Nährboden.

Zur Komplettierung des ROBERTSschen Minimal-Nährbodens C wurden Hefeextrakt (Difco), Pepton (Witte) und Rattenknochenmark benutzt.

II. *Stoffwechselhemmung:* (a) Zur Untersuchung der Anoxiewirkung nach der Bestrahlung wurde zwischen die Ausbreitung der bestrahlten Bakteriumsuspensionen auf festem Nährboden und die übliche aerobe Inkubation bei 37° C, eine Inkubation in Stickstoffatmosphäre während 30 min. bei derselben Temperatur eingefügt. (b) Bei der Chloramphenicolbehandlung gingen wir folgendermaßen vor: Die bestrahlten und verdünnten Suspensionen wurden in zwei Fraktionen geteilt, die eine in das komplette Nährmedium, die andere in den »Minimal«-Nährboden eingemessen und 20 min. bei 37° C inkubiert. Hiernach teilten wir beide Kulturen wiederum in zwei Fraktionen und setzten der einen Chloramphenicol zu (Endkonzentration 5 μg/ml), wonach eine 30 min. dauernde Inkubation bei 37° C folgte. Nach dreimaligem Waschen mit physiol. NaCl-Lösung und Verdünnung erfolgte die Ausbreitung auf Nähragar bzw. »ROBERTS C«-Agar.

III. *Temperaturwirkungen:* Bei den Postirradiations-Vorinkubationen in verschiedenen Temperaturen erwies sich nach unseren Vorversuchen die Dauer von 12 Stunden als optimal.

Ergebnisse

(1) *Wirkung der Präirradiations-Nährbodenverhältnisse.* Wie Abb. 1 zeigt, war die Strahlenempfindlichkeit der auf dem »Minimal«-Nährboden gezüchteten *E. coli* B-Zellen größer als die der ebenso lange (14 Stunden) auf komplettem Nährboden kultivierten.

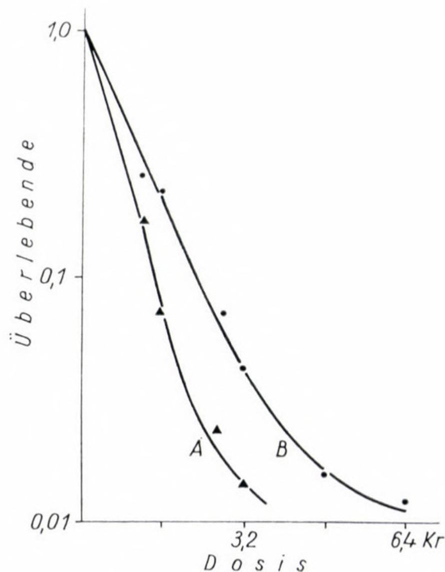

Abb. 1. Veränderung der Strahlenempfindlichkeit von *E. coli* B-Zellen in Abhängigkeit von den Präirradiations-Züchtungsbedingungen. A = ROBERTS C-Nährboden; B = Bouillon

(2) *Veränderung der Bestrahlungsverhältnisse.*

I. Wirkung der Temperatur während der Bestrahlung. Von übereinstimmenden *E. coli* B-Bouillonkulturen haben wir bei drei verschiedenen Wärmegraden Dosiswirkungskurven aufgenommen (Abb. 2).

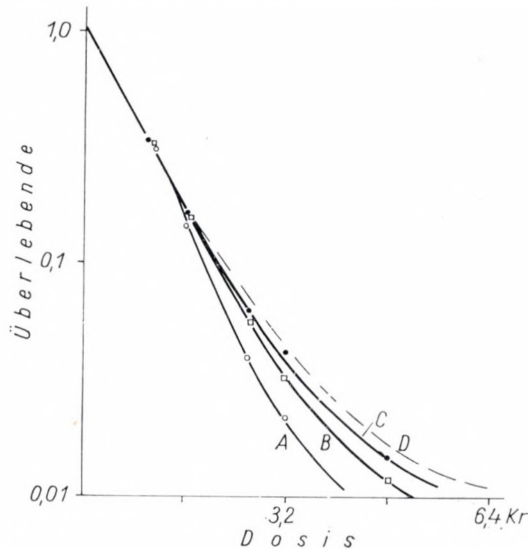

Abb. 2. Wirkung der Temperatur während der Bestrahlung auf die Strahlenempfindlichkeit von *E. coli* B-Suspensionen. A = 260 r/min bei 37° C; B = 260 r/min bei 21° C; C = 260 r/min bei 0° C; D = 3200 r/min bei 21° C

Bei niedriger Dosisleistung (260 r/min) nimmt die Strahlenempfindlichkeit der *E. coli* B-Kulturen mit der Temperatur ab. Im Falle hoher Dosisleistung (3200 r/min) kommt die Strahlenempfindlichkeit der bei 21° C bestrahlten Zellsuspensionen der mit niedriger Dosisleistung bei 0° C bestrahlten nahe.

II. Wirkung der Anoxie während der Bestrahlung. Anoxie bewirkt eine stark verminderte Strahlenempfindlichkeit der *E. coli* B-Zellen (Abb. 3).

(3) *Postirradiations-Behandlungen.*

I. Wirkung der Nährbodenverhältnisse. Durch die Postirradiations-Züchtung auf dem lediglich eine anorganische Stickstoffquelle enthaltenden Minimal-Nährboden wird das prozentuale Überleben gesteigert, wobei sich aus der »b«-artigen Dosiswirkungskurve eine »c«-artige entwickelt (Abb. 4). Wird der Minimal-Nährboden mit Bestandteilen ergänzt, die eine organische Stickstoffquelle gewährleisten (1,0% Hefeextrakt, 0,5% Pepton, $1{,}25 \times 10^7$ Zellen/ml Knochenmark), so wird diese die Strahlenempfindlichkeit herabsetzende Wirkung fast ganz aufgehoben (Abb. 5 und 6).

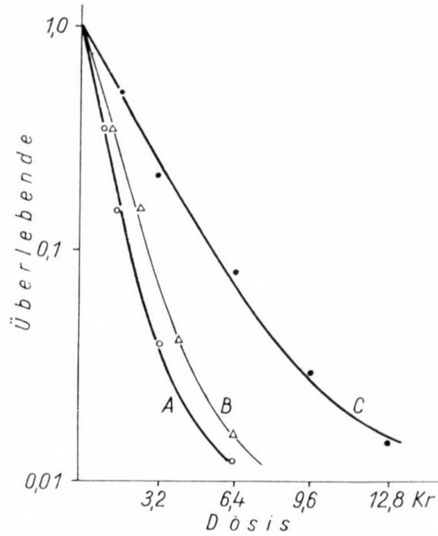

Abb. 3. Wirkung der Bestrahlung unter anoxischen Bedingungen auf das Überleben von *E. coli* B-Zellsuspensionen. A = unter aeroben Bedingungen; B = partieller Sauerstoffmangel; C = anoxische Bedingungen

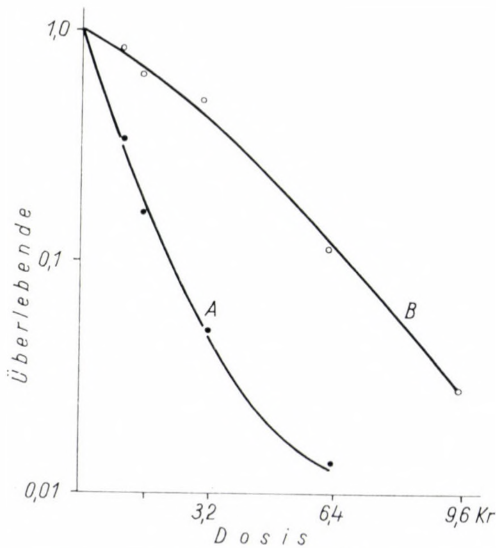

Abb. 4. Wirkung der postirradiativen Nährbodenverhältnisse auf das Überleben. A = Nähragar; B = fester ROBERTS C-Nährboden

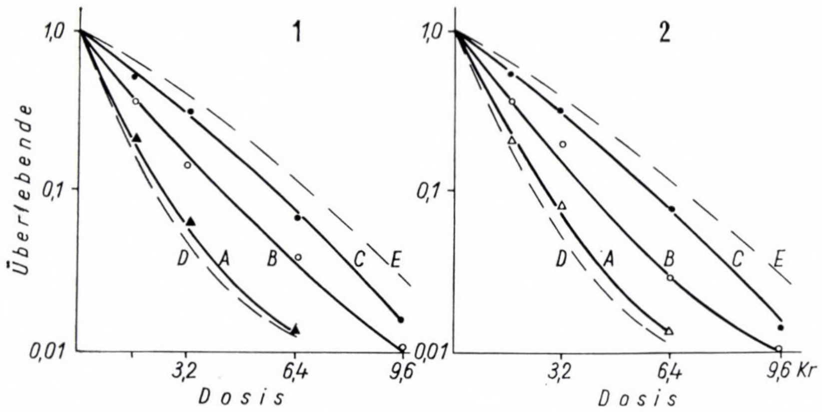

Abb. 5. Wirkung der Komplettierung des Minimal-Nährbodens mit organischen Bestandteilen auf das Überleben von *E. coli* B. A = Roberts C + 1,0%iger Hefeextrakt; B = Roberts C + 0,5%iger Hefeextrakt; C = Roberts C + 0,3%iger Hefeextrakt. A = Roberts C + 0,5% Pepton; B = Roberts C + 0,3% Pepton; C = Roberts C + 0,1% Pepton. D = Nähragar; E = fester Roberts C-Nährboden.
(1) Hefehaltiger Nährboden; (2) peptonhaltiger Nährboden

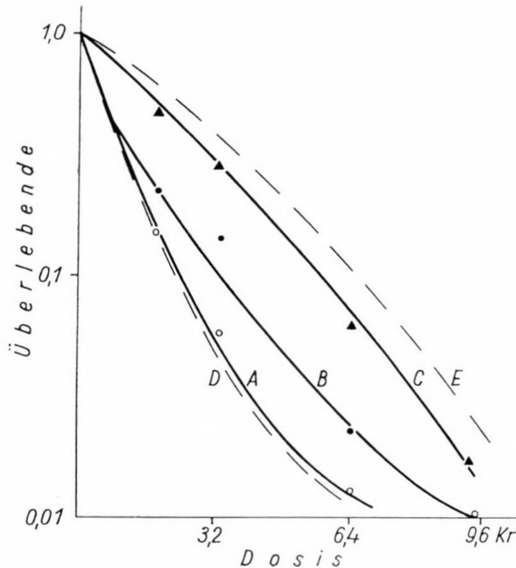

Abb. 6. Wirkung von Knochenmark auf das postirradiative Überleben von *E. coli* B-Zellen. A = $2,5 \times 10^8$ Knochenmarkzellen/20 ml Roberts C-Nährboden, B = $1,0 \times 10^8$ Knochenmarkzellen/20 ml Roberts C-Nährboden; C = $0,5 \times 10^8$ Knochenmarkzellen/20 ml Roberts C-Nährboden; D = Nähragar; E = fester Roberts C-Nährboden

II. Stoffwechselhemmung. (a) Die Postirradiations-Anoxie untersuchten wir in Versuchen, die folgendermassen dargestellt werden können:

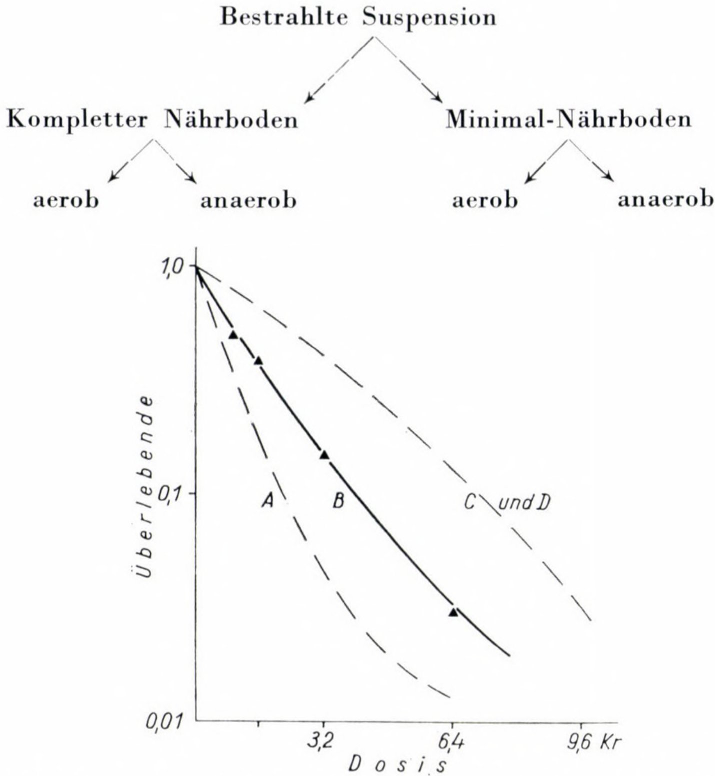

Abb. 7. Wirkung der postirradiativen anoxischen Vorinkubation auf das Überleben von *E. coli* B-Zellen. A = Nähragar; B = Nähragar + anoxische Vorinkubation; C = ROBERTS C-Nährboden; D = ROBERTS C-Nährboden + anoxische Vorinkubation

In den Vorversuchen beobachteten wir, daß das zunehmende Überleben eine Zeitlang von der postirradiativen anoxischen Vorinkubationszeit abhängt. Wird die Vorinkubationszeit über 30 min verlängert, so tritt keine Steigerung des prozentualen Überlebens mehr ein. Wie in Abb. 7 ersichtlich, wird von der 30 min unter anoxischen Bedingungen erfolgenden Vorinkubation nach der Bestrahlung nur das Überleben der auf den kompletten Nährboden ausgebreiteten Suspensionen gesteigert.

(b) Wirkung von Chloramphenicol. Durch die Vorinkubation während 30 min in dem 5 μg/ml Chloramphenicol enthaltenden Bouillon-Nährmedium wird das Überleben von *E. coli* B-Suspensionen nach der Bestrahlung erhöht. Die das Überleben steigernde Wirkung der Züchtung auf »Minimal«-Nährboden wird durch Behandlung mit Chloramphenicol nicht gesteigert (Abb. 8).

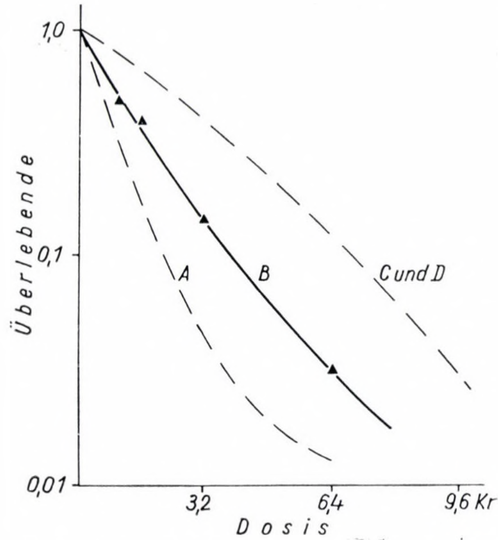

Abb. 8. Wirkung von Chloramphenicol auf das postirradiative Überleben von *E. coli* B-Suspensionen. A = in Bouillon vorinkubiert; B = in 5 μg/ml Chloramphenicol enthaltendem Bouillon vorinkubiert; C = in ROBERTS C vorinkubiert; D = in 5 μg/ml Chloramphenicol enthaltendem ROBERTS C-Nährboden vorinkubiert

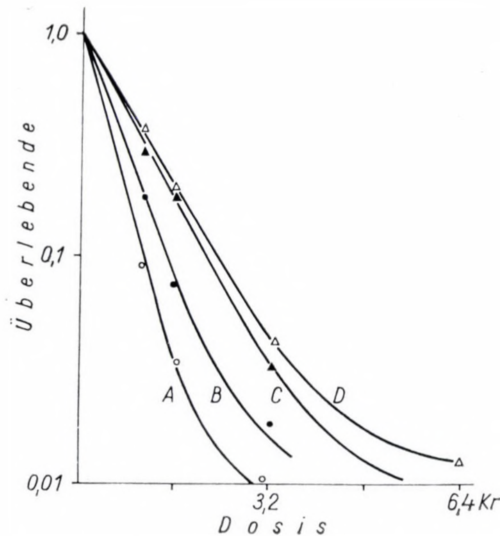

Abb. 9. Wirkung der postirradiativen Vorinkubationstemperatur auf das Überleben von *E. coli* B-Suspensionen. A = 27° C; B = 21° C; C = 0° C; D = 37° C

III. Wirkung der Temperatur der Postirradiations-Vorinkubation (Abb. 9). Wie Abb. 9 zeigt, wirkt die Vorinkubation bei 37° C optimal auf das Überleben der *E. coli* B-Zellen nach der Bestrahlung. Beinahe ebenso wirkt die Vorinkubation bei 0° C. Durch die Vorinkubation bei 21° bzw. 27° C wird das prozentuale Überleben reduziert.

Besprechung

Nach den Versuchsergebnissen ist die Strahlenempfindlichkeit der auf Minimal-Nährboden 14 Stunden gezüchteten *E. coli* B-Zellen größer als die der auf komplettem Nährboden 14 Stunden gezüchteten Zellsuspensionen. Zur Erklärung des Unterschiedes bieten sich zwei Hypothesen: 1. Die unterschiedliche Strahlenempfindlichkeit beruht einfach auf dem abweichenden Wachstumstempo. 2. Bei den auf dem Minimal-Nährboden gewachsenen *E. coli* B-Zellen handelt es sich um qualitativ andere als bei den auf dem kompletten Nährboden gewachsenen. Die auf dem vorigen Nährboden gewachsenen Zellen beanspruchen zur Reproduktion eine qualitativ andere, reichhaltigere Enzymgarnitur. Bei der Bestrahlung kann die Synthese dieser adaptiven Enzyme eine Schädigung erleiden und der Unterschied in der Strahlenempfindlichkeit darauf zurückzuführen sein.

Zwecks Klarstellung dieser Frage untersuchten wir das Wachstumstempo der *E. coli* B-Kultur nebeneinander auf Bouillon und auf ROBERTS C-Nährboden. Die Wachstumskurven zeigten einen abweichenden Verlauf. In der 14. Stunde befand sich die auf dem kompletten Nährboden wachsende *E. coli* B-Kultur bereits am Ende der Log-Phase, in einem resistenteren Stadium, zugleich jedoch die Kultur der auf dem Minimal-Nährboden gewachsenen Zellen in einem früheren, strahlenempfindlicheren Stadium der Log-Phase.

Aus den Versuchen über die Temperatur während der Bestrahlung können wir die Schlußfolgerung ziehen, daß die im Augenblick der Bestrahlung vorliegenden Temperaturverhältnisse auf physiko-chemischer Ebene an und für sich keinen Einfluß auf die Strahlenempfindlichkeit ausüben. Die Temperaturverhältnisse vermögen die Strahlenempfindlichkeit nur dann zu verändern, wenn die Temperaturveränderung eine Stoffwechselwirkung auf das Testobjekt ausübt und die Veränderung der Strahlenempfindlichkeit im Grunde infolge des veränderten Stoffwechsels zustande kommt. Wahrscheinlich beobachteten wir deshalb bei niedriger Dosisleistung und höherer Temperatur gesteigerte, bei hoher Dosisleistung jedoch unveränderte Strahlenempfindlichkeit.

Die mittels N_2-Durchströmung vorgenommenen Anoxieversuche bewiesen die die Strahlenempfindlichkeit herabsetzende Wirkung der Anoxie und deuteten gleichzeitig darauf hin, daß es wichtig sei, den Sauerstoff auszuschlies-

sen. Begann die N_2-Durchströmung lediglich eine Minute vor der Bestrahlung, so war die Verringerung der Strahlenempfindlichkeit minimal.

Von optimalen Züchtungsverhältnissen nach der Bestrahlung wird das prozentuale Überleben herabgesetzt, von suboptimalen Züchtungsverhältnissen gesteigert. So hat die Züchtung der Zellsuspensionen nach der Bestrahlung auf Minimal-Nährboden unter anaeroben Bedingungen bzw. die Vorinkubation auf dem Chloramphenicol enthaltenden kompletten Nährboden — vermutlich in beiden Fällen durch die Verzögerung des Beginns der Stoffwechselprozesse — das Überleben der *E. coli* B-Suspensionen nach der ionisierenden Strahlung erhöht. Anscheinend geht durch die verzögerte Einleitung der Stoffwechselprozesse eine Restitution von Biomolekülen vor sich, die in der Reproduktion der Zellen eine wichtige Rolle spielen.

Die günstige Chloramphenicolwirkung hing auch davon ab, in welchem Augenblick das Mittel dem kompletten Nährboden zugesetzt wurde. In unseren Versuchen mußte das Chloramphenicol den Zellen 20 min. nach der Bestrahlung zugegeben werden. Es wird angenommen [9], daß in dieser Zeit eine gewisse Nukleinsäure und Eiweißsynthese in Gang kommt, die zur Restitution des geschädigten DNS-synthetisierenden Systems erforderlich ist.

Die Vorinkubation bei niedrigerer Temperatur als 37° C ergibt keine die Strahlenempfindlichkeit verringernde Wirkung, sondern führt zur gesteigerten Vernichtung der *E. coli* B-Zellen nach der Bestrahlung. Diese Wirkung der niedrigen Temperatur vermögen wir einstweilen nicht zu erklären. Der Gedanke liegt nahe, daß im Falle der benutzten Nährböden die niedrigere Temperatur für das Wachstum der *E. coli* B nach der Bestrahlung nicht als suboptimal angesehen werden kann.

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SENSITIVITY OF E. COLI B TO IRRADIATION

IV. DEPENDENCE OF THE PROTECTIVE EFFECT OF CYSTEINE-CYSTEAMINE-TYPE COMPOUNDS ON PREIRRADIATION OXYGENATION AND ON PRE- AND POSTIRRADIATION CONDITIONS OF CULTURING

By

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(Received September 21, 1965)

Summary. Factors influencing the X-ray sensitivity of *E. coli* B have been studied. When pre- or postirradiation incubation of *E. coli* B with cysteine-cysteamine type compounds was omitted, these latter substances, as compared to the anoxic control, did not or only slightly increase the resistance of the cells to irradiation under anoxic conditions. The protective effect of the substances in question was not increased during optimal pre- or postirradiation incubation periods. It has been concluded that during irradiation cysteine-cysteamine derivatives exert a physicochemical, while before and after irradiation a metabolic, effect.

The radioprotective effect of cysteine-cysteamine type compounds under hypoxic or anoxic conditions is a disputed problem in radiobiology.

According to a number of authors, radioresistance under anoxic irradiation conditions is increased only slightly, if at all, by the above compounds and, conversely, the protective effect of these substances ceases or at least decreases in the absence of oxygen.

Due to their autoxidation capacity, the radioprotective effect of the aforesaid compounds was found to be based on oxygen deprivation [1—3]. Thus the protective effect of these compounds ceases when oxygen has been eliminated.

On the other hand, Hollaender and Stapleton [4], Wood [5], Lothe and Devik [6] were able to achieve additional radioresistance under anoxic conditions, by means of cysteine compounds. These results were confirmed by Valdstein [7] and Elias [9] who also demonstrated that these substances exerted an increased protective effect under anaerobic as compared to aerobic conditions. In the opinion of these investigators, oxygen and air by their presence as radiosensitors, decrease the protective effect of cysteamine.

The radiosensitivity of microorganisms, as has been pointed out by a number of authors [8, 10] including ourselves[11], is considerably affected not only by oxygenation during irradiation, but also by conditions of culture prevailing before and after exposure to X-rays. Thus, when examining cysteine-cysteamine type compounds, the possibility of the protective effect being influenced by the aforesaid conditions of culture has also been taken into consideration.

The above assumptions have been confirmed by KOHN and GUNTER [12], who have shown that the radioprotective effect of cysteine involved certain metabolic processes of the microorganisms, and that the extent of radioprotection afforded by cysteine was determined by the temperature prevailing in the cell suspensions during the preirradiation period as well as by their H ionic concentration.

In previous papers [13, 14] the fact has been stressed that metabolic factors were also involved in the protective effect exerted by radioprotective compounds belonging to the cysteine-cysteamine group.

In planning our experiments, the above discussed possibilities, *i.e.* the role played by anoxia and metabolic factors in the mechanism of action of the radioprotective substances, have been considered. This is the reason why we have concentrated on experiments allowing to ascertain the protective effect of cysteine-cysteamine type compounds as well as the role of these compounds during the pre- and postirradiation periods.

Materials and methods

E. coli B strain was grown at 37° C in broth and Roberts C minimal medium [15].

Particulars concerning the preparation of cell-suspensions and the periods during which the latter were kept in suspension, have been described previously [16].

The suspensions were irradiated under aerobic and anaerobic conditions, using a Siemens Stabilivolt X-ray apparatus, as described previously [11].

Anaerobic conditions were maintained by constant nitrogen bubbling for 10 minutes before and during the whole period of irradiation.

After irradiation the suspension was plated on nutrient agar and Roberts C agar plates. After 24 hours incubation at 37° C, the colonies grown from the surviving cells were counted.

The compounds involved in the experiments were l-cysteine-HCl (Reanal), cysteamine (Fluka) and d-penicillamine (prepared in our laboratory), at concentrations of 10^{-2} *M*.

Results

First, the radioprotective effect of the above-mentioned compounds was compared under aerobic and anaerobic conditions.

In the experiments presented in Fig. 1 the "reaction" and "postirradiation" periods were practically omitted; the protective substances were added to the suspensions immediately before irradiation and the suspensions were diluted and plated directly after exposure.

Part A of Fig. 1 indicates that the lethal doses of ionizing radiation were reduced by a factor of 1.4 DRF by penicillamine, 1.5 DRF by cysteine, and 2.9 DRF by cysteamine.

Part B of Fig. 1 shows that anoxia *per se* decreased the sensitivity by 2.4 DRF. In the case of cell-suspensions irradiated under anoxic conditions the radioprotectors in question were, however, found to exert no or only a very slight protective effect. To quote an example, the 2.9 DRF arrived at

by irradiating cell-suspensions under aerobic conditions in the presence of cysteamine, and the 2.4 DRF obtained under aerobic conditions, failed to exert a cumulative effect.

In the second part of the experiments, the radioprotective substances were applied after an "optimal reaction" period.

It has been shown in previous studies[16] that the effectiveness of protective substances increased when, instead of applying them to *E. coli* B suspensions immediately before irradiation, they were added 10 to 60 minutes

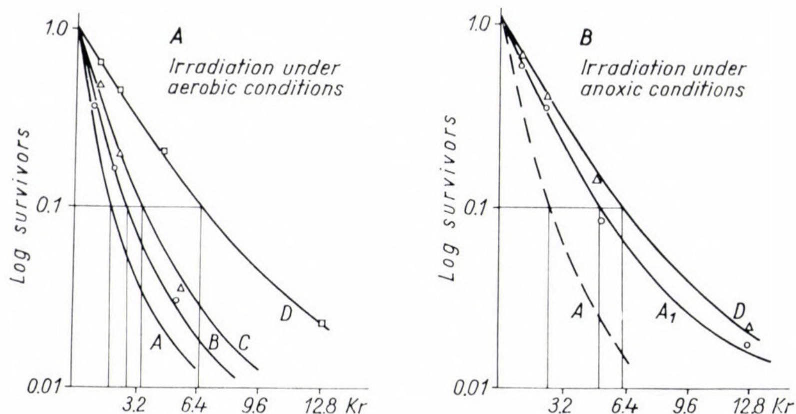

Fig. 1. Radioprotective effect of cysteine-cysteamine compounds on *E. coli* B. A: aerobic control; A_1: anoxic control; B: penicillamine; C: cysteine; D: cysteamine

earlier and then the suspensions were incubated at 27° C and 37° C, respectively. In these experiments the protective effect of cysteamine increased from 2.9 to 3.8 DRF, that of cysteine from 1.5 to 3.4 and that of penicillamine from 1.4 to 2.4, DRF. All incubations were effected at 37° C for periods of 15, 30 and 30 min., respectively.

Fig. 2 shows that the radioprotective substances exerted a protective effect in excess of that afforded by the anoxic control marked A_1. Hence the increased effect obtained in the course of an "optimal reaction" period could be observed in these experiments, too.

In the third series of experiments the bacteria irradiated under anoxic conditions were plated after observing an "optimal postirradiation" period. In these experiments the "reaction" period was omitted and the radioprotective substances were added to the suspensions 1 min. prior to irradiation.

Fig. 3 shows that the number of surviving *E. coli* B cells increased when incubated in the presence of cysteine-cysteamine compounds after having been irradiated under anoxic conditions for 30 min. at 37° C. During the postirradiation period, the effect of cysteine and of penicillamine increased considerably while that of cysteamine, only slightly.

The protective effect of cysteine-cysteamine derivatives added to the cell-suspensions 1 min. after irradiation under anoxic conditions has confirmed the above findings.

When the protective substances had been added to the suspensions 1 min. after irradiation, the effect was similar to that observed during postincubation periods. As in the above experiments, the effect was most pronounced when the cell-suspensions had been incubated at 37° C.

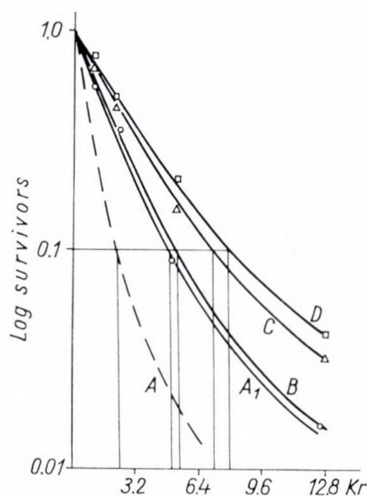

Fig. 2. Protective effect of cyteine-cysteamine compounds against irradiation under anoxic conditions after an optimal reaction period. A: aerobic control; B: penicillamine; C: cysteine; D: cysteamine; A_1: anoxic control

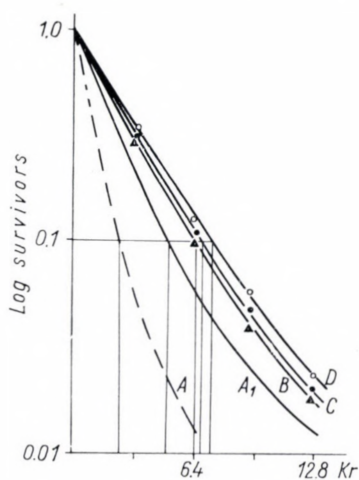

Fig. 3. Protective effect of cysteine-cysteamine compounds against irradiation under anoxic conditions after an optimal postirradiation period. A: aerobic control; A_1: anoxic control; B: penicillamine; C: cysteine; D: cysteamine

Next, the effect on radiation sensitivity of preirradiation conditions of culture was examined.

Fig. 5 indicates that the control curves representing the radiosensitivity of cultures grown for 14 hours in broth and for 18 hours in Roberts C medium, were similar. Both these cultures were measured at the end of their log-phase. Likewise, *E. coli* B control suspensions grown in nutrient broth for 10 hours and synthetic medium for 14 hours showed approximately identical radio-

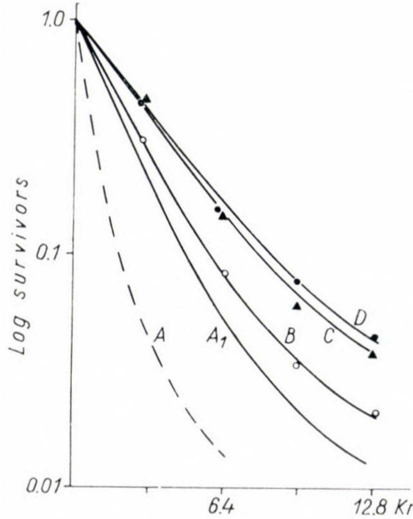

Fig. 4. Protective effect of cysteine-cysteamine compounds added 1 min. after irradiation under anoxic conditions. A: aerobic control; A_1: anoxic control; B: cysteamine; C: penicillamine; D: cysteine

sensitivity at mid-log phase. The radio-sensitivity of samples taken from cell-suspensions at mid-log phase were decreased by 1.5 DRF by cysteine at a concentration of 10^{-2} *M*, provided that the substance had been added just before irradiation and that this was immediately followed by plating. These results have not been included in Fig. 5.

Similar curves were obtained when an "optimal reaction" period was observed after the addition of cysteine. The four curves representing the dose response of the cell-suspensions protected by cysteine ran a similar course. A comparison of the radioprotection afforded by cysteine in the cell-suspensions with the controls, revealed that by observing an "optimal reaction" period and temperature, a DRF of 3.4 was obtained if the samples had been taken at the end of the log-phase from suspensions including cells previously found to possess the greatest radioresistance. On the other hand, with cells manifesting a greater radiosensitivity in the course of their growth, the DRF was 5.0.

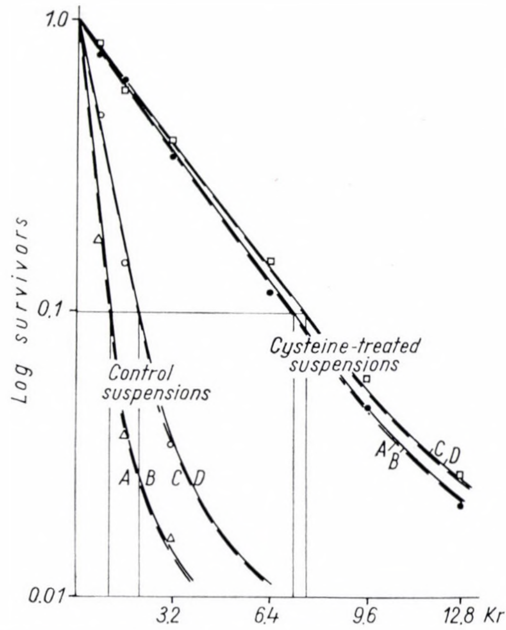

Fig. 5. Influence of preirradiation conditions of culture on the effect of cysteine. A: 10 hour broth; B: 14 hour Roberts C minimal medium; C: 14 hour broth; D: 18 hour Roberts C minimal medium cultures

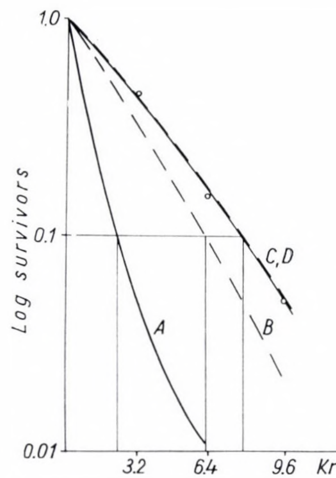

Fig. 6. Influence of postirradiation conditions of culture on the effect of cysteine. A: nutrient agar control; B: Roberts C agar control; C: nutrient agar with cysteine; D: Roberst C agar with cysteine

Finally, it was examined whether the effect of cysteine exerted in the postirradiation period remained unchanged when postirradiation incubation had been shifted in an optimal direction as regards survival, *i.e.* when after irradiation the bacteria were plated on Roberts C minimal agar.

Fig. 6 indicates that the experimental conditions which had previously been found to increase the protective effect of cysteine, exerted no favourable effect on the survival of bacteria. The survival of *E. coli* B failed to increase after having been cultured subsequently to irradiation on a minimal medium.

Discussion

The results allowed the following conclusions. The metabolic state of *E. coli* B which depends on the preirradiation conditions of culture, determines the degree of radioprotectivity. The radioprotection afforded by cysteine-cysteamine derivatives in the case of an optimal reaction period involved a DRF of 5.0 when the bacteria were removed at mid log-phase, while DRF considerably decreased when the cells were obtained at the most radioresistant phase. It follows that both the process taking place in the cells during the "optimal reaction" period as a result of the action of cysteine-cysteamine derivatives, and the physiological process occurring in the cells as a concomitant of their growth, are diminishing the radiosensitivity.

The role of cysteine-cysteamine derivatives in biosynthetic processes responsible for the development of radiation resistance has been stressed by several authors [12, 17].

Considering that, provided a 1 min. reaction- and postirradiation period was observed, the radioprotective effect of cysteine was diminished and that of cysteamine was abolished after irradiation under anoxic conditions, (a) the above substances act by giving rise to anoxia or hypoxia during irradiation, or also, (b) oxygen and cysteine-cysteamine compounds operate at an identical site of action.

Postirradiation conditions of culture may increase the percentage survival of *E. coli* B if a 1 min. postirradiation period is observed. This, however, fails to occur when the bacteria are diluted from the solutions of cysteine-cysteamine derivatives after an optimal postirradiation period. This would indicate that the site of action is identical with the one mentioned above. Hence the modification by cysteine-cysteamine compounds of the radiosensitivity of *E. coli* B involves:

(i) a physicochemical effect during irradiation; (ii) a metabolic effect before and after irradiation.

ad (i) In the course of irradiation the physicochemical effects are lessened or abolished by the lack of O_2 acting at the same level, while their metabolic effect exerted before and after irradiation is not affected.

ad (ii) Prevailing culture conditions decreased or abolished metabolic effect before and after irradiation, and thus neutralized those exerted by the compounds in question. These factors, however, did not influence the physico-chemical protective effect of cysteine-cysteamine compounds during irradiation.

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SENSITIVITY OF E. COLI B TO IRRADIATION

VI. EFFECT OF CYSTEINE ON DNA BREAKDOWN BY IONIZING IRRADIATION

By

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(Received April 12, 1965)

Summary. The effect of 0.1—0.001 *M* cysteine solutions on the irradiation-induced breakdown of DNA in *E. coli* B, and on the number of surviving cells has been studied. DNA breakdown was inhibited by 0.1—0.01 *M* but not by 0.001 *M* cysteine. No direct correlation has been revealed between DNA content and viable counts.

Ionizing radiation results in a partial breakdown of DNA in bacteria [1, 2]. The degree of the effect depends primarily on the dosage and on the post-irradiation metabolic state of the organism. When protein metabolism is inhibited after irradiation, DNA breakdown is greatly enhanced [2]. In contrast, citrate [1], glutamic and succinic acids [3] and high concentrations of salts (NaCl, KCl, $CaCl_2$, $MgSO_4$, sodium oxalate) [4] inhibit the degradation of DNA.

Cysteine is a protective substance which, when applied prior to irradiation, increases the rate of survival. From data in the literature [5, 6] and our own results [7] it has been concluded that the protective action of cysteine is due not merely to a physico-chemical effect during irradiation, but also to its active part in metabolism.

Accordingly, the purpose of the present investigation was to examine the postirradiation effect of cysteine in the irradiation-induced DNA breakdown and colony-forming ability of *E. coli* B.

Materials and methods

E. coli strain B was cultured aerobically in a synthetic medium [8] at 37° C until the optical density of the culture had reached 0.500 (Beckman model DU spectrophotometer). Then the cells were washed in physiological saline (pH 7.0) and resuspended in acetate buffer (pH 7.0).

Irradiation was performed at room temperature with a Siemens Stabilivolt apparatus operating at 180 KV, 12 mA and 3200 r/min. dose rate.

After irradiation the DNA content and viable count of part of the suspension was determined. Other parts were incubated aerobically at 37° C for 90 minutes in acetate buffer and in acetate buffer supplemented with 1-cysteine HCl at different concentrations (Reanal, Budapest). At 0, 10, 30, 60 and 90 minutes the suspensions were sampled for DNA content and viable count determination.

Extraction and determination of DNA were performed as described by Burton [9]. Viable cell counts were determined on agar plates incubated for 24 hours at 37° C.

Results

Fig. 1 presents the dose-response curves. No alteration was observed in the DNA content of control (non-irradiated) bacteria throughout the whole incubation period.

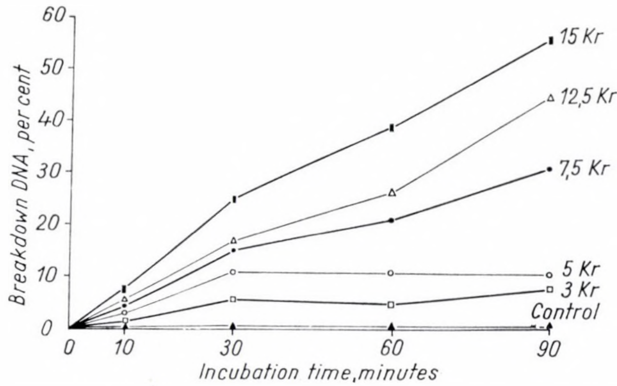

Fig. 1. Dose-response curves showing the degree of DNA breakdown in irradiated as compared to non-irradiated bacteria

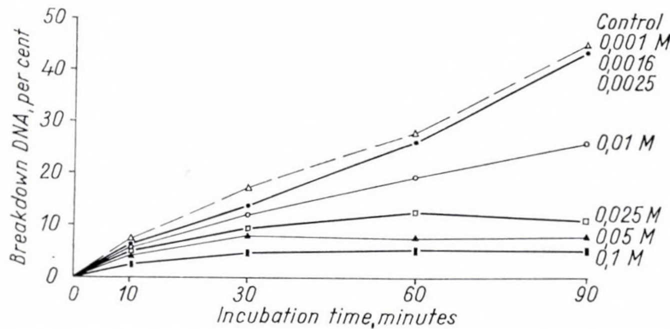

Fig. 2. DNA breakdown in bacteria irradiated with a dose of 12.5 Kr, then suspended in acetate buffer containing cysteine at different concentrations. Control: irradiated bacteria incubated in acetate buffer

Fig. 2 indicates the effect of 0.1, 0.05, 0.025, 0.01, 0.0025, 0.0016 and 0.001 *M* cysteine on DNA breakdown. It is seen that 0.1 *M* cysteine definitely inhibited the postirradiation decrease in DNA content (breakdown of only 4 per cent was observed). In the presence of 0.01 *M* cysteine a breakdown of 25 per cent occurred. At 0.001 *M* concentration, cysteine exerted no effect on DNA breakdown.

Fig. 3 shows the effect of cysteine on the number of surviving cells. During the 90 minutes incubation cysteine concentrations inhibiting DNA breakdown (0.1 and 0.01 *M*) exerted practically no effect on viable counts.

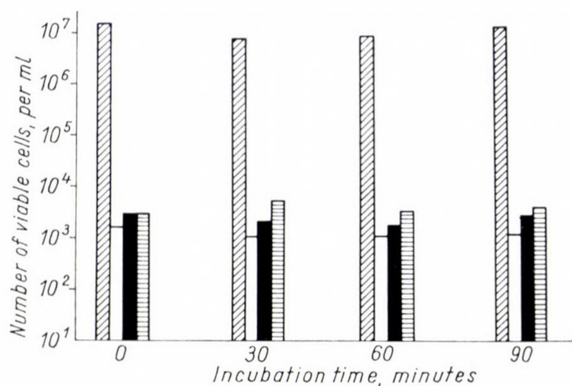

Fig. 3. Viable counts obtained after exposure to 12.5 Kr irradiation. ▨ = non-irradiated bacteria incubated in acetate buffer without cysteine or with 0.1 and 0.01 *M* cysteine; □ = irradiated bacteria incubated in acetate buffer; ■ = irradiated bacteria incubated in acetate buffer with 0.1 *M* cysteine; ▤ = irradiated bacteria incubated in acetate buffer with 0.01 *M* cysteine

Discussion

During 90 minutes incubation in acetate buffer no change occurred in the number of viable cells and DNA content of non-irradiated bacteria. In the irradiated culture the DNA content decreased by 45 per cent and the bacterial count fell from 10^7 to 10^3 per ml.

The degree of DNA breakdown can be modified by incubating the irradiated bacteria in the presence of cysteine at different concentrations. As DNA breakdown is inhibited at high cysteine concentrations only, it may be assumed that the phenomenon is aspecific and similar to that observed in concentrated solutions of various substances. Due to a dehydrating effect, the osmotic pressure of the milieu influences the radiosensitivity of cells by altering enzymic reactions responsible for irradiation-induced DNA breakdown [4].

No correlation was revealed between DNA breakdown and the number of surviving cells. In experiments where DNA breakdown was maximal during the 90 minute incubation period, the viable cell count remained unchanged. When cysteine inhibited the breakdown of DNA, the viable count slightly increased.

The slight but significant increase in the bacterial count of irradiated suspensions incubated in the presence of cysteine is due to a postirradiation effect of this substance [10], the biochemical mechanism of which is still unknown.

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BREAKDOWN OF AMINO ACIDS BY ENTEROBACTERIACEAE

V. BREAKDOWN OF GLYCINE IN SIMPLE MEDIUM

By

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(Received July 9, 1965)

Summary. The frequency of glycine positivity among enteric bacteria increases when instead of a complex medium a solution containing only glycine and phosphate is used. The method seems suitable for the differentiation of enteric bacteria and for revealing biotypes within some species, as with alkalization macrotest only *S. paratyphi A*, Shigella and Proteus-Providencia, with ninhydrin test only certain Shigella strains may react negatively. With alkalization microtest, *S. paratyphi A*, Shigella, Morganella and Providencia gave no early positive result.

Our investigations into the breakdown of amino acids have so far been carried out in media containing inorganic and organic substances in addition to the substrate. These media allowed multiplication of certain bacteria [3, 4] or the formation of coloured complex compound from the produced keto acids and the iron salt reagent [5]. As in alkalization and ninhydrin tests made with large inocula these requirements might be neglected, we have attempted to perform the reaction in a simple amino acid solution.

Materials and methods

In preliminary experiments the following media have proved suitable.

Alkalization test (AT): KH_2PO_4, 0.10 g; distilled water, 100 ml; glycine, 0.20 g; fresh phenol red solution, 2.5 ml (phenolsulphonphthalein, 0.01 g; *N* NaOH, 0.40 ml; distilled water, 4.6 ml); the medium was adjusted to pH 5.4.

Ninhydrin test (NT): KH_2PO_4, 0.10 g; distilled water, 100 ml; glycine 0.05 g; pH 7.2—7.4. The control medium for NT was prepared by adding 1 ml 1 per cent merthiolate (ethylmercurithiosalicylate) to 100 ml sterilized medium.

After sterilization at 115° C for 15 minutes the pH is checked and corrected if necessary. The media can be stored in the refrigerator for 3 weeks.

Technical procedures for macro and micro methods of AT and for NT and evaluation of results were the same as described in reference 6.

Results

By examining 618 strains with macro and micro methods of AT, the results presented in Table I were obtained. With the macrotest, considerably more strains (including numerous Shigella cultures) were glycine positive in

Table I

Result of alkalization test in simple glycine medium

	No. of strains	Macrotest					Microtest				
		—	(+)	+	Of the positives		—	(+)	+	of the positives	
					(++)	++				(++)	++
E. coli	74	—	6	68	32	34	3	18	53	61	4
S. paratyphi A	7	3	4	—	—	—	6	1	—	—	—
Other Salmonella (S. paratyphi B, typhi-murium, bareilly, typhi)	115	—	9	106	75	36	—	32	83	105	4
Klebsiella	25	—	1	24	9	14	2	4	19	20	—
Citrobacter	23	—	—	23	18	1	1	1	21	17	5
Enterobacter	31	—	2	29	8	21	1	3	27	23	4
Serratia	30	—	—	30	8	19	—	4	26	17	9
Arizona	15	—	—	15	10	5	—	—	15	8	6
Sh. dysenteriae 1—10	24	8	8	8	4	7	22	2	—	—	—
Sh. flexneri 1	36	31	4	1	—	—	33	3	—	—	—
Sh. flexneri 2	30	29	1	—	—	—	28	2	—	—	—
Sh. flexneri 3	21	16	—	5	—	5	21	—	—	—	—
Sh. flexneri 4	15	6	1	8	1	8	14	1	—	—	—
Sh. flexneri 5	7	7	—	—	—	—	7	—	—	—	—
Sh. flexneri 6	23	9	2	12	3	10	14	9	—	—	—
Sh. boydii 1—15	29	18	3	8	1	7	20	9	—	—	—
Sh. sonnei	15	6	4	5	—	5	4	11	—	2	—
P. vulgaris	20	1	—	19	1	16	—	6	14	19	—
P. mirabilis	20	3	10	7	13	—	1	3	16	16	2
Morganella	20	15	—	5	2	3	18	2	—	—	—
Rettgerella	18	2	1	15	—	15	1	12	5	10	—
Providencia	20	2	1	17	3	14	4	16	—	6	—
Total	618	156	57	405	188	220	200	139	279	304	34

Key: Macrotest
— = No red colour within 7 days
(+) = Red colour within 4—7 days
+ = Red colour within 3 days
(++) = Intensive red colour within 4—7 days
++ = Intensive red colour within 3 days

Microtest
— = No red colour within 24 hours
(+) = Red colour within 5—24 hours
+ = Red colour within 4 hours
(++) = Intensive red colour within 5—24 hours
++ = Intensive red colour within 4 hours

the simple medium than in the complex medium. The macrotest, however, seems suitable also for differentiation, as glycine negative organisms occur only among *S. paratyphi A*, Shigella and Proteus-Providencia strains. The microtest is less sensitive; *S. paratyphi A*, Shigella, Morganella and Providencia cultures gave no early positive results.

With the macrotest, some Shigella strains showed rapid alkalization; thus within the same serotype glycine negative and strongly glycine positive biovariants were encountered. Our *Sh. flexneri* 6 strains corresponded to the Manchester biotype according to carbohydrate fermentation reactions; therefore, on the basis of glycine breakdown a further subdivision may be performed.

The question arose whether differences in the breakdown of glycine were not associated with the virulence of shigellae. Conjunctival infection of guinea pigs [1, 2] was carried out with 42 strains. No association was found between virulence and glycine reaction. Out of the examined 37 glycine negative strains, 26 were avirulent and 11 were virulent; among 5 glycine positive, ++ strains, 2 avirulent and 3 virulent cultures occurred.

Most positive results were obtained with NT, which was positive with all *S. paratyphi A* and Proteus-Providencia strains. Among shigellae more cultures reacted positively with this test as with AT. A negative NT was found to be definitely indicative of Shigella.

Discussion

Preliminary experiments have shown that the breakdown of glycine ensues later and less frequently when the amino acid-phosphate medium contains substances other than those used in the previous studies [6]. This effect was particularly evident when $MgSO_4$ had been incorporated into the medium. As various glycine solutions gave different results, the question arose whether in routine work the simple or the combined medium should be used.

In our opinion the microtest performed in simple glycine-phosphate solution is the more suitable one. This method is non-laborious and yields readable results often within 4, but at most in 24 hours. Most members of Enterobacteriaceae can be differentiated from *S. paratyphi A*, Shigella, Morganella and Providencia. The macrotest carried out in the simple medium requires a 7 day incubation period. This reaction, especially when NT is performed in parallel, allows a further differentiation.

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STABILITY AND PURIFICATION OF ANTIPHOSPHORYLASE

By

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(Received July 29, 1965)

Summary. Stability of antibodies formed in roosters against rabbit muscle phosphorylase *b* and exposed to various acid and alkaline pH values and temperatures, has been investigated. Purification of antiphosphorylase with a yield of 60—75 per cent could be accomplished by dissolving the washed specific precipitate in a pH 11.0 glycine-sucrose buffer, followed by heat treatment at 56°C.

Purification of antibodies from immune sera can be achieved along two lines [1], namely, by physico-chemical fractionation or by means of specific immuno-chemical methods. In the latter case, the washed specific antigen-antibody complex is dissociated and the antibody molecules are separated from the antigen. The method of choice for dissociation and especially for antibody separation depends on the specific physico-chemical properties of the given antigen and antibody. The arising difficulties can be partially overcome by employing specific immunosorbents [2—4]. Preparation of these may, however, be complicated, particularly in the case of labile antigens.

Since no data on the stability of avian antibodies have been available, as the first step the stability of antibodies produced in roosters against rabbit muscle phosphorylase *b* [5] was determined, employing at varying temperatures different acid and alkaline buffer solutions. The higher stability of antiphosphorylase as compared to that of the homologous antigen allowed antibody separation by denaturation and removal of antigen from the dissociated specific antigen-antibody complex.

Materials and methods

Immunization of roosters with crystalline rabbit muscle phosphorylase *b*, harvesting of antisera and determination of phosphorylase and antiphosphorylase activity was carried out as described previously [5]. Factors influencing phosphorylase activity in a non-specific fashion (shift in pH values, materials of low molecular weight) were taken into correction as detailed elsewhere [6]. Antibody content of the antiserum tested equalled 320 μg-N per ml.

By checking the stability of antiphosphorylase and by calculating the yield of antibodies from the specific precipitate, the inhibitory effect of antibodies on the antigen's enzymatic activity was determined and expressed as the percentage of control values.

Heat-treatment was performed on antisera diluted with equal amounts of physiological saline or 40 per cent sucrose solution, and incubated for 10 minutes in a water-bath of varying temperature.

Stability at different pH values was estimated with antisera diluted 1 : 10 with 0.1 *M* glycine buffer of corresponding pH, and complemented by half volume of physiological saline or 60 per cent sucrose solution. Aliquots of each test mixture were incubated at 20°C for 40 minutes, other aliquots at 56°C for 20 minutes and, subsequently, at 20°C for 20 minutes. After incubation, the systems were neutralized with tris-buffer (tris-hydroxymethyl-amino-methane) or acetic acid pH 6.8, and tested for antiphosphorylase activity.

In order to determine the yield of antiphosphorylase-antibodies from the specific precipitate, to antiphosphorylase sera inactivated previously at 56°C, equivalent amounts of crystalline rabbit muscle phosphorylase *b* were added. The mixtures were distributed in identical volumes in centrifuge tubes and incubated at 37°C for 30 minutes and at 0°C for 60 minutes. The resulting precipitate was centrifuged off and washed with chilled physiological saline. Precipitation of antibodies was carried out in physiological saline containing 1 *mM* ethylenediaminetetraacetate. High salt concentrations were avoided in order to decrease the quantity of macroglobulins co-precipitating non-specifically with the antigen-antibody complex [7]. Precipitate samples were dissolved in 0.1 *M* glycine buffer solution of varying pH, with or without the addition of sucrose, and incubated at 20°C for 30 minutes or at 56°C for 20 minutes and at 20°C for 10 minutes. The samples were then neutralized with tris-buffer or acetic acid, centrifuged, and the supernatant was tested for antiphosphorylase activity.

Final separation was achieved by concentrating the supernatants by dialysis against concentrated dextran solution followed by dialysis against physiological saline. The resulting precipitate was discarded.

Results and discussion

Fig. 1 demonstrates the residual antiphosphorylase activity after 10 minutes treatment at different temperatures, expressed in the percentage of control values. At 65°C, antiphosphorylase activity of the sera decreased by about 50 per cent, while treatment at 75°C completely abolished the inhibitory activity of antisera. The addition of 20 per cent sucrose exerted a protective effect against heat-denaturation of the antiphosphorylase.

Fig. 2 demonstrates the stability of antiphosphorylase antibodies in acid buffer solutions. Antiphosphorylase activity decreased by about 15 per cent at pH 3.0, while only about 38 per cent of the original activity remained after dilution with 0.1 *N* hydrochloric acid, incubation and neutralization. Treatment at 56°C at acid pH completely abolished antiphosphorylase activity. As can be seen, results obtained at acid pH values were not influenced by the presence of sucrose.

Fig. 3 shows the effect of treatment with glycine buffer solutions of alkaline pH values. Alkaline medium up to pH 12.5 was found to be far less deleterious to antiphosphorylase than acid pH. The protective effect of sucrose against heat-denaturation was also marked.

Comparing the stability of rooster antibodies to that of rabbit antibodies, precipitin activity of certain rabbit antibodies was completely abolished by heat-treatment at 75°C for 10 minutes, although these antibodies retained most of their complement binding capacity and biological activity [8, 9]. It was shown by Farah *et al.* [10] that heat-treatment at 70°C for 30 minutes of rabbit antibodies in the presence of serum proteins resulted in a partial trans-

formation to univalent antibodies, caused by the interaction of antibody molecules with serum albumin due to heat-treatment.

With knowledge of the stability of antiphosphorylase, its purification from the specific precipitates was attempted. Since phosphorylase was found

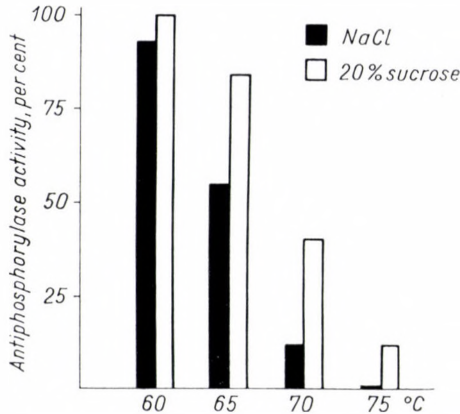

Fig. 1. Effect of heat treatment on antiphosphorylase activity

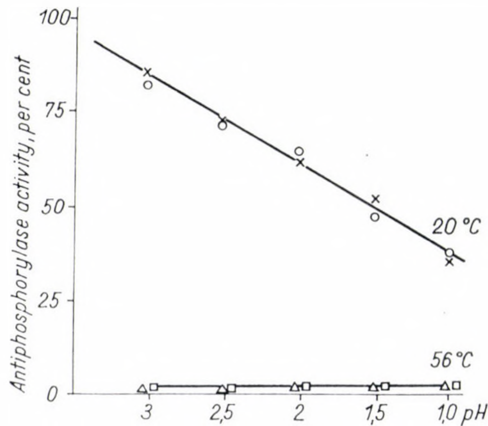

Fig. 2. Stability of antiphosphorylase at acid pH values
x—x NaCl at 20°C; o—o 20 per cent sucrose at 20°C;
△—△ NaCl at 56°C; □—□ 20 per cent sucrose at 56°C

to be especially sensitive to acid pH values, the specific precipitate was dissolved in acid glycine buffer. After neutralization the precipitates were partly re-formed, a significant part of antiphosphorylase activity, however, was left in solution. This was explained by the fact that, in the course of dissolving and dissociating the precipitate, the phosphorylase underwent irreversible denaturation and precipitation. After neutralization, a certain amount of antibody was

again bound by the denaturated phosphorylase, a significant portion of neutralizing antibodies, however, remained in solution. The yield of anti-phosphorylase was essentially the same whether centrifugation was carried out prior to or after neutralization. Although the lower the pH, the more complete the dissociation of antigen-antibody complex, below pH 3.5 the destruction

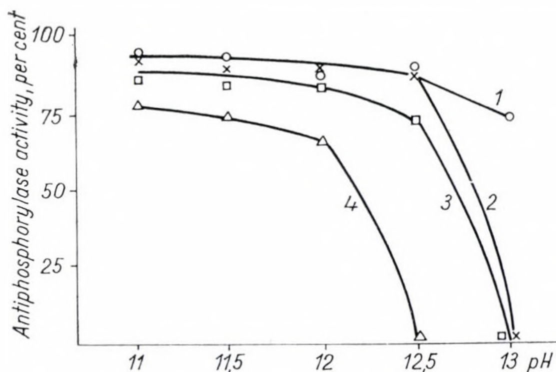

Fig. 3. Stability of antiphosphorylase at alkaline pH values. 1 : 20 per cent sucrose at 20°C; 2: NaCl at 20°C; 3 : 20 per cent sucrose at 56°C; 4 : NaCl at 56°C

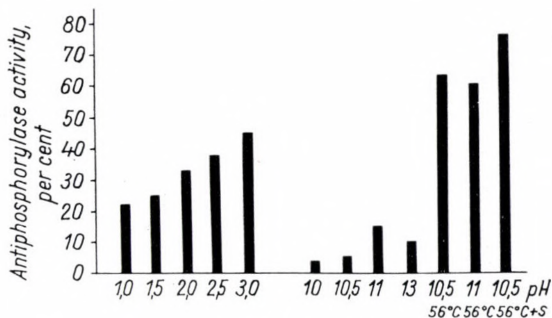

Fig. 4. Antiphosphorylase activity recovered from the specific precipitate

of antibodies was also more marked. Thus, at pH 3.0 more antibody could be recovered (45 per cent), than at pH 1.0 (20 per cent). During the formation of precipitates after neutralization, the co-precipitation of certain macroglobulins of non-antibody nature, adsorbed into the original precipitate, had also to be taken into consideration [7].

Alkaline pH values favoured the dissolution of precipitates, and the deterioration of antibodies was less striking. After neutralization, however, only a minute portion of antiphosphorylase activity could be recovered, due to the inadequate denaturation of the antigen. Thus a combined heat-treatment was attempted. Notably, 56°C heat-treatment for 20 minutes resulted in a

80—95 per cent yield of antiphosphorylase activity from the dilute, not centrifuged phosphorylase-antiphosphorylase complex, due to irreversible denaturation of phosphorylase and elution of antibodies. Heat-treatment of the undiluted, washed precipitates failed, however, to elute antiphosphorylase. Consequently, the washed precipitate was dissolved in a glycine buffer of pH 10.5—11.0 and subjected to 56°C heat-treatment for 20 minutes. The 60 per cent yield thus achieved was increased to 76 per cent on the addition of 20 per cent sucrose to the buffer solution. Sucrose acted by protecting antibodies against heat-denaturation and by facilitating dissolution of the specific precipitate (see Fig. 4).

The procedure proved to be most appropriate for isolating antiphosphorylase. The preparations thus obtained retained their precipitating ability after concentration and dialysis against NaCl, and contained 90 per cent of homogeneous antibody of 7.5 S sedimentation constant in the ultracentrifuge. The inhomogeneous rest of about 10 per cent probably represented aggregates of antigen-antibody complexes of higher sedimentation rate.

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REDUCTION OF HEAT AND RADIATION RESISTANCE OF BACILLUS CEREUS SPORES BY INITIATING GERMINATION

By

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(Received October 27, 1965)

Summary. After heat-activation at 60°C for 30 minutes, 90 per cent of *B. cereus* spores germinated within 50 minutes at 30°C in an aqueous solution containing adenosine and l-alanine at 0.4 mg/ml concentration. The activating effect of 60°C heat was evident after 4 minutes and increased proportionally to the time of incubation.

Germinated spores became less refractive and less resistant to heat and radiation.

Addition of adenosine and l-alanine increased the rate and frequency of germination in pea-extract and in a medium of green peas placed in a liquor containing salt and sugar. The effect of additives stimulating germination was manifest in a few minutes incubation at 30°C. The reduction of heat and radiation sensitivity of the population was in good agreement with phase microscopic observations of the cell-state distribution.

Combined use of heat-activation and germinants initiated germination and increased sensitivity in an overwhelming majority of spores even at cell counts of 10^8 to 10^9/ml, and in the medium used for spore production.

The complicated development of bacterial spores into vegetative cells is characterized by two main stages. The first step consists of "germination" [4] in the strict sense, the second, also referred to as the "postgerminative stage" [23], corresponds to the outgrowth of vegetative forms from the germinated spores [20].

In the course of germination the highly refractive dormant spore changes in 15—30 minutes into a dark, germinated form [17, 24, 27, 29] then swells gradually and ruptures the spore coat and finally the vegetative cell grows out in some hours. Conditions inducing germination do not necessarily improve the outgrowth, since temperature, hydrogen ion concentration, nutrient and water requirements are usually different in the two stages [3, 13, 14]. Thus the first stage may take place at temperatures which inhibit the outgrowth [18, 19]. Vas and Proszt [35] showed that the influence of temperature on germination was a function of the medium's pH. The stage of outgrowth is more sensitive to nutritional requirements than the stage of germination.

During germination a considerable amount of organic material is released and the spore loses about 1/3 of its dry weight. The majority of the dipicolinic acid content is also excreted into the medium [25, 26].

Germination is initiated by certain environmental factors (specific substances, thermal and mechanic effects). Evans and Curran [7] showed that sublethal exposure to higher temperatures (60—110°C) stimulates germination of bacterial spores.

HILLS [11, 12] was the first to observe that germination is induced in solutions containing certain amino acids and nucleosides. Several authors have then shown that, depending on the species, strain and previous conditions [21], germination may be initiated with or without parallel "heat-activation" by l-alanine, glucose, adenosine [32], various small molecule fatty acids, amines, surface active quaternary ammonium compounds [30, 31] and chelating agents such as ethylenediamine-tetraacetate or even calcium dipicolinate [28]. Mechanical injury of the spore wall causing a release of spore peptides and dipicolinic acid also results in a decrease of refractivity and thermal resistance [29].

Our previous comparative studies on *Bacillus cereus* revealed that the resistance of spores dropped practically to that of vegetative cells in the first phase of germination [8]. Introduction of a method for lowering the resistance of dormant spores would undoubtedly be a great progress especially in industrial food preservation. Although germinant substances and heat treatment inducing germination obviously offer this possibility, experiments as to this problem have scarcely been published [1, 16]. In the present studies we examined the effect and practical applicability of some substances known as germination-initiating agents and exposure to mild heat as well as the combination of the two procedures. As a test organism we used *B. cereus*, which is a common member of the aerobic bacterial flora of food [2] and bears some hygienic importance [22]. In order to render our studies applicable to the irradiation method of food preservation, changes in the radiosensitivity of *B. cereus* have also been examined.

Materials and methods

Organism. The *B. cereus* strain isolated from green peas and extensively studied in our heat and radiation sensitivity experiments [9, 10] was used throughout.

Culturing of the test strain and preparation of dormant spore suspensions. Cultivation and maintenance of the test strain were performed by use of the universal medium [36] employed either in liquid form or as a solid medium prepared with agar. This medium contained the following ingredients: non-acid whey, 200 ml; yeast extract (1 : 10), 100 ml; meat extract, 4 g; peptone, 2 g; glucose, 10 g; water, 700 ml. The medium was adjusted to pH 7.2 and sterilized at 115°C for 30 minutes. During sterilization the pH decreased to about 6.4.

In order to obtain dormant spores, 1000 ml of universal medium was supplemented prior to sterilization with 4 per cent agar and 16.5 ml of the following solution: Na_2HPO_4, 0.01 *M*; KH_2PO_4, 0.002 *M*; NaCl, 0.01 *M*; $MgSO_4$, 0.00025 *M*; $CaCl_2$, 0.0001 *M*; $MnSO_4$, 0.00001 *M*; $FeSO_4$, 0.00001 *M* dissolved in distilled water. Each plate of this medium was inoculated by streaking evenly on the surface 0.1 ml suspension containing 10^8 cells per ml. The seeded media were incubated at 30°C, which was the optimal temperature for the multiplication of the test organism. As shown with phase microscopy, after 5 days' incubation most vegetative cells disintegrated and the population contained 90 to 100 per cent dormant spores. The spores were then harvested by the aid of a bent glass rod and suspended in sterile water, washed 4 times in sterile water by centrifugation and finally resuspended in 1/15 *M* pH 7.0 or pH 6.0 phosphate buffer so as to contain 10^{11} spores per ml. The spore suspension was stored at 4°C; in this manner the majority of the spores remained viable for 2 years. However, in the experiments suspension stored not more than for 1 month were only used. When 50 plates were seeded, the above method yielded approximately 1.5 g or 10^{12} spores.

Total counts were usually estimated by the slide-chamber method [34], sometimes a Helber chamber was used. As at least 200—500 cells were counted in each preparation, the 95 per cent confidence limits for $\bar{x}$ microscope fields were less than $\bar{x} \pm 0.15\,\bar{x}$ [5].

Viable counts were determined by preparing 3 parallel plates for each dilution of the suspension. The pea-extract agar used in these experiments was prepared as follows: 1 part of green peas was autoclaved with 1 part of distilled water for 60 minutes, after adding 2 per cent agar to the filtered extract, sterilization was carried out at 0.5 atm. for 30 minutes.

The plates were read after 48 hours incubation at 30°C. Only plates showing 30 to 300 colonies were used for the calculation of viable counts. The total error (standard deviation of the mean) of the arithmetic average counts ($\bar{x}$) for the corresponding dilution was estimated according to the formula

$$s\,(\%) = \pm \sqrt{(\text{dilution error } \%)^2 + (\text{distribution error})^2} \qquad 1.$$

The error of viable counts in the pour plate method originates from the dilution error and from the distribution error of colonies (viable cells) [15].

Distribution error of colonies can be expressed with the standard error of the average counts of parallel plates:

$$\text{Distribution error} = \pm \sqrt{\frac{\sum x_i^2 - \frac{(\Sigma x_i)^2}{n}}{n-1}} \qquad 2.$$

where x_i is the number of colonies on plate "i" and n is the number of parallel plates. The distribution error expressed as the percentage of the arithmetic mean of colonies ($\bar{x}$) can be substituted in equation *1.*

In order to estimate dilution errors, at first the standard deviations for 1 ml graded pipettes and for the 9 ml dilution blanks were determined. Both values were approximately ± 3 per cent (± 0.03 ml and ± 0.3 ml, respectively). From the degree of dilution and standard deviations the percentage dilution error at the given level was obtained from the table of JENNISON and WADSWORTH [15] and substituted in equation *1.*

Thus the calculated total error according to the definition of standard deviation means a probability of 68 per cent that the actual viable count is within $\pm\, s\,\%$ of the value calculated from the arithmetic average of the number of colonies.

Irradiation was performed without filters by means of a "Stabil 250" X-ray apparatus operating at 250 kV peak voltage and 15 mA tube current. The dose rate as measured by use of the ferrous sulphate dosimeter [37] at the same site where the samples were placed, was 3.4 krad/min. Irradiations were performed at room temperature.

Heat treatment. The suspensions were measured into thin-walled vials 20 mm in diameter. Care was exercised not to touch with pipettes the upper parts of the containers. The vials were then placed in a thermostatically regulated glycerol bath so that the surface of the suspension was always some centimetres below the bath surface. In order to increase heat exchange, the vials were kept in constant movement throughout incubation. Thus the samples reached the temperature of the glycerol bath within 1 minute.

Cell-state distribution was examined by a phase contrast microscope supplied with a 90× immersion objective at 2025× total magnification in petrolatum-sealed preparations 0.01 mm thick [27]. When nutrient medium was used as a suspending liquor, germination or growth was stopped by the addition of 0.05 ml 4 per cent mercuric chloride per ml of suspension. Cell-state distribution was determined by observing some hundreds of cells distributed in 20—30 microscope fields. Germinating spores and various stages of postgerminative development were recorded as follows.

Bright spores: highly refractive spores corresponded to dormant spores.

Less refractive spores were not so shiny as dormant spores, but were still more refractive than vegetative cells; in size they were similar to dormant spores.

Dark spores were similar in refractivity to vegetative cells, but they were not larger than dormant spores.

Outgrowing cells. This term included all stages between dark spores and vegetative cells (swollen, dark spores, elongated cells and cells losing their spore coats).

Results

Germination of B. cereus spores in nutrient medium. First, the germination rate and frequency of untreated dormant spores were examined. The spores were incubated at 30°C in various media at approximate counts of 10^8/ml. The suspensions were sampled at 5 minute intervals and examined under the phase contrast microscope. Repeated examination of dormant spores stored for different periods of time showed germination in universal medium only in 30

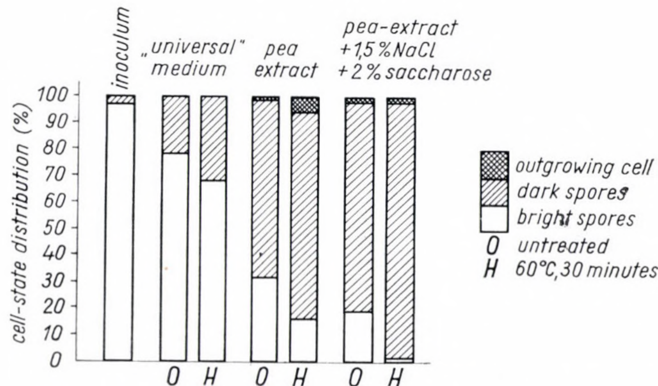


Fig. 1. Germination of spores in different media after 10 minutes at 30°C

to 60 per cent. In pea-extract the frequency of germinated spores was 45 to 80 per cent after 20 to 30 minutes incubation; no further increase was observed in the number of germinating spores even after 80 minutes. When, however, the concentrated aqueous or phosphate buffer suspension of dormant spores had been kept prior to inoculation into nutrient medium at 60°C for 30 minutes or at 80°C for 10 minutes, the change from dormant to germinated (dark) spores exceeded 90 per cent in both nutrient media. If pea-extract, in order to correspond to canned pea liquor, was supplemented with 1.5 per cent NaCl and 2 per cent sucrose, the proportion of germinated cells was further increased. Fig. 1 presents the result of an experiment revealing a typical cell-state distribution after 10 minute incubation.

Initiation of spore germination by exposure to mild heat and stimulating substances. On the basis of data in the literature [11, 12, 38], it was examined whether in media deficient in nutrient materials spore germination was induced by mild heating, d-glucose, l-alanine, adenosine or with the combination of these agents. The substances were added to spores suspended in pH 7.0 phosphate buffer. Aliquots of the suspension were pre-heated to 60°C for 30 minutes, other aliquots were not exposed to heat. Incubation temperature was 30° C and the total counts were approximately 10^8 per ml. Results are shown in Table I.

Table I

Percentage frequency of dark spores

Additive	Concentration, mg/ml	Heat activation	Incubation 30	60	90	120	24 hours
			minutes				
—	—	—	.	2.9	3.3	3.1	3.7
—	—	+	.	4.4	.	2.6	.
d-glucose	0.9	—	.	.	.	.	3.7
d-glucose + l-alanine	0.9+0.1	—	.	.	4.5	.	6.4
l-alanine	0.1	—	.	.	.	2.8	.
l-alanine	0.4	—	.	6.0	.	4.7	.
l-alanine	0.1	+	.	.	.	4.0	.
l-alanine	0.4	+	.	.	.	3.8	.
Adenosine	0.1	—	.	.	.	4.2	.
Adenosine	0.4	—	.	.	.	5.8	.
Adenosine	0.1	+	11.6	12.7	7.4	.	.
Adenosine	0.4	+	15.3	.	18.3	29.1	.
l-alanine + adenosine	0.4+0.4	—	21.7	25.8	.	36.9	.
l-alanine + adenosine	0.4+0.4	+	85.3	91.9	93.7	89.7	.

. = Not examined.
Proportion of dark spores in the inoculum was 2.4 per cent.

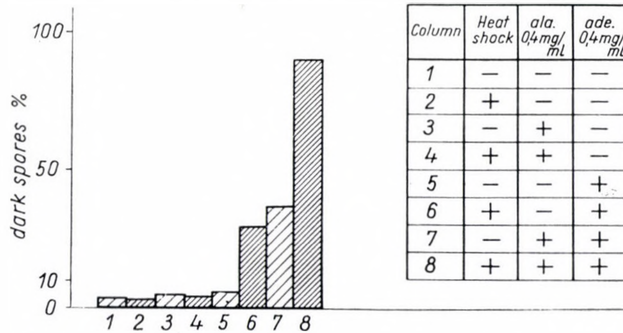
Table I indicates that in the concentrations used neither d-glucose nor l-alanine stimulated the germination of dormant spores. In the absence of nutrient materials a previous heating was also ineffective. In the presence of adenosine the number of dark spores in pre-heated suspension increased. With a combination of l-alanine and adenosine about one third of the spores showed signs of germination even without previous heating. Pre-heating and subsequent treatment with these substances resulted in 90 per cent germination in the absence of other nutrients. For the sake of a better comparison, characteristic values found after 120 minutes incubation are presented in Fig. 2.

Minimum period of heat-activation. In these experiments the minimum time of exposure to heat needed for stimulating germination in the presence of adenosine and l-alanine was determined. The spores were suspended in pH 6.0 buffer containing both germinants at 4 mg/ml concentration. Results are shown in Fig. 3.

It is seen that the activating effect of heating to 60° C was evident after 4 minutes and within the 30 minute observation period it increased proportionally to the time of exposure.

In further experiments it was examined whether there was a decrease in the radiation and thermal resistance of spores that had been subjected to the above initiating effects.

Change in radiation resistance due to initiation of germination. In the first experiment dormant spores after an exposure to 60°C for 30 minutes were



Column	Heat shock	ala. 0,4mg/ml	ade. 0,4mg/ml
1	–	–	–
2	+	–	–
3	–	+	–
4	+	+	–
5	–	–	+
6	+	–	+
7	–	+	+
8	+	+	+

Fig. 2. Initiating effect on germination of previous heat-activation at 60°C for 30 minutes and adenosine and l-alanine treatment. Per cent of dark spores after 120 minutes at 30°C

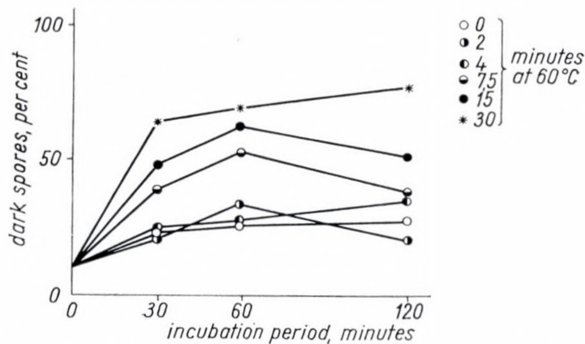


Fig. 3. Effect of the time of previous heat-activition on the germination of *B. cereus* spores in the presence of l-alanine and adenosine

suspended in distilled water and in aqueous solutions of adenosine and l-alanine. The suspensions were incubated at 30°C for 60 minutes then irradiated with a dose of 200 krad. The percentage of dark spores and viable counts was determined in all phases of the experiment. In samples containing no adenosine and l-alanine the frequency of dark spores remained at the starting level (8.5 per cent), while in those supplied with germinants 95.5 per cent of the dormant spores underwent germination. Irradiation caused a considerably higher reduction of viable counts in the initiated suspension than in the suspension containing spores not "compelled" to germinate. The findings are presented in Fig. 4.

As compared to the control, the reduction in the number of viable cells receiving stimulating treatment before irradiation, was higher by 3 exponents. Heating *per se* did not change the viability and radiation resistance of dormant spores. These data were in good agreement with the 200 krad points of survival curves obtained in previous experiments [9].

Reduction of resistance of B. cereus spores in pea-extract after initiation of germination. In order to study the reduction of resistance in food model, sterile pea-extract was seeded with dormant spores. Part of the extract was supplemented with adenosine and 1-alanine to final concentrations of 0.2 mg per ml.

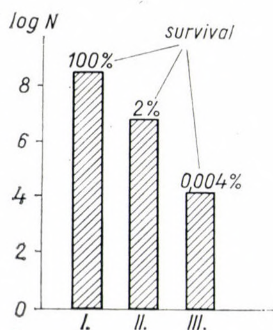


Fig. 4. Effect of germination initiation on the radiation resistance of spores. I = 60°C, 30 minutes; II = 60°C, 30 minutes + 200 krad; III = 60°C, 30 minutes + Ade, Ala + 200 krad

The inoculated media were partly heated at 60°C for 30 minutes, then incubated at 30°C for 50 minutes, partly kept at 30°C for 80 minutes. Subsequently the samples were heated to 90°C for 5 minutes or irradiated with 200 krad doses. Viable counts and cell-state distribution are presented in Fig. 5. Squares including the viable count value points of the graph express the limits of total error.

It is seen that dormant spores germinated in approximately 90 per cent without pre-heating. Heating to 60°C had instead of a stimulating a delaying effect, as germination during heat-activation took place probably considerably slower than at 30°C. KNAYSI [19] found that the maximum temperature for the first stage of germination in *B. cereus* strain C_3 was 59°C. A combination of adenosine and 1-alanine increased the rate of germination in every experiment and therefore reduced the heat and radiation resistance of the population.

To approach commercial canning conditions, quick-frozen green peas after thawing were mixed with an equal part of the sugar and salt-containing liquor, and inoculated with dormant spores to give final counts of 10^7 per g. The used quick-frozen peas contained no bacterial spores, other micro-organisms present in small numbers exerted no influence on the results. Ali-

quots of the seeded medium were incubated at 30°C, other aliquots were heated at 80°C for 10 minutes then cooled to 30°C and incubated further at this temperature.

To some samples 0.2 mg per g of adenosine and l-alanine were added after 10 minutes heating or incubation at 30°C. Samples containing no additives served as controls. Thus, incubation, including the time of heating, lasted

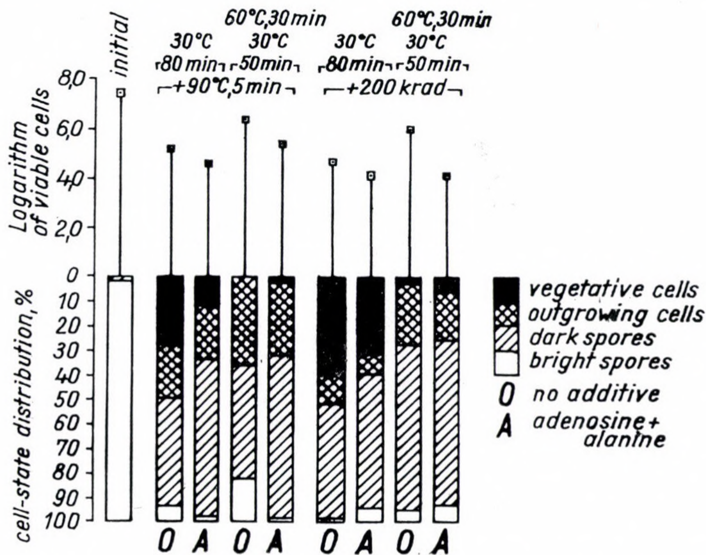


Fig. 5. Reduction of heat and radiation resistance of spores as a result of germination initiation. The lower part of the figure represents the cell-state distribution at the end of treatment; the upper part shows the number of cells surviving 90°C for 5 minutes and 200 krad irradiation after incubation

altogether for 15 minutes. Then the cells which had become heat-sensitive, were destroyed by heating to 80°C for 10 minutes and the number of surviving spores was determined by the pour plate method. Subsequently, the remaining suspension was irradiated with a 400 krad dose and the number of surviving spores was again determined. Results are shown in Fig. 6.

Adenosine and l-alanine stimulated the germination of spores also in the liquor used in these experiments, as samples containing the germinants yielded always lower numbers of surviving bacteria than the control did. Microscopic examination of the cell-state distribution was in good agreement with differences found in the resistance of the populations. The stimulating effect of additives on germination was evident in 5 minutes, from the decrease in the resistance of the spore population. It should be noted that in samples subjected to a 80°C pre-incubation heat shock, the reduction of the number of resistant cells was not higher than in suspensions incubated at 30°C. This was probably

due to the fact that no germination occurred during heating and this delay was only just compensated by the higher spore germination rate taking place in the pre-heated suspension.

Initiation of germination and reduction of resistance in the medium where spore formation had taken place. It was desirable to examine whether germination of *B. cereus* spores could be initiated in the same medium where they had

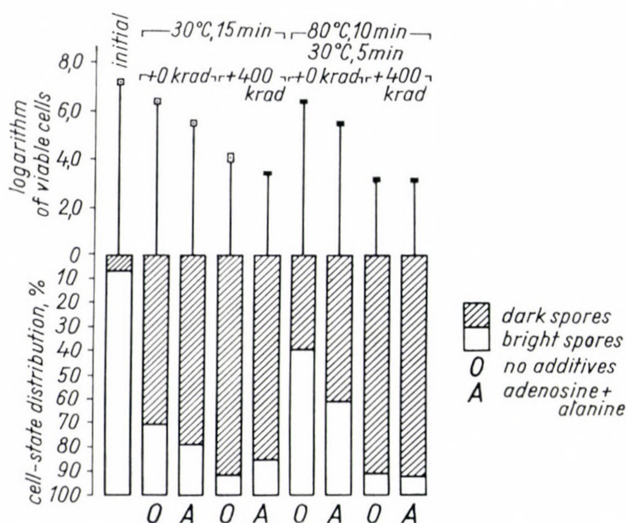


Fig. 6. Stimulation of spore germination in a mixture of sugar and salt-containing liquor and green peas

been formed. Pea-extract containing the sporulation salt mixture and liquid universal medium was inoculated with *B. cereus*. The cultures were shaken for 6 days at 30°C. After 6 days both cultures consisted practically of sporulated forms. While in universal medium the autolysis of sporangia was almost perfect, in pea-extract cultures 58.8 per cent of the cells contained sporangia. (Prior to these examinations the suspensions were homogenized in an "Atomix" blendor.)

Aliquots of the cultures were centrifuged, then other aliquots were diluted 1 : 10 with the clear supernatant. Then the diluted and undiluted suspensions were heated to 60°C for 30 minutes as well as treated with combined germinants (both adenosine and l-alanine were used at 0.2 mg per ml concentrations). The suspensions were incubated at 30°C. Changes in the cell-state distribution are presented in Fig. 7.

Heat-activation at 60°C for 30 minutes caused no germination, although in samples incubated for 24 hours most spores were less refractive than in the starting suspensions. The effect of germinants was evident after 1 hour and considerably increased after 24 hours incubation. Whether the cell-state

distribution observed in 24 hour samples was reached in some hours or later, has not been elucidated, as no examinations were performed in that interval. The thermal resistance of the spores was, however, established immediately after the 30 minutes heat activation at 60°C, as well as after 3 and 27 hours incubation. Fig. 8 shows the heat resistant cell count in pea-extract cultures diluted with supernatant.

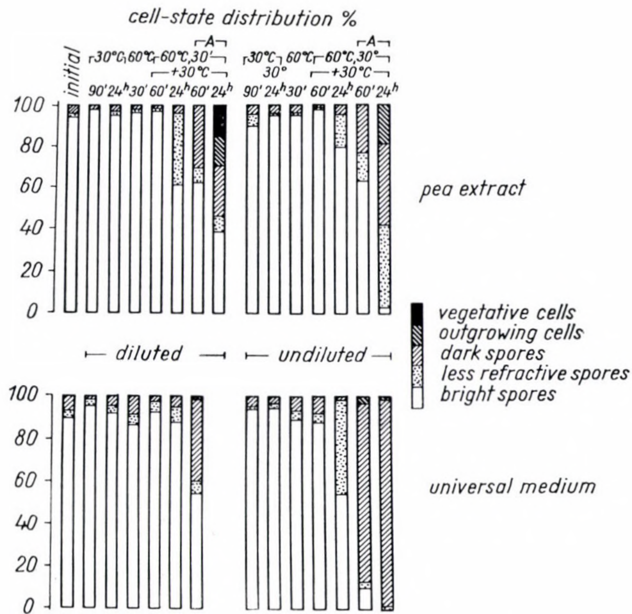


Fig. 7. Initiation of spore germination in the medium where they had been formed. To samples marked with "A" l-alanine and adenosine at 0.2 mg per ml concentration were added after heat-activation at 60°C for 30 minutes

Discussion

The above results are indicative of considerable individual differences in bacterial spores originating from suitably synchronized cultures, as to regards the effect of germination-initiating agents and the velocity of germination [17, 32]. In agreement with other authors' experience [6, 11, 12, 33], l-alanine and adenosine are capable of inducing germination in media free from other organic substances and of accelerating the germination rate in nutrient media. The effect of germinants could be increased by pre-heating, at 60° to 80°C.

The reduction in the refractivity of spores went parallel not only with the decrease in heat-tolerance [17, 24] but also with a decrease in radiation resistance. Initiation of germination therefore effectively increases the number of spores destroyed by heating as well as by radiation.

Germination inducing factors act rapidly at 30°C. The proportion of resistant spores was considerably decreased by incubation following initiation and prior to processing.

By the use of the above described physical and chemical treatments, the overwhelming majority of spores can be induced to germinate. In consequence,

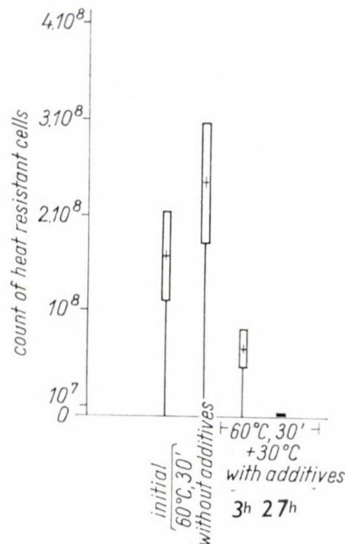


Fig. 8. Decrease in the number of heat resistant cells in sporulated cultures under the effect of germinants

the spores become less resistant even when they are present in high counts, which are otherwise unfavourable for germination [36] and even when they are in the same medium in which they had developed.

Thus it seems realizable that by means of suitably chosen blanching and pre-sterilization temperatures and periods and addition of harmless, even nutrient germinants, resistant spores can be changed into sensitive cells in processed foods previous to sterilization. While this new, fractionated sterilization of foods is more effective and causes smaller damages in the material, it is also advantageous in that it requires less energy than the usual processing methods.

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INTESTINAL MICROFLORA OF THE LARVAE OF ST. MARK'S FLY

I. A COMPARATIVE STUDY OF STREPTOMYCES STRAINS BELONGING TO THE GRISEUS GROUP ISOLATED FROM THE INTESTINAL CANAL

By

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Summary. Streptomyces strains belonging to the griseus-group isolated from the intestinal tract and excrement of individuals of three larval populations of St. Mark's fly *(Bibio marci L.)* have been identified as *Act. vulgaris* Nikitina et al. 1960 and *Act. citreofluorescens* Koreniako et al. 1960. Griseus-strains isolated from A_H horizon situated under the activity area of *Bibio* populations were partly identical with *Act. levoris* Koreniako et al. 1960, partly closely related to *Str. michiganensis* Corbaz et al. 1957. In connection with this work a key has been established for the determination of species and varieties belonging to the *Streptomyces griseus* group.

Actinomycetes play an important part in the intestinal tract of earthworms and other soil-inhabiting animals feeding on organic substances present in the soil. In the fresh excrement of these animals, the important biological function of which is well known, there occurs a large number of actinomycetes [1, 2]. Recently it has been shown that insects such as the larvae of *Bibio marci* (Diptera), also carry these microorganisms in their intestinal flora in considerable proportions (20 to 50 per cent) [3]. The systematic position of the actinomycetes occurring in the intestine of soil animals has scarcely been investigated in spite of the finding [1, 3] that in the intestinal tract and fresh excrement of soil-inhabiting animals certain microorganisms become predominant by selection or, at least, occur in larger numbers than in soil. In contrast, some other species commonly found in soil and in the consumed food disappear rapidly from the intestinal tract of these animals.

In the present experiments we have examined *Bibio marci* larvae, as these, in view of their large numbers and high frequency, play an outstanding biological role in soil [4]. From the intestinal tract and excrement of these animals Streptromyces strains belonging to the griseus group have been isolated and the classification of these microorganisms has been attempted.

Materials and methods

Soil examined. Forest mull-like rendzina (humid rendzina) formed on limestone.

Bibio marci larvae were inhabiting the horizons A_{00} and A_F in populations consisting of 500 to 2000 individuals. The animals fed on miscellaneous materials and thus, in addition to the litter, they passed through their intestinal tract considerable amounts of soil matter

(partly humified organic materials, partly fine mineral particles, mainly of calcite). The consumed food contained several different species of actinomycetes of the soil microflora.

Isolation of strains. The larvae were dissected aseptically under stereomicroscope, then the intestinal contents were ground in saline. Serial dilutions prepared from the homogenate and suspensions prepared from fresh excrement collected from under the larval colonies were plated onto glucose-asparagine, glycerol-arginine (EL NAKEB and LECHEVALIER) and casein-glucose agar. The plates were incubated for 2 weeks at 28°C. The frequency of the different colonial types was examined under the microscope at low power. The colonies were transferred to agar slants and the growth was checked for purity. Microbiological examination of the intestinal contents was performed partly by use of individual larvae, partly by use of mixed intestinal specimens obtained from large numbers of larvae.

Cultural and physiological properties of the isolates and of authentic (mainly "type") strains, unless otherwise indicated, were examined as recommended in 1964 by the Subcommittee on Actinomycetes of the International Committee on Bacteriological Nomenclature, International Association of Microbiological Societies [5].

Authentic strains were maintained on agar media or by freeze-drying.

Results

Incidence of griseus strains and grouping of isolates. Griseus strains were isolated from the intestinal tract of individuals of 3 larval populations (No. 3, 4 and 6; 50, 41 and 220 dissected animals, respectively). The proportion of bacteria and actinomycetes in mixed intestinal samples from these populations varied between 10:2.5 and 10:1.8. It should be noted that in these cases the observed proportion of actinomycetes was the lowest that has so far been revealed in *Bibio marci*. In other examinations the corresponding rate was generally higher than 20 per cent. Among actinomycetes the fertile and asporogenic forms of *Str. finlayi* [6] exceeded 80 per cent in all three larval populations. Griseus strains, the "accessory" members of the intestinal flora, were present at most in 4 to 5 per cent of all actinomycetes. Among "accessory" members, however, these organisms were the commonest. In the intestinal tract of the individuals of other larval populations, in which streptomycetes *(Str. olivaceus, Str. antibioticus, Str. aureofaciens*, etc.) other than *Str. finlayi* predominated the presence of griseus strains was not revealed. Among the few species of actinomycetes that become predominant in the intestinal flora by selection [3], symbiotic and antibiotic connections are probably formed, thus when *Str. finlayi* constitutes the predominating organism, a moderate griseus population may develop. On the other hand, when other species occur at high proportions, colonization of griseus strains is completely inhibited. The incidence of griseus strains may be higher in fresh excrements where, as a result of microbial succession, the proportion of actinomycetes in the total faecal flora increases gradually up to 70—80 per cent; the number of species also increases and the flora becomes different from that present in the intestine [3]. Occurrence and frequency of griseus strains were determined by microscopical examination of the agar plates seeded with the samples. The aerial mycelium of surface colonies showed a typical "griseus" colour [7] and typical sporophores.

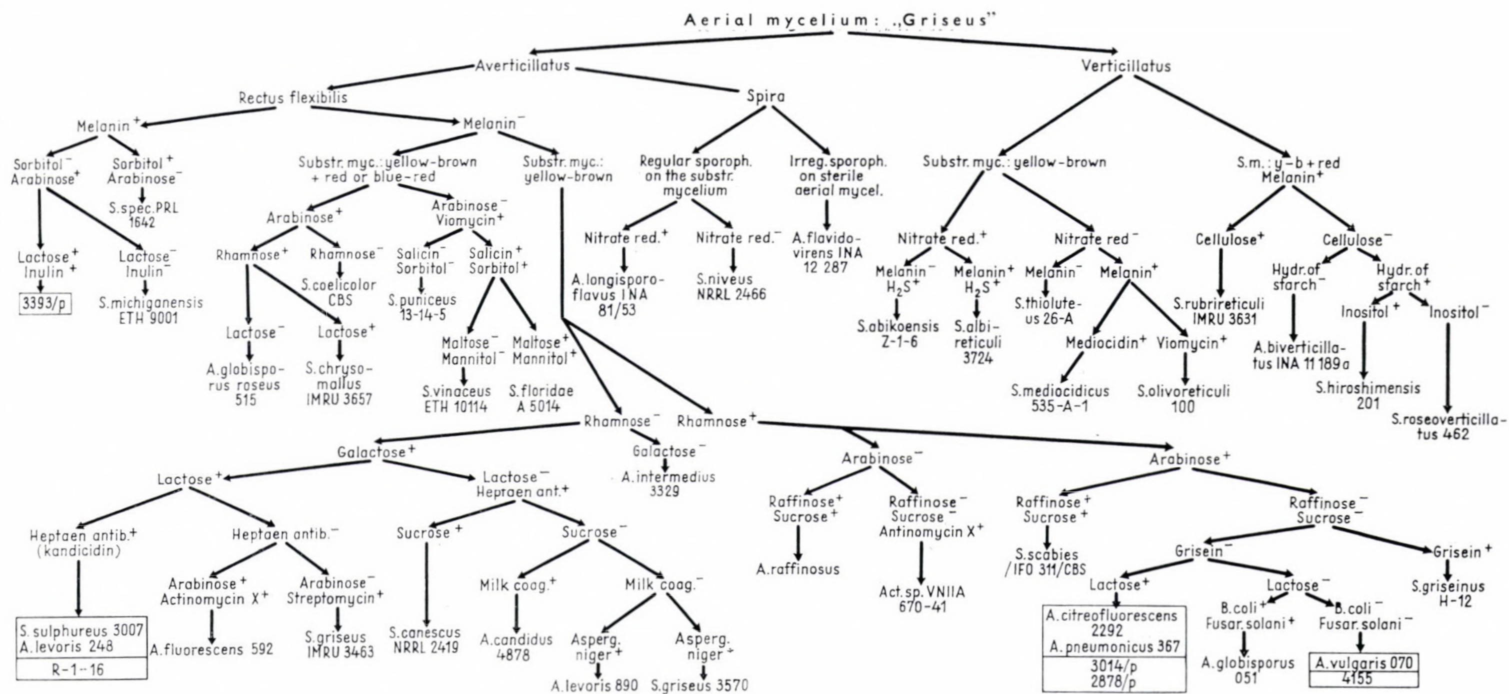
Of large numbers of griseus isolates 28 intestinal and faecal and 23 soil strains were subjected to taxonomic analysis. These strains were divided into 4 groups: (1) melanin positive variant; (2) melanin negative, heptaen antibiotic-producing variants; (3) and (4) melanin negative, heptaen-non-producing variants differing in some physiological properties. Variants 1 and 2 were isolated from control soil, variants 3 and 4 were revealed in intestinal contents and excreta. Prior to discussing the taxonomic position of the isolates, it seems advisable to summarize the classification of the griseus group.

Classification of the griseus group. On the basis of comparative examinations performed with authentic strains and of data in the literature, the griseus group was divided into 36 taxa. These units may be regarded as species, although criteria of species-differentiation in actinomycetes have not yet been decided. Table I shows a key to the 36 taxa, each of which except *Act. raffinosus* is represented by one authentic strain. As shown by HÜTTER [7], on the basis of the type of branching of aerial hyphae (verticillatus and averticillatus) streptomycetes producing a "griseus" aerial mycelium can be divided into 2 main groups. By the use of a dichotomic key system based on sporophore morphology, melanin production, carbon source utilization spectrum, and some other morphological and cultural properties, a further simple and practical diagnostic differentiation scheme has been established. The properties of *Act. raffinosus*, which is included in Table I without designation, are given according to data in the literature, as no authentic strain of this organism could be obtained. In addition, one taxon is represented by one of our isolates (strain 3393/p), which is closely related to *Str. michiganensis*. In our opinion the present key has the advantage of including defined strains instead of generalized species. Therefore, on the one hand it reflects the richness in different forms and physiological heterogeneity of the griseus group, and on the other, it serves as a guide for the identification of new isolates. Unfortunately, at present the number of available authentic or wild strains representing the different taxa is small (a taxon is often represented by one strain only); there is therefore no basis for estimating differential value and stability of physiological and cultural characters.

Identification of isolates. Table I shows in framed areas the designation of our griseus strains representing 4 different types and the corresponding authentic strains. Strain 3393/p is a single representative of the melanin positive griseus type related to *Str. michiganensis*. This culture with the exception of its action on lactose and inulin, is similar in carbon source utilization spectrum to *Str. michiganensis* ETH 9001 type strain. Both 3393/p and ETH 9001 are l-xylose, l-arabinose, d-fructose, d-galactose, maltose and d-mannitol positive, and l-rhamnose, sucrose, meso-inositol, raffinose and d-sorbitol negative. In view of its similar cultural and physiological characters, strain 3393/p can be regarded as a variant of *Str. michiganensis*.

Table I

Key to the differentiation of the Streptomyces griseus group



Numerous griseus strains exerting an inhibitory action on yeasts have been isolated also from horizon A_H. These organisms belonged to *Act. levoris* and showed the properties described for the laboratory type strain of this species (Int. Common. Exp.: 5202). The following carbon utilization spectrum is characteristic of *Act. levoris:* l-arabinose, d-xylose, d-mannitol and d-fructose positive; sucrose, i-inositol, rhamnose, raffinose and cellulose negative. The strains were identical in every respect with members of a griseus population designated R-1-16, which had been isolated from rendzina soil of similar origin and described as *Act. levoris* [8].

Griseus strains belonging to the third group are represented in Table I by strain 4155. This culture is identical with *Act. vulgaris* Nikitina et al. [9], an organism commonly found in different soil types.

The fourth group of our griseus strains is represented in Table I by strains 3014/p and 2878/p. These two strains, which differed in some less important characters, were closely related to, or identical with, *Act. citreofluorescens* and somewhat less closely related to *Act. pneumonicus.* These cultures were typical averticillatus, melanin or Tresner-Danga negative, rhamnose and arabinose positive, raffinose and sucrose negative organisms.

Discussion

Streptomycetes belonging to the griseus group isolated from the intestinal tract and excrements of *Bibio marci* larvae were different from the griseus-strains occurring in the A_H-horizon. *Act. levoris* [10], an organism commonly occurring in the deeper layer of the horizon A_H, has not been revealed in the intestinal tract of *Bibio* larvae. In contrast, *Act. vulgaris*, which is also a characteristic soil inhabiting microorganism occurred relatively frequently in the intestinal canal. The frequency of distribution of actinomycetes varies considerably according to horizons of different soil types [11]. Therefore, only the spores or vegetative forms of certain species consumed together with organic soil substances are subject to the selective process taking place in the intestinal flora of soil inhabiting animals. In horizons A_{00} and A_F and in the top (1—2 cm) layer of the A_H horizon of the examined mull-like rendzina, the organic substances of which are continuously passed through the intestinal tract of *Bibio marci* larvae, *Act. vulgaris* and *Act. citreofluorescens* were only revealed, whereas *Act. levoris* tended to occur more frequently only in horizon A_H, especially in the deeper layer of calcium carbonate accumulation [8].

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INCIDENCE OF RHODOTORULA SPECIES IN URBAN AIR

By

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Summary. In connection with investigations into urban air pollution, the yeast flora of air has been examined. The most common organisms occurring in urban air were Rhodotorula and Cryptococcus species.

No seasonal difference was observed in the incidence of the isolated 100 Rhodotorula strains representing 12 species. Of the isolates, 34 per cent belonged to *Rh. glutinis*, 26 per cent to *Rh. mucilaginosa*, 16 per cent to *Rh. rubra* and 11 per cent to *Rh. minuta*.

The findings indicate that in the search for sources of human mycoses the microflora of air should also be considered.

Although recent extensive investigations have considerably increased our knowledge of the ecology of yeasts, few authors have examined the incidence of these organisms in air. Yeasts have been detected in altitudes exceeding 2000 metres, in the air of oceans and north polar regions and especially frequently in tropical air [4]. Recent studies have shown that the incidence of yeasts shows a diurnal variation, namely, these organisms can mainly be detected at night and in the early morning hours [9]. As yeasts frequently occur in soil [1, 10, 13, 14], they probably get into the air on dust particles. In the literature there are no detailed references as to the species distribution of yeasts occurring in air, but for DI MENNA's report [12] on several species isolated from indoor air.

The present studies were begun in 1964 as part of an investigation into the pollution of outdoor air in Budapest. Rhodotorula and Cryptococcus were the most commonly encountered yeast species. The present paper deals with the examination of Rhodotorula strains.

Routine experience and data described in the literature indicate that carotinoid pigment-containing yeasts are frequently isolated from human material. The surface of the skin contains most frequently *Rhodotorula mucilaginosa*, *Rh. minuta* and *Rh. glutinis* [2, 8]. These species were also detected in the respiratory tract [24]. *Rh. mucilaginosa* and *Rh. minuta* are common members of the genital yeast flora [19]. From the gall bladder and duodenal juice mainly Rhodotorula species can be isolated, usually *Rh. mucilaginosa* and *Rh. rubra*, less frequently *Rh. glutinis*, *Rh. aurantiaca* and *Rh. minuta* [2, 21]. Although these organisms are generally regarded as non-pathogenic,

some yeasts isolated from infected gall bladders belonged to Rhodotorula [22], and Rhodotorula fungiaemia has been described as a complication of staphylococcal endocarditis [23]. *Rh. mucilaginosa* may give rise to experimental mycosis in white mice [14a].

Our investigations indicate that the air's yeast flora should not be neglected in the search for infective sources. The present paper gives an account of Rhodotorula species.

Materials and methods

During a 14 month period between April, 1964, and August, 1965, with the exception of the cold months January, February and March, two samples were taken daily (one in the morning and one at noon) by the staff of our laboratory. Petri dishes containing molasses agar were exposed for 15 minutes on the top of a 20 metre high building situated at a busy traffic centre (Nagyvárad tér, Budapest IX). The plates were incubated in a dim place at room temperature for 2 to 4 days. Rhodotorula colonies were transferred to molasses agar slants then, in order to obtain pure cultures, were streaked onto PAGANO—LEVIN—TREJO plates [17].

Identification of the cultures was based on methods described in LODDER and KREGER-VAN RIJ's monograph [11]. The following examinations were carried out: pseudomycelium production on potato agar, macrocolony formation and size of cells on malt extract agar, size of cells in malt extract cultures, carbon source assimilation, nitrogen source assimilation, turbidimetric measurement of growth in ethanol as a sole source of carbon, cleavage of arbutin, production of starch.

Results and discussion

During the 14 months observation period, 100 Rhodotorula strains were isolated. The incidence (also percentage distribution) of pink pigment-producing yeast strains was as follows. *Rh. glutinis* (Fres.) Harrison, 34; *Rh. mucilaginosa* (Jörg.) Harrison, 26; *Rh. rubra* (Demme) Lodder, 16; *Rh. minuta* (Saito) Harrison, 11; *Rh. zsoltii* Galgóczy et Novák, 4; *Rh. graminis* di Menna, 2; *Rh. slooffii* Novák et Vörös-Felkai, 1; *Rh. aurantiaca* (Saito) Lodder 1; *Rh. marina* Phaff, Mrak et Williams, 1; *Rhodotorula peneai* Phaff, Mrak et Williams 1; *Rh. laryngis* Reiersöl, 1; Flavotorula sp., 2.

Table I shows monthly incidence of the isolated cultures. It is seen that there was no seasonal difference in the distribution of species.

Table II presents the morphological and physiological characters of the strains.

Classification of the 100 Rhodotorula strains was based on LODDER and KREGER-VAN RIJ's monograph [11] and on the studies of HASEGAWA, BANNO and YAMAUCHI [6, 7] and NOVÁK and ZSOLT [15].

LODDER and KREGER-VAN RIJ [11] included 7 species and 1 variety in the genus Rhodotorula. These were distinguished on the basis of nitrate assimilation, sugar utilization, morphology and size of cells and colour of colonies *(Rh. glutinis, Rh. glutinis* var. *rubescens, Rh. aurantiaca, Rh. pallida, Rh. minuta, Rh. rubra, Rh. mucilaginosa, Rh. flava).*

Table I

Monthly distribution of Rhodotorula species isolated from air
Observation period, April, 1964 — August, 1965

	April	May	June	July	August	September	October	November	December	April	May	June	July	August	No. of strains
Rhodotorula glutinis	1	1	1	3	1	8	4	1	1	3	2		3	5	34
Rhodotorula mucilaginosa	3	2	4	2				4		3			4	4	26
Rhodotorula rubra	1	3	1	2	1	3				2	1		1	1	16
Rhodotorula minuta	2		1					2	3	2	1				11
Rhodotorula graminis		1	1												2
Rhodotorula zsoltii		1					1				2				4
Rhodotorula slooffii		1													1
Rhodotorula aurantiaca											1				1
Rhodotorula laryngis												1			1
Rhodotorula marina								1							1
Rhodotorula peneai											1				1
Flavotorula sp.		1					1								2
Total	7	10	8	7	2	11	6	8	4	10	8	1	8	10	100

Hasegawa in 1958 [5] united *Rh. mucilaginosa* and *Rh. rubra* into one species *(Rh. rubra)*. He regarded *Rh. aurantiaca* as a variant of *Rh. glutinis*. On the basis of pigment production, Hasegawa, Banno and Yamauchi [6, 7] recommended the establishment of two subgenera: 1. Rubrotorula subgen. Hasegawa, Banno et Yamauchi including yellowish-red and orange rhodotorulae; 2. Flavotorula subgen. Hasegawa, Banno et Yamauchi including yellowish rhodotorulae. Some Cryptococcus species were also classified in the latter subgenus. Subgenus Rhodotorula contained 7 species: *Rh. glutinis*, *Rh. rubra*, *Rh. macerans*, *Rh. lactosa*, *Rh. marina*, *Rh. texensis* and *Rh. pallida*.

Including into their system all known rhodotorulae, Novák and Zsolt in 1961 [15] distinguished 12 species *(Rh. pallida, Rh. minuta, Rh. texensis, Rh. graminis, Rh. crocea, Rh. mucilaginosa, Rh. rubra, Rh. glutinis, Rh. marina, Rh. flava, Rh. peneai* and *Rh. macerans)*.

Our 34 *Rh. glutinis* strains were easy to classify. All cultures produced smooth, mucoid, orange coloured colonies, intracellular fat globules 5.2—7.8 μ in diameter, and uniform nitrate and sugar reactions. Arbutin was decomposed by all strains. Ethanol was assimilated by about 50 per cent of the isolates. According to Lodder and Kreger-van Rij, and Novák and Zsolt, this

Table II

Physiological and morphological propertie

Rhodotorula species	Sugar assimilation						NO_3 ass.	Starch prod.	Ethanol ass.	Arbutin decomp.
	D	G	S	M	L	R				
Rh. glutinis	+	+	+	+	—	+	+	—	d	+
Rh. mucilaginosa ..	+	+	+	+	—	+	—	—	d	+
Rh. rubra	+	+	+	+	—	+	—	—	+	d
Rh. minuta	+	+	+	—	—	+	—	—	—	—
Rh. graminis	+	+	+	—	—	+	+	—	d	±
Rh. zsoltii	+	+	+	—	—	—	—	—	—	+
Rh. slooffii	+	+	+	—	+	—	—	—	—	+
Rh. aurantiaca	+	+	+	+	—	—	+	—	—	+
Rh. laryngis	+	+	+	+	—	—	—	—	—	+
Rh. marina	+	+	+	+	+	+	—	—	—	+
Rh. peneai	+	+	+	+	+	+	—	+	—	+
Flavotorula sp. ...	+	+	+	+	—	+	+	—	d	d

Abbreviations: D = dextrose, G = galactose, S = sucrose, M = maltose, L = lactose, R = raffinose, d = different

species utilizes ethanol; in contrast, Hasegawa et al. described *Rh. glutinis* as acting on ethanol poorly or not at all.

More difficulty was encountered in identifying *Rh. mucilaginosa* and *Rh. rubra* strains. In sugar reactions and nitrate negativity all strains were uniform. According to colonial morphology after 30 days incubation, the strains were divided into 2 groups: on malt agar 26 strains produced smooth, mucoid, glistening light orange coloured colonies; 16 strains formed non-mucoid, waxy, rugose colonies red in colour. The size of cells was similar in both groups. The 26 smooth, mucoid strains uniformly decomposed arbutin and acted variably upon ethanol; the 16 non-mucoid, rugose strains assimilated ethanol uniformly, but exercised a variable effect on arbutin. On the basis of Lodder and Kreger-van Rij's data, the first group of isolates should be included into *Rh. mucilaginosa*. Members of the second group, except the size of cells, show the properties of *Rh. rubra*. According to Hasegawa [5], the united *Rh. rubra* species decomposes arbutin, utilizes ethanol weakly and produces smooth, glistening, mucoid colonies. Our observations indicate that

of yeasts isolated from air

Colonial morphology and size of malt agar culture cells, μ	Size of malt broth culture cells, μ
Smooth, mucoid, glistening, orange colonies; 2—3.9×3.9—5.2	2—3.2×3.9—5.2 fat globules, 5.2—7.8 μ ⌀
Smooth, mucoid, glistening, light orange colonies; 2.6—3.9×3.9—6.5	2.6—3.9×3.9—7.8
Waxy, rugose, red colonies; 2.6—3.9 × 3.2—5.2	2.6—3.9×3.9—5.2 fat globules, 2.6—5.2 μ ⌀
Somewhat mucoid, glistening, light red colonies; 2.6—3.2×3.9—5.7	2.6—3.9×3.9—6.5
Mucoid, glistening,flesh coloured colonies; 2.6—3.2×3.9—5.7	3.2—3.9×3.9—5.2
Flesh coloured, waxy colonies; 2.6—3.9×3.9—6.5	2.0—3.9×3.9—5.2
Flesh coloured, waxy colonies; 2.6—3.2×3.2—5.2	2.0—3.2×3.2—5.2
Glistening, somewhat mucoid, red colonies; 2.6—3.9×5.2×7.8	2.6—3.9×5.2—7.6
Glistening, mucoid red colonies; 3.2—5.2×5.2—10.4	2.6—3.2×5.2—7.8
Glistening, waxy, red colonies; 3.2—5.2×5.6—7.8	2.6—3.9×5.2—7.8
Glistening, waxy, red colonies; 2.6—5.2×3.9—7.8	2.6—5.2×3.9—7.8
Waxy, yellow or flesh coloured colonies;	

these properties are characteristic of *Rh. mucilaginosa* only; therefore, this species should be separated from *Rh. rubra*, though both give similar sugar and nitrate assimilation reactions.

Eleven cultures were classified as *Rh. minuta;* these strains assimilated raffinose in addition to glucose, galactose and sucrose, and did not attack arbutin and ethanol. HASEGAWA *et al.* regarded *Rh. minuta* as a variant of *Rh. texensis*. According to GALGÓCZY and NOVÁK [3], classification of the lactose-non-assimilating *Rh. minuta* into the species *Rh. texensis* is hardly justifiable. As none of our 11 strains assimilated lactose, all of them were identified as *Rh. minuta*.

One strain belonged to *Rh. slooffii* [16], 4 strains to *Rh. zsoltii* [3]. Members of these recently described two species produced waxy, non-mucoid, flesh coloured colonies. The two species differed in the assimilation of lactose.

HASEGAWA *et al.* regarded *Rh. aurantiaca* as a variant of *Rh. glutinis*. Our strain, unlike other *Rh. glutinis* cultures, did not assimilate raffinose.

One of our strains corresponded to *Rh. laryngis* described by REIERSÖL [18].

Two strains isolated from air produced yellowish flesh-coloured colonies. These starch-non-producing cultures, on the basis of their pigment production, were classified as belonging to subgenus Flavotorula. They were not identical with *Rh. flava* of HASEGAWA *et al.* [7], because they assimilated nitrate but not lactose. In assimilation properties both strains resembled *Rh. glutinis.*

It is interesting that air pollution examinations yielded most frequently Rhodotorula and Cryptococcus species. These organisms produce carotinoid pigment and are often encapsulated. The capsule as well as the pigment may protect the cells against harmful environmental effects. Elucidation of this question, *i. e.* whether these species are or are not more resistant to temperature or humidity alterations, drying, and ultraviolet rays, than other yeasts, awaits further investigations.

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ORGANIC AND AMINO ACID ASSIMILATION BY YEASTS AS STUDIED BY THE REPLICA PLATING TECHNIQUE

By

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Summary. By use of the replica plating technique the assimilation and utilization as carbon and nitrogen sources of 10 organic acids and 11 amino acids have been examined. The results obtained with one strain of each of 25 different yeast species were evaluated in view of taxonomical and physiological importance. The replica plating technique, owing to the low nitrogen requirement of the majority of the examined species, was found unsuitable for nitrogen source determination.

Examination of nutrient sources of yeasts is important both physiologically and taxonomically. In view of the practical importance of these organisms, extensive investigations have been performed into their carbohydrate utilization. Although Ehrlich [2] in 1907 already pointed out the role of amino acid fermentation in the production of fusel oils, less attention has so far been paid to other carbon and energy sources. As to amino acids, studies have mainly been focussed on their utilization as nitrogen sources [3, 14]. Less extensive examinations have been carried out on amino acids as carbon sources [17]. Investigations into the mechanism of utilization have been limited to a small number of species. Results of some orientation experiments performed with larger numbers of species were found unsuitable by Lodder and Kreger-van Rij for classification purposes [8].

Assimilation experiments with organic acids have mainly been restricted to substances involved in the citrate cycle and in glycolysis. In classifying ascosporogenic yeasts, Kudrjawzew [5, 6] was the first to take organic acid assimilation into consideration. Wickerham and Burton [23] recommended the extension of the spectrum of assimilation tests for diagnostic purposes: in addition to sugars, glucosides and alcohols they used organic acids. This method, with some modifications, was adopted by other investigators for the classification of new species and for the monographic description of certain genera [19–21, 4].

The present experiments had two main purposes, *viz.* (1) to check on a larger material the reliability of the replica plating technique originally described for genetic studies by Lederberg and Lederberg [7] and used for testing sugar assimilation in yeasts by Shifrine *et al.* [15]; and (2) to obtain orientation data for further taxonomical and physiological experiments by

examining the utilization of 10 organic and 11 amino acids by 25 yeast species. It has, however, been thought advisable to deal separately with the role of amino acids as carbon and nitrogen and simultaneous carbon-nitrogen sources.

Materials and methods

Assimilation experiments were performed with the replica plating method described by LEDERBERG and LEDERBERG [7] for the isolation of bacterial mutants. The same technique was used by SHIFRINE *et al.* [15] for the examination of carbon source assimilation by yeasts. Our experiments were carried out as follows.

Cultures representing 25 different yeast species were inoculated by needle point onto one Sabouraud glucose agar plate 9 cm in diameter, so as to obtain isolated colonies after two days' incubation at 26°C. By means of a velveteen stamp attached to a plexiglass block, from this "master" plate the 25 colonies were replicated to agar plates containing different test substances. One master plate was sufficient for the preparation of 5 replica plates. When more transfers were required, the first master plate was replicated to several Sabouraud glucose agar plates and these secondary master plates were used for inoculating the test plates.

Cultures. The following yeast species isolated from human sources were used: *Procandida albicans* 85/57, *Procandida tropicalis* 570/60, *Azymocandida rugosa* 463/60, *Candida krusei* 387/60, *Candida reukaufii* Nr. 1, *Candida famata* 375/60, *Candida solani* 488/60, *Candida parapsilosis* 474/60, *Candida utilis* 25/58, *Candida guilliermondii* 372/59, *Candida pseudotropicalis* 61/60, *Cryptococcus neoformans* 343/60, *Cryptococcus diffluens* 255/59, *Cryptococcus albidus* 373/59, *Rhodotorula mucilaginosa* 373/60, *Rhodotorula glutinis* 518/59, *Rhodotorula flava* 289/58, *Hansenula anomala* 54/59, *Dekkeromyces fragilis* 500/59, *Geotrichum linkii* 132/57, *Geotrichum candidum* 468/60, *Geotrichum matalense* 92/58, *Torulopsis glabrata* 465/60, *Torulopsis inconspicua* 435/60, *Trichosporon cutaneum* 436/59.

Organic acids and amino acids. Acetic, lactic, succinic, fumaric, malic, tartaric, citric, oxalic, malonic and 1-ascorbic acid, glycine, 1-alpha-alanine, 1-cysteine, 1-aspartic acid, 1-asparagine, 1-glutamic acid, 1-glutamine, 1-valine, 1-methionine, 1-arginine and 1-histidine. Organic acids were used at 0.2 *M* concentration and pH 5.9—6.1. Amino acids were used as carbon sources at 0.2 *M*, as nitrogen sources at 0.02 *M* (nitrogen), as simultaneous carbon = nitrogen sources at 0.2 *M* concentration at pH 6.0—6.2. The test medium employed for auxanographic sugar assimilation experiments for identification of yeasts [8, 24] served as a basal medium. In this medium the above carbon sources were substituted for glucose and the above nitrogen sources were substituted for ammonium sulphate. When amino acids were tested as simultaneous carbon and nitrogen sources, the corresponding amino acid was substituted for both glucose and ammonium sulphate. Control plates were also prepared from the basal medium. In carbon source examinations one control was represented by the original, the other by a glucose-free medium. In nitrogen source determinations, in addition to the complete medium, an ammonium sulphate-free medium was employed. In simultaneous carbon-nitrogen utilization experiments the controls were a complete, a glucose-free, an ammonium sulphate-free and a glucose-ammonium sulphate-free medium. The media were prepared from washed agar. A scheme showing the employed test and control media is presented in Table I.

Two experiments were made in duplicate on two occasions. Colony development was observed daily and the degree of growth as compared to the control was expressed on the basis of an arbitrary scale. Readings were recorded as: 1 = very weak, 2 = weak and 4 = good growth.

Results

Table II presents the result of organic acid utilization after 6 days incubation. The three kinds of examinations with amino acids are shown in Tables III/a, III/b and III/c.

It should be mentioned that on ammonium sulphate-free control plates several strains exhibited a poor growth; some strains, however, grew well on this medium. Thus the evaluation of nitrogen source experiments in the case of the latter was difficult or sometimes even impossible.

Table I

Scheme of assimilation experiments

Test media			Control media			
			Negative control			Positive control
C source	N source	Simultaneous C and N source	C source	N source	Simultaneous C and N source	Complete
Organic or amino acid	Glucose	Amino acid	—	Glucose	—	Glucose
$(NH_4)_2SO_4$	Amino acid	Amino acid	$(NH_4)_2SO_4$	—	—	$(NH_4)_2SO_4$
Inorganic salts	Inorganic salts	Inorganic salts	Inorganic salts	Inorganic salts	Inorganic salts	Inorganic salts

Discussion

Table II indicates that among the acids acetic acid was the one assimilated most commonly and effectively. Lactic, succinic, citric, malic, ascorbic, fumaric, malonic, tartaric and oxalic acids in that succession were attacked less commonly and effectively. This order, however, expresses neither the degree of energy yielded by, nor the number of carbon atoms contained in, these organic acids. The results may be explained partly by a difference in the uptake of various organic acids [1], partly by a different metabolic role (substrate or inhibitor) of the incorporated substances [13]. The role of malonic and oxalic acids as metabolic inhibitors should particularly be considered. With the exception of *Hansenula anomala*, which utilized malonic acid, the examined cultures assimilated weakly both substances. Thus it seems doubtful whether results obtained with these organic acids in a quantitatively less exact test, are reliable. If, however, it is taken into account that there was a good agreement among parallel and repeated experiments and that by a suitable evaluation scale negative and positive results were well distinguishable, it is evident that even if the possibility of assimilation is rejected, the finding is still worth of consideration. Were these substances inhibitory, they would inhibit the metabolism fed from the endogeneous reserve of cells and, consequently, the multiplication of the culture. A similar result, *i. e.* the absence of

Table II

Organic acid utilization by yeasts

	Acetic acid	Lactic acid	Succinic acid	Fumaric acid	Malic acid	Tartaric acid	Citric acid	Oxalic acid	Malonic acid	Ascorbic acid
Procandida albicans	4	2	1	0	0	0	0	0	1	1
Procandida tropicalis	4	2	2	2	2	1	2	0	1	1
Azymocandida rugosa	0	1	0	0	0	0	1	0	1	0
Candida krusei	4	4	4	0	1	2	2	0	1	2
Candida reukaufii	0	0	0	0	0	0	0	0	0	0
Candida famata	4	1	4	0	2	0	2	0	0	0
Candida solani	2	0	1	0	0	0	2	0	0	1
Candida parapsilosis	4	2	4	2	2	0	2	0	0	1
Candida utilis	4	4	4	2	4	1	4	0	1	2
Candida guilliermondii	4	2	4	4	4	1	2	1	1	1
Candida pseudotropicalis	2	1	2	0	0	0	0	0	1	0
Torulopsis glabrata	1	0	0	0	0	1	0	0	1	0
Torulopsis inconspicua	1	1	1	0	1	0	1	0	0	1
Hansenula anomala	4	4	4	0	4	2	4	0	4	2
Dekkeromyces fragilis	2	1	2	0	1	1	1	1	1	1
Geotrichum linkii	4	4	4	1	4	0	2	0	0	0
Geotrichum candidum	1	0	0	0	0	0	0	0	0	0
Geotrichum matalense	4	4	4	1	4	0	2	0	0	0
Trichosporon cutaneum	0	0	0	0	0	0	0	0	0	0
Cryptococcus neoformans	0	1	0	0	1	1	1	1	0	1
Cryptococcus diffluens	0	1	0	0	1	1	1	0	0	1
Cryptococcus albidus	4	2	4	4	4	1	2	0	1	1
Rhodotorula mucilaginosa	2	2	2	2	2	0	1	1	0	0
Rhodotorula glutinis	2	2	2	2	2	0	0	0	0	0
Rhodotorula flava	2	4	4	4	4	0	2	0	0	0

inhibition and even the assimilation of the inhibitory agent was revealed earlier for malonic acid [9, 11, 12]. These findings may explain the good growth of *Hansenula anomala* in the presence of malonic acid. It should be noted that recent observations indicate that malonic acid assimilation by microorganisms is a less frequent but not an exceptional phenomenon [13]. No similar findings have been reported for oxalic acid; this substance is often inhibitory even to conidial germination [9], although it is known that some fungi are able to oxidize it when formed as a metabolic product [22].

Table IIIa

Amino acid utilization by yeasts

	l-aspartic acid			l-asparagine			l-glutamic acid			l-glutamine		
	C	N	C-N	C	N	C-N	C	N	C-N	C	N	C-N
Procandida albicans	1	1	2	1	1	1	2	0	4	0	0	2
Procandida tropicalis	2	1	4	4	2	4	4	2	4	4	1	4
Azymocandida rugosa	0	2	0	0	1	1	2	2	2	0	1	2
Candida krusei	0	2	0	0	2	0	2	2	2	0	2	0
Candida reukaufii	0	0	0	0	0	0	1	0	1	0	0	0
Candida famata	4	2	4	4	2	4	4	4	4	4	2	4
Candida solani	1	0	1	0	2	0	2	0	4	0	0	4
Candida parapsilosis	4	2	4	4	2	4	4	2	4	4	1	4
Candida utilis	0	0*	0	0	0*	1	2	0*	2	4	0*	2
Candida guilliermondii	4	1	4	4	2	4	4	0	4	4	0	4
Candida pseudotropicalis	0	0	4	0	0	4	4	0	4	0	0	4
Torulopsis glabrata	0	0	0	0	0	0	1	0	1	0	0	0
Torulopsis inconspicua	0	0	0	0	1	0	2	0	2	0	0	0
Hansenula anomala	1	0*	1	1	0*	1	2	0*	2	0	0*	2
Dekkeromyces fragilis	0	0	0	0	0	0	1	0	1	0	0	0
Geotrichum linkii	4	2	4	4	4	4	4	4	4	4	4	4
Geotrichum candidum	0	0	0	0	0	0	1	0	1	0	0	0
Geotrichum matalense	4	4	4	4	4	4	4	4	4	4	4	4
Trichosporon cutaneum	0	0	0	0	0	1	1	0	1	0	0	0
Cryptococcus neoformans	0	2	0	0	2	0	0	0	0	0	2	1
Cryptococcus diffluens	0	0	0	0	0	0	1	0	1	0	0	0
Cryptococcus albidus	4	4	4	4	4	4	4	4	4	2	2	4
Rhodotorula mucilaginosa	1	1	1	1	1	2	2	2	2	1	1	2
Rhodotorula glutinis	1	1	1	1	1	2	2	2	2	1	1	2
Rhodotorula flava	4	1	1	4	1	2	2	2	2	4	1	2

Our orientation experiments are unsuitable for a detailed analysis; it is nevertheless evident that organic acids which are assimilated either by many or by a few species are the most advantageous for differential diagnostic purposes.

As regards organic acid assimilation, it should be mentioned that *Candida guilliermondii*, *Candida utilis*, *Cryptococcus albidus* and *Procandida tropicalis* were polyphagous, *Geotrichum candidum* was monophagous, and *Azymocandida rugosa*, *Torulopsis glabrata* were oligophagous organisms. Members of other species were mesophagous.

Table IIIb

Amino acid utilization by yeasts

	l-valine			l-methionine			l-arginine			l-histidine		
	C	N	C-N	C	N	C-N	C	N	C-N	C	N	C-N
Procandida albicans	2	0	2	0	2	0	4	4	4	0	0	0
Procandida tropicalis	2	0	2	0	2	0	4	4	4	0	0	0
Azymocandida rugosa	2	2	2	0	2	0	1	1	1	0	0	0
Candida krusei	4	2	4	0	4	0	0	2	0	0	1	0
Candida reukaufii	1	0	1	0	0	0	0	0	0	0	0	0
Candida famata	2	0	2	0	1	0	4	2	4	0	0	0
Candida solani	2	2	2	0	0	0	4	1	0	0	0	0
Candida parapsilosis	2	2	2	0	2	0	4	2	4	0	1	0
Candida utilis	2	0*	4	0	0*	0	0	0*	0	0	0*	0
Candida guilliermondii	2	2	2	0	4	0	4	2	4	0	1	0
Candida pseudotropicalis	2	0	2	0	0	0	0	0	4	0	0	0
Torulopsis glabrata	1	0	2	0	0	0	0	0	0	0	0	0
Torulopsis inconspicua	2	0	1	0	0	0	0	0	0	0	0	0
Hansenula anomala	4	0*	4	0	0*	0	4	0*	2	0	0*	0
Dekkeromyces fragilis	2	0	2	0	0	0	0	0	0	0	0	0
Geotrichum linkii	4	4	4	0	4	0	2	4	1	0	0	0
Geotrichum candidum	2	1	2	0	0	0	0	0	0	0	0	0
Geotrichum matalense	4	4	4	0	4	0	2	4	1	0	0	0
Trichosporon cutaneum	2	0	2	0	0	0	0	0	0	0	0	0
Cryptococcus neoformans	2	0	4	0	0	0	0	2	0	0	2	0
Cryptococcus diffluens	1	0	2	0	1	0	0	0	0	0	0	0
Cryptococcus albidus	2	1	2	0	0	0	4	4	4	0	2	0
Rhodotorula mucilaginosa	4	2	4	0	4	0	0	2	1	0	1	0
Rhodotorula glutinis	4	2	4	0	4	0	0	2	1	0	1	0
Rhodotorula flava	4	2	4	0	4	0	0	2	1	4	1	0

As to the amino acids, carbon source examinations and simultaneous carbon-nitrogen source examinations yielded somewhat dissimilar results. As fungi usually require carbon sources poor in nitrogen content, the results were expected to be similar. The difference in the order of amino acids used as carbon sources and in the order of amino acids used as carbon and nitrogen sources is indicated in Table IV. Accordingly, certain amino acids were assimilated better as carbon sources and others were utilized more intensively as simultaneous carbon-nitrogen sources. The first of these findings may be explained by an improved assimilation of the amino acid when an extraneous nitrogen source is provided (NH_4^+ ions in carbon source examinations); the second might be

Table IIIc

Amino acid utilization by yeasts

	Glycine			l-α-alanine			l-cysteine		
	C	N	C-N	C	N	C-N	C	N	C-N
Procandida albicans	0	1	1	1	1	1	0	0	0
Procandida tropicalis	0	1	0	4	2	4	0	0	0
Azymocandida rugosa	0	1	0	0	0	1	0	0	0
Candida krusei	0	2	0	0	2	0	0	2	0
Candida reukaufii	0	0	0	0	1	0	0	0	0
Candida famata	2	2	2	4	2	4	0	0	0
Candida solani	0	0	0	0	2	0	0	0	0
Candida parapsilosis	0	2	0	4	2	4	0	0	0
Candida utilis	0	0*	0	4	0*	4	0	0*	0
Candida guilliermondii	4	2	4	4	2	4	0	0	0
Candida pseudotropicalis	0	0	0	0	0	4	0	0	0
Torulopsis glabrata	0	0	0	0	0	0	0	0	0
Torulopsis inconspicua	0	0	0	0	0	0	0	0	0
Hansenula anomala	0	0*	0	0	0*	0	0	0*	0
Dekkeromyces fragilis	0	1	0	0	0	1	0	0	0
Geotrichum linkii	0	2	0	4	4	4	0	1	0
Geotrichum candidum	0	0	0	0	0	0	0	0	0
Geotrichum matalense	0	4	0	4	4	4	0	2	0
Trichosporon cutaneum	0	0	0	0	0	0	0	0	0
Cryptococcus neoformans	0	0	1	0	2	0	0	0	0
Cryptococcus diffluens	0	0	0	0	0	0	0	0	0
Cryptococcus albidus	2	2	2	4	4	4	0	0	0
Rhodotorula mucilaginosa	1	2	1	1	2	1	0	1	0
Rhodotorula glutinis	1	2	1	1	2	1	0	1	0
Rhodotorula flava	0	2	0	4	2	2	0	1	0

Note to Tables IIIa, b, c: 0* = Because of a heavy growth on glucose-containing control medium, assimilation as a nitrogen source could not be estimated.

due to the very presence of NH_4^+ ions, which inhibit the uptake or decomposition of the amino acid. Thus the replica plating technique seems adequate for studying uptake, decomposition, or assimilation of substances. It is suitable for a rapid and broad spectrum collection of interesting data which may constitute a starting point of further detailed analyses.

As to the assimilation of amino acids as carbon and simultaneous carbon-nitrogen sources, about 50 per cent of the species examined were definitely polyphagous; 7 species were practically monophagous.

Table IV

Ord er of amino acids on the basis of the degree and species-spectrum of assimilation

Utilization as C source	Utilization as C and N source
Valine	Valine
Glutamic acid	Glutamic acid
Alanine	Glutamine
Arginine	Asparagine
Aspartic acid	Alanine
Asparagine	Aspartic acid
Glutamine	Arginine
Glycine	Glycine
Histidine	Methionine
Methionine	Histidine
Cysteine	Cysteine

Our results are in accordance with those of SCHULTZ *et al.* [17]; some of our yeasts that assimilated glutamic acid weakly or not at all, were, however, able to utilize other amino acids.

It should be noted that neither of the examined strains assimilated cysteine and methionine as carbon and carbon-nitrogen sources, and that with histidine only one positive result was obtained. As unfortunately our histidine-assimilating *Rhodotorula flava* strain has been lost, no further investigations can be performed with this organism.

Because of the orientative character of the experiments on amino acid utilization as carbon and simultaneous carbon-nitrogen source, we do not wish to go into details as regards the taxonomic usefulness of our data. Some findings, however, deserve to be mentioned. It was striking, for example, that glycine was strongly assimilated by *Candida guilliermondii;* there was a sharp difference between *Cryptococcus albidus* and *Cryptococcus diffluens* or *Cryptococcus neoformans* and also between *Geotrichum candidum* and *Geotrichum linkii* or *Geotrichum matalense.* As regards the latter, it should be mentioned that *Geotrichum candidum* and *Trichosporon cutaneum* exhibited very similar properties. The difference between *Dekkeromyces fragilis* and its imperfect form, *Candida pseudotropicalis*, was also interesting.

Cryptococcus neoformans grew better on amino acid media free from NH_4 ions, therefore this organism was considered to be sensitive to ammonium compounds.

The difficulty arising in consequence of the good growth on control plates of amino acid nitrogen source experiments has already been mentioned. This finding was probably due to the fact that fungi multiply better in the

Table V

Comparison of the assimilation of metabolically related amino and organic acids

Species	Aspartic acid	Asparagine	Succinic acid	Fumaric acid	Malic acid	Tartaric acid
Hansenula anomala	1	1	4	0	4	2
Dekkeromyces fragilis	0	0	2	0	1	1
Geotrichum linkii	4	4	4	1	4	0
Geotrichum candidum	0	0	0	0	0	0
Geotrichum matalense	4	4	4	1	4	0
Trichosporon cutaneum	0	0	0	0	0	0
Cryptococcus neoformans	0	0	0	0	1	1
Cryptococcus diffluens	0	0	0	0	1	1
Cryptococcus albidus	4	4	4	4	4	1
Rhodotorula mucilaginosa	1	1	2	2	2	0
Rhodotorula glutinis	1	1	2	2	2	0
Rhodotorula flava	4	4	4	4	4	0
Procandida albicans	1	1	1	0	0	0
Procandida tropicalis	2	4	2	2	2	1
Azymocandida rugosa	0	0	0	0	0	0
Candida krusei	0	0	4	0	1	2
Candida reukaufii	0	0	0	0	0	0
Candida famata	4	4	4	0	2	0
Candida solani	1	0	1	0	0	0
Candida parapsilosis	4	4	4	2	2	0
Candida utilis	0	0	4	2	4	1
Candida guilliermondii	4	4	4	4	4	1
Candida pseudotropicalis	0	0	2	0	0	0
Torulopsis glabrata	0	0	0	0	0	1
Torulopsis inconspicua	0	0	1	0	1	0

presence of carbon sources poor in nitrogen [5, 6]. Thus, results cannot be compared properly and the method itself is inadequate for nitrogen source examinations. Consequently, our data cannot be compared with the findings of SCHULTZ and POMPER [16].

The role of methionine as a nitrogen source for some species exerting a degree 4 assimilation is worth mentioning. This amino acid was not assimilated by any of the examined cultures as a carbon or simultaneous carbon-nitrogen source. Thus it may be assumed that its sulphur content inhibits the utilization

of its carbon content but not that of its amino group. Results obtained with cysteine and histidine as nitrogen sources could not be evaluated because of the poor growth (degree 2 or 1) and the above-mentioned tolerance of nitrogen deficiency. Our results are in accordance with those of SCHULTZ and McMANUS [18], in that none of their yeasts utilized methionine and cysteine as carbon sources.

It is interesting to compare the results yielded by four carbon atom-containing dicarboxylic acids with those obtained with aspartic acid and asparagine as carbon sources. Table V shows that the accordance of the degree of dicarboxylic acid utilization with aspartic acid and asparagine utilization tends to fall in the order: succinic, malic, fumaric and tartaric acid. That the results obtained with tartaric acid considerably differed from those given by the other three acids may be explained by the fact that, in contrast to the others, tartaric acid plays no part in intermediary metabolism.

In comparing the results concerning organic acids and amino acids, it is evident that with the exception of some species our cultures generally showed a polyphagous or monophagous character with respect to both groups of nutrient materials. It is interesting that when an exceptional behaviour was noted, the monophagous character appeared always with amino acids and the polyphagous character always with organic acids.

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INTRAVASCULAR PRECIPITATE FORMATION DURING ANAPHYLACTIC SHOCK IN THE GUINEA PIG

By

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Summary. Guinea pigs, sensitized actively or passively to or hyperimmunized with, ferritin, were shocked by intrajugular antigenic challenge. Light and electron microscopic studies disclosed the formation of precipitates corresponding to a ferritin-antiferritin complex throughout the pulmonary capillary bed. Shock was prevented and intravascular precipitate formation was suppressed by hypothermia. Although chlorpromazine fully protected the animals from anaphylactic shock, the formation of precipitates of ferritin-antiferritin complex in the pulmonary capillary bed was not significantly affected. Guinea pigs with a high serum antibody level formed only a moderate amount of precipitate in their small pulmonary vessels. The results indicate that in the guinea pig during anaphylactic shock intravascular precipitate formation takes place, due to antigen-antibody reaction. The precipitates thus formed, however, lack any significant pathogenetic role.

The occurrence *in vivo* of specific immune precipitation demonstrable *in vitro*, and its possible pathogenetic role have been intriguing problems to those concerned with experimental anaphylaxis. McKinnon *et al.* [1] observed precipitate formation throughout the pulmonary capillary bed during anaphylactic shock in the rabbit, and assigned to the phenomenon a particular significance in the mechanism of anaphylactic symptoms. The studies outlined below had the aim to clarify whether pulmonary intravascular precipitate formation during anaphylaxis in the guinea pig, had any anaphylactogenic role. Ferritin, being of high electron-density and thus particularly suitable for electron microscopic studies, was used throughout the experiments.

Materials and methods

Crystalline bovine spleen ferritin was prepared according to Keiderling and Wöhler [2], with slight modifications.

In active anaphylaxis experiments, guinea pigs were injected 8 mg ferritin subcutaneously, twice with an interval of two days. Three weeks after the first sensitizing dose, the animals were shocked with 15 mg ferritin by the intrajugular route.

Guinea pigs were sensitized passively with rabbit anti-ferritin immune serum administered intravenously in doses of 500 μg antibody-N per kg body weight, and challenged after 48 hours intrajugularly with 15 mg ferritin.

Both the actively and passively sensitized animals were protected from anaphylactic shock by chlorpromazine [3], or by hypothermia [4, 5]. Chlorpromazine was administered subcutaneously in doses of 30 mg per kg body weight, 24 and 2 hours prior to challenge. Guinea pigs anaesthetized with 90 mg per kg body weight of phenylethylbarbiturate were cooled to 26—27° C with ice bags.

Finally, guinea pigs were hyperimmunized intramuscularly for 5 weeks with a total dose of 50 mg ferritin and challenged in the 7th week with 15 mg antigen by the intrajugular route. Serum antibody levels were determined by quantitative precipitation.

Lung specimens taken for light microscopic study were processed by conventional methods and stained with hematoxylin and eosin. Specimens for electron microscopy were fixed partly in glutaric aldehyde followed by osmium tetroxide, partly in Dalton's fixative, then dehydrated and embedded in Araldite. Ultra-thin sections were contrasted with lead citrate and examined with a Zeiss D—2 electron microscope.

Each type of experiment was carried out in 5 guinea pigs. Guinea pigs in fatal shock were sacrificed immediately prior to death, *i.e.* 4—6 minutes after the eliciting dose. In the rest of experiments, the animals were killed 5 or 20 minutes after challenge.

Results

As the first step, the ultrastructural appearance of the ferritin-antiferritin complex produced *in vitro*, was studied. Precipitates prepared in the zone of equivalence were repeatedly washed with physiological saline, dehydrated and embedded. Ultra-thin sections revealed the picture characteristic of antigen-antibody complex, as defined by EASTY and MERCER [6] in their extensive work on ferritin (Fig. 1).

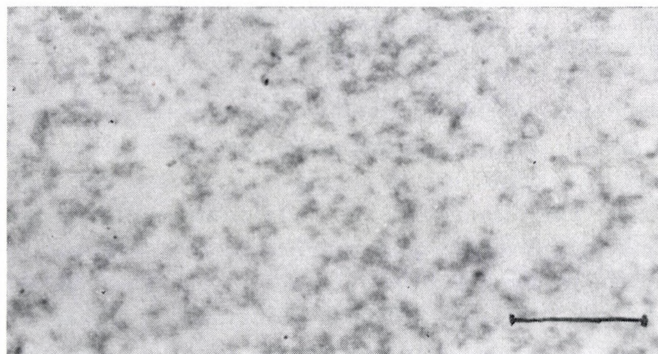

Fig. 1. Ferritin-antiferritin precipitate formed *in vitro*. Fixed in glutaric aldehyde, postfixed in osmium tetroxide, embedded in Araldite. Ultra-thin section, contrasted with lead citrate, ×15,000

Subsequently, control specimens were excised from lungs of guinea pigs not subjected to pretreatment, 5 to 10 minutes after a single intrajugular dose of 15 mg ferritin. Light and electron microscopy disclosed no ferritin-antiferritin precipitates in the pulmonary capillary bed.

In active anaphylaxis experiments, at gross examination the lungs were found characteristic of shock. Light microscopy revealed a marked dilatation of alveoli and small vessels, the latter being occluded in all lobes by plugs containing, among others, cellular elements and corresponding to the amorphous thrombi described by SABESIN [7] (Fig. 2).

Under the electron microscope these plugs consisted of ferritin-antiferritin precipitate and clusters of cellular elements (Fig. 3).

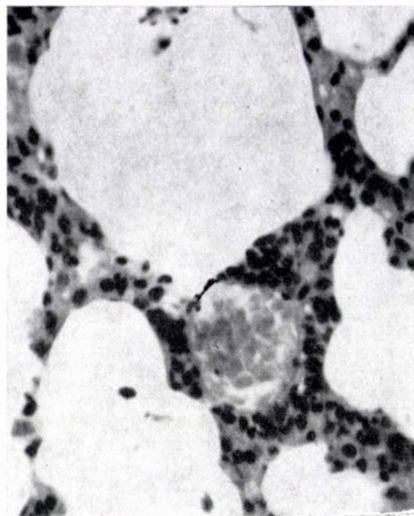

Fig. 2. Active anaphylactic shock. Plug of cellular structure in a small vessel. Haematoxylin and eosin stain, ×370

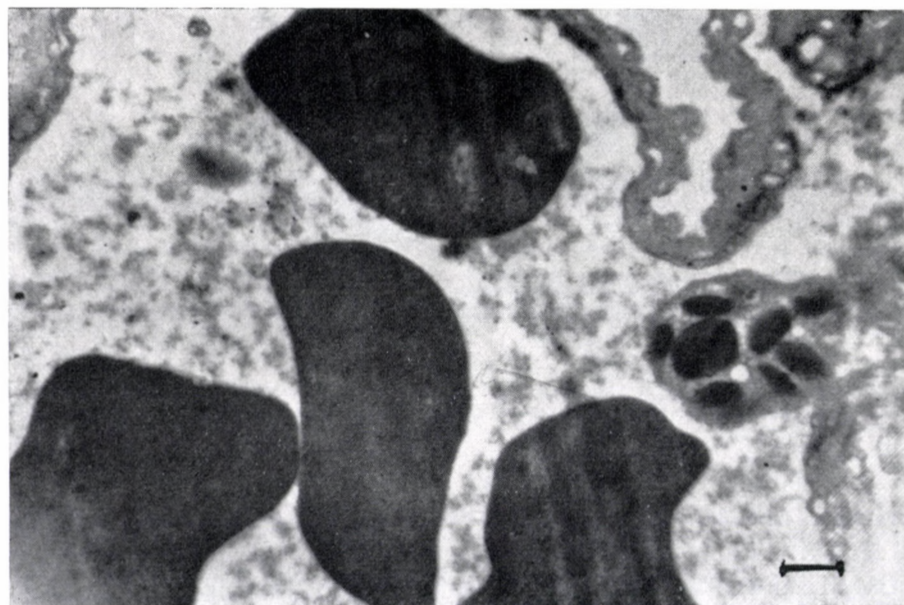

Fig. 3. Active anaphylactic shock. Erythrocytes and electron-dense ferritin particles in a pulmonary capillary loop. Light areas around particles correspond to the precipitate's antibody component, ×7,800

Serum antiferritin levels prior to challenge varied between 10 and 20 μg antibody-N per ml. Light and electron microscopy of lungs from passively sensitized shocked guinea pigs revealed pictures identical to those described above.

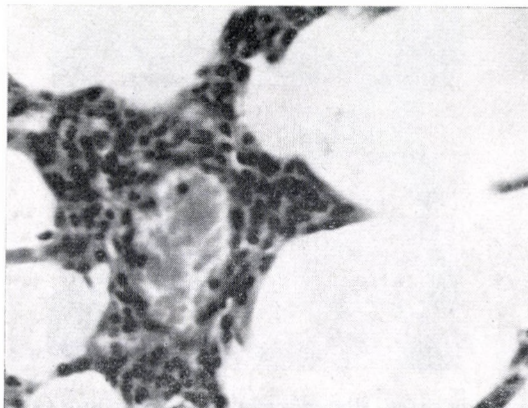

Fig. 4. Active anaphylactic shock prevented by chlorpromazine. Amorphous plug in a distended vessel, alveolar dilatation. Haematoxylin and eosin stain, ×370

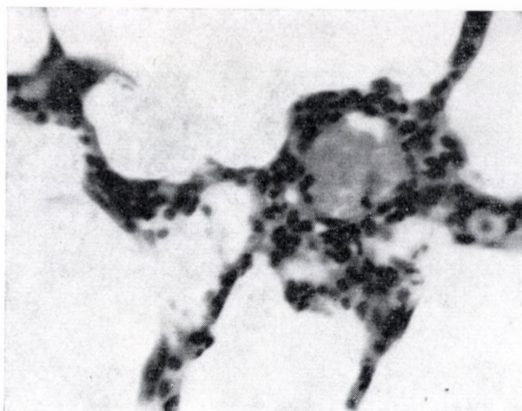

Fig. 5. Passive anaphylactic shock prevented by chlorpromazine. Amorphous plug in a distended vessel, alveolar dilatation. Haematoxylin and eosin stain, ×370

Guinea pigs sensitized actively or passively and treated with chlorpromazine, manifested no shock symptoms after challenge. Light microscopy still displayed the occlusion of pulmonary capillary loops by granulated (Fig. 4) or less granulated thrombi (Fig. 5), and alveolar distension.

As seen in Fig. 6, pulmonary capillary loops of the latter guinea pigs contained numerous electron-dense particles, but less than in shocked animals not pre-treated with chlorpromazine.

Our previous studies demonstrated a definite protective effect of deep hypothermia (26°—27° C body temperature) against active and passive anaphylactic shock in the guinea pig [4, 5]. Light microscopy of the lungs of actively or passively sensitized guinea pigs, challenged with ferritin under hypothermia, revealed no pulmonary capillary occlusion, but displayed the

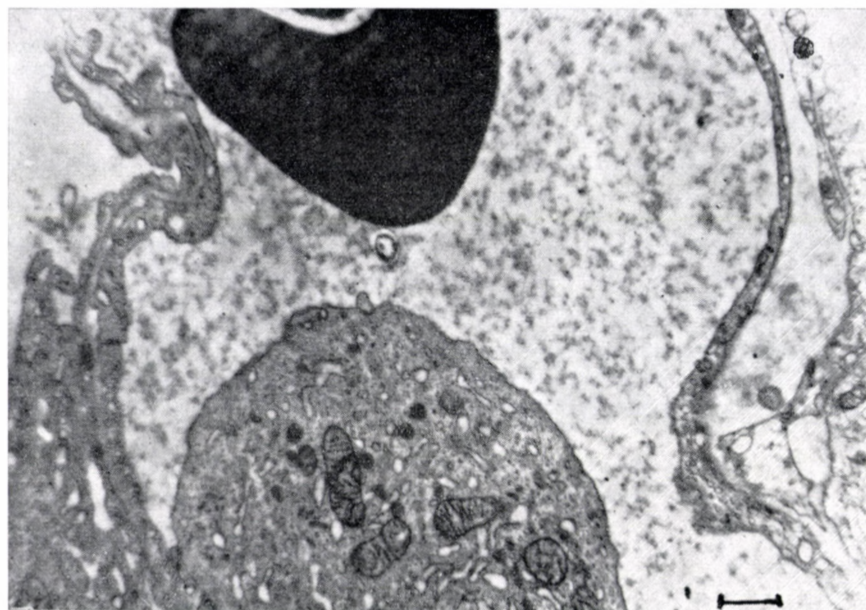

Fig. 6. Passive anaphylactic shock prevented by chlorpromazine. Electron-dense particles in the pulmonary capillary loop distributed more sparsely than in Fig. 3. ×7,800

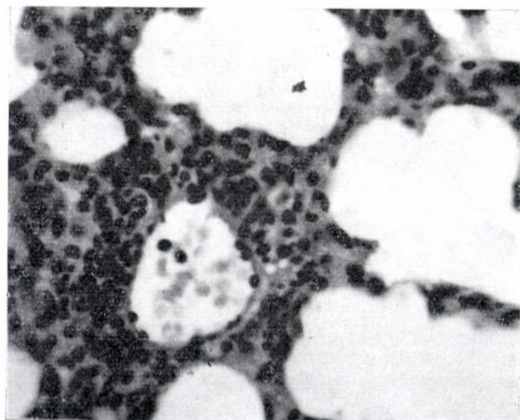

Fig. 7. Active anaphylactic shock prevented by hypothermia. Isolated erythrocytes and white blood cells in a blood vessel. Lumen and alveoli of normal width. Haematoxylin and eosin stain, ×370

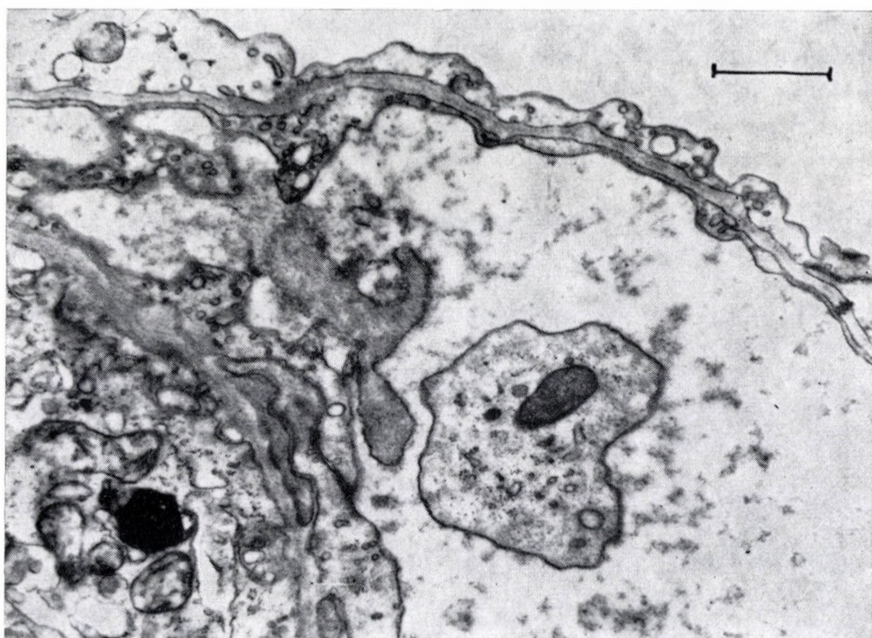

Fig. 8. Passive anaphylactic shock prevented by hypothermia. Scanty distribution of electron-dense particles in a pulmonary capillary loop, ×15,600

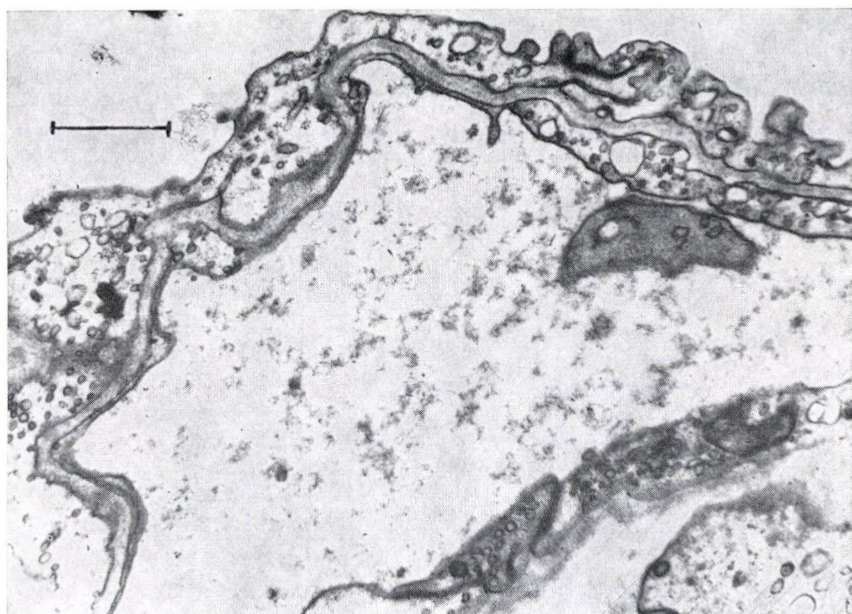

Fig. 9. Guinea pig immunized for 5 weeks, shocked in 7th week. Serum antibody level 110 μg antibody-N per ml. Sparsely distributed electron-dense particles in a pulmonary capillary loop, ×15,600

same picture as seen after the injection of ferritin into nonsensitized animals. As seen in Fig. 7, if shock was prevented by hypothermia, no dilatation of capillary loops and alveoli could be observed. The distribution of electron-dense particles in the small vessels was rather scanty, as demonstrated by electron microscopy (Fig. 8).

Guinea pigs hyperimmunized with ferritin for several weeks exhibited serum antibody titres between 60 and 110 μg antibody-N per ml. Animals with a high serum antibody content displayed slight, if any, shock symptoms after challenge. Electron microscopy disclosed a minor degree of intravascular precipitation (Fig. 9). In the case of a relatively low serum antibody level, the guinea pigs responded with severe or fatal shock to the reinjection of ferritin and the microscopic picture in this case was identical with that seen in active anaphylaxis.

Discussion

Precipitate formation in the pulmonary capillary bed during anaphylactic shock in the guinea pig was demonstrated by SABESIN in 1964 [7]. In these studies ferritin was employed as antigen and the active anaphylactic shock was prevented by antihistamines. KENT and ECKER [8] elicited anaphylactic shock with horse or human serum and bovine serum albumin. Under the light microscope the authors found no, or only minimal, intravascular occlusion in the lungs during anaphylaxis. The present studies yielded definite proof of the development of thrombus-like precipitates throughout the pulmonary capillary bed during both active and passive anaphylaxis in the guinea pig.

As demonstrated earlier, the anti-anaphylactic effect of chlorpromazine was based on its antihistaminic property [3], while hypothermia was operative *via* the inhibition of histamine liberation [9, 10]. The present light and electron microscopical findings supported the validity of the former statements. Namely, in the case of anaphylaxis prevented by chlorpromazine, certain histamine effects could be observed, such as the dilatation of alveoli and capillary loops, while in hypothermic animals no signs of histamine liberation were present.

Chlorpromazine was found to influence only slightly the intravascular precipitation due to antigen-antibody reaction. Hypothermia, on the other hand, significantly suppressed or completely inhibited precipitate formation. This might have been due partly to the slowing down of blood circulation, partly to the impairment of antigen-antibody reaction [11, 12].

The absence or mild course of anaphylactic shock in guinea pigs with a high serum antibody level validates the cellular theory of anaphylaxis. The slight amount of intravascular precipitates, as observed in such cases, might

have its explanation in the partial dissolution of the precipitates in the excess antibody.

In the light of our findings, intravascular precipitate formation during anaphylactic shock in the guinea pig should be regarded as a secondary phenomenon with no substantial pathogenetic role. The above observations made in guinea pigs, however, do not allow conclusions as to the effect of precipitate-thrombi in other animal species. In the rabbit, for instance, pulmonary circulation undergoes deep changes during anaphylactic shock, while the parameters of pulmonary circulation during anaphylaxis in the guinea pig still awaits elucidation [8].

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LIPID COMPOSITION OF TREPONEMAL STRAINS

By

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Summary. Fatty acid and phospholipid composition of pathogenic *T. pallidum* (strain Budapest) and of various cultivable Treponema strains *(T. reiteri, kazani 5, minutum* and *refringentis)* were analysed by means of gas and thin layer chromatography. The fatty acid spectra were simple and nearly identical in all strains. They contained chiefly palmitic and unsaturated (oleic and oleinic) fatty acids, the latter in relatively high proportions. Cyclopropane-ring-containing fatty acids were not detected. The phospholipid composition was quite complex. Concerning their unsaturated fatty acid content, treponemes and streptococci were similar. The findings indicate an increased permeability of their cell membrane and thereby their higher vulnerability by environmental influences.

Treponemes in comparison with other bacteria are relatively rich in lipids. Our knowledge concerning their chemical composition, and their structural and biological role is scanty. In the present paper the fatty acid and phospholipid composition of pathogenic and various cultivable treponemes is compared.

Materials and methods

Pathogenic *T. pallidum* (strain Budapest) was obtained from early orchitis of rabbits. The animals were infected with $2—2.5 \times 10^7$ cells per testicle. To enhance the yield of treponemes, the animals were injected with 10 mg of prednisone (Diadreson, Organon) daily. After 9 days the animals (as a rule four on one occasion) were bled by heart puncture, then the testicles were removed aseptically, sliced and extracted for one hour in saline (5 ml per testicle) under N_2 atmosphere. After changing the saline, extraction was continued for 6 hours. To remove tissue debris, the treponemal suspension was centrifuged at 1500 *g* for 10 minutes. After discarding the sediment, the treponemes were centrifuged at 5000 *g* for 30 minutes. The treponemal sediment was resuspended in about 40 ml of saline by shaking with glass beads and the procedure was repeated twice. Finally the sediment was taken up in 1 ml of saline and lyophilized.

As a control, lyophilized homogenate of syphilitic rabbit testicles was used.

Cultivable treponemes *(T. minutum, T. refringentis* and cultivable strains of *T. reiteri, T. kazani* 5) were grown in fluid thioglycollate medium of the following composition. Lact-amino-acid 10 g, sodium chloride 2.5 g, dl-cysteine hydrochloride 0.75 g, thioglycollic acid 0.4 ml, dextrose 5.0 g, cellamine (yeast extract) 5.0 g, distilled water, to 1000.0 ml.

The ingredients were dissolved by boiling, and the pH was adjusted to 7.8 with 20 per cent NaOH. After filtration, the solution was sterilized in an autoclave at 126° C for 30 minutes. The medium (8.5 parts) was supplemented with inactivated horse serum (1 part) and ultra-filtered yeast extract (0.5 part). The ingredients were sterilized by filtration. The medium was dispensed in 1000 ml flasks and inoculated with seed cultures grown in the same medium, containing rabbit serum as supplement. After incubation at 35°C for 5—7 days the treponemes were harvested by centrifugation at 4000 g for 15 minutes. The sediment was washed with saline until in the supernatant no traces of protein could be detected with 20 per cent sulphosalicylic acid. The washed sediment was resuspended in saline and lyophilized.

Concerning the methods of lipid analysis we refer to a previous paper [1].

Results

The fatty acid composition of the treponemes, expressed in per cents of the extracted total fatty acids is shown in Fig. 1. All strains examined contained the same fatty acids: the bulk was composed of palmitic acid. Unsaturated fatty acids were present in the following percentages: *T. pallidum*, 17; *T. kazani* 5, 28; *T. reiteri*, 32; *T. minutum*, 34; and *T. refringens*, 8. Fatty acids with cyclopropane ring have not been detected.

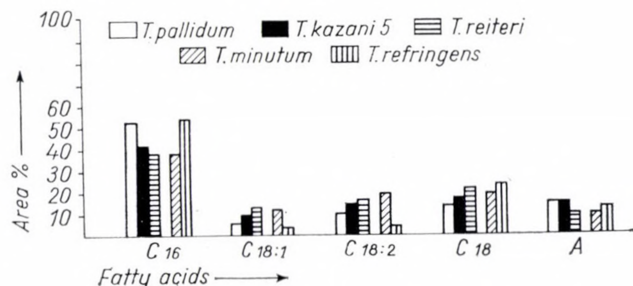

Fig. 1. Fatty acids in treponemes
A: C_{10}; C_{11}; $C_{12:2}$; C_{12}; C_{13}; $C_{14:1}$; C_{14}; C_{15}; ai C_{15}; $C_{16:1}$ C_{17} total

Fig. 2 shows the gas chromatograms of fatty acids in two species *(T. pallidum* and *Str. pyogenes)* highly sensitive to antibiotics, and in two polyresistant organisms *(P. vulgaris* and *Ps. pyocyanea)*. Sensitive bacteria, in contrast to polyresistant organisms, were comparatively rich in unsaturated fatty acids but completely devoid of fatty acids with cyclopropane ring. Fig. 3 shows the percentage distribution of fatty acids (unsaturated, cyclopropane-ring containing, and saturated) of various bacteria differing in sensitivity to

Table I

The phospholipid composition of treponemes

Organism	Aceton insoluble phosphatide-fraction	
	Percentage of total lipid	Percentage of ether insoluble fraction
T. pallidum	↑ 37.4	↑ 89.3
T. kazani 5	31.7	66.7
T. reiteri	19.5	62.1
T. minutum	29.0	64.8
T. refringens	19.8	63.1

antibiotics. The close resemblance in fatty acid composition between *Str. pyogenes* group A and C (upper part) and various species of treponemes (middle part) was remarkable; both are rich in unsaturated fatty acids and completely devoid of cyclopropane-ring compounds. This fact was quite conspicuous,

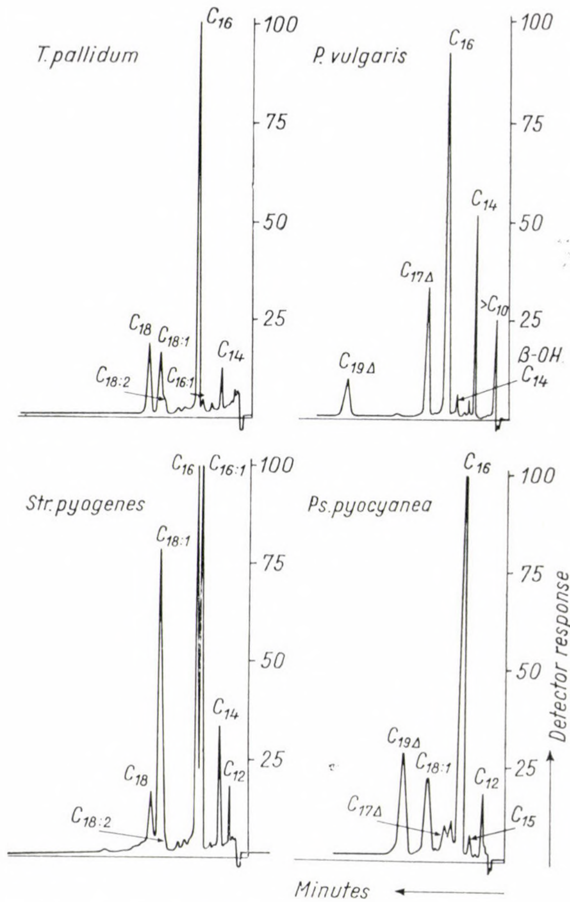

Fig. 2. Fatty acid composition of two highly sensitive and two highly resistant microorganisms

because the latter are formed from unsaturated fatty acids. *Staph. aureus* strain 53 and *P. vulgaris*, which are both highly resistant to antibiotics (lower part), contain chiefly cyclopropane-ring-containing and saturated fatty acids.

The phospholipid composition of treponemes, as demonstrated by the preliminary results, is rather complex. Phospholipids were separated into fractions soluble and insoluble in acetone. The bulk of phosphatides was

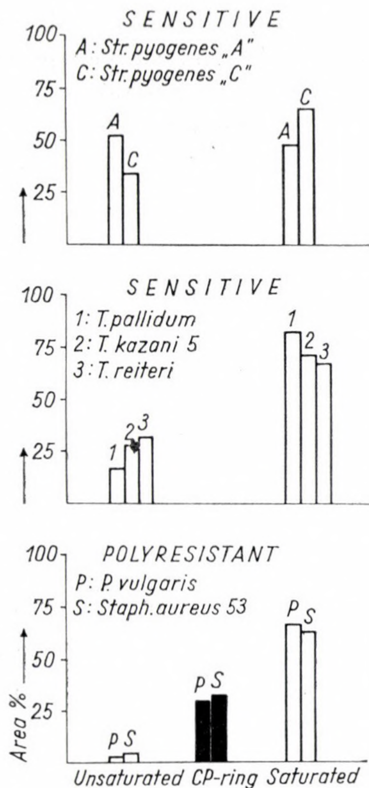

Fig. 3. Percentage distribution of fatty acids of various microorganisms

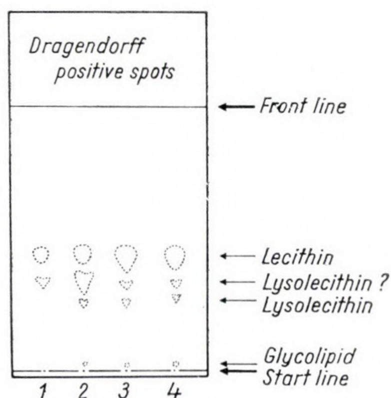

Fig. 4. Thin layer chromatogram of ether insoluble phospholipids.
1. Rabbit testicle; 2 *T. pallidum;* 3. *T. kazani;* 4. *T. minutum.*
Solvent, chloroform : methanol : water = 70 : 22 : 13

contained in the latter fraction. The phosphatide content of *T. pallidum* was the highest, that of nonpathogenic species was lower (Table I). The acetone-insoluble fraction was divided according to solubility in ether. Of the phospholipids of *T. pallidum* about 90 per cent, while of other treponemes about 65 per cent were insoluble in ether. The ether soluble and insoluble fractions were separated by thin-layer chromatography. Independently from species, each treponema consisted of 11—13 components including lecithin, cephalin, cardiolipin and the corresponding lyso-compounds. The treponemes differed mainly in the components of the ether-insoluble fraction developed by Dragendorff's reagent. A chromatogram is shown in Fig. 4. In comparison with rabbit testicles and non-pathogenic treponemes *T. pallidum* obviously contained a significant quantity of lysolecithin.

Discussion

According to FROMM [2] and PILLOT and FAURE [3], the lipid content of treponemes varies between 14—20 per cent of the dry weight. FAURE and PILLOT [4] found that 55—65 per cent of the total lipid consisted of phosphatide, 33 per cent of the latter being lecithin and 5 per cent cardiolipin. According to HEYMANN and SIEFERT [5], the lipid content of *T. reiteri* and *T. pallidum* is 16.6 and 18.2 per cent, respectively. The effect of different fatty acids on the growth of treponemes was investigated by POWER and PELCZER [6] and OYAMA, STEINMAN and EAGLE [7], who emphasized the significant role of oleic acid. Synthesis of short-chain fatty acids by oral and genital treponemes was investigated by MOUREAU [8], who found differences in the degree of synthesis.

Our aim was to examine the degree of differences in total lipids and fatty acid spectrum of treponemal strains. On the basis of this fatty acid composition, treponemal species cannot be distinguished from each other. The fact that their lipids are composed of the same fatty acids indicates a common genetic origin and a resemblance of their lipid metabolism. The fatty acid spectrum of treponemes is characterized by the presence of great amounts of unsaturated and a total absence of cyclopropane-ring-containing fatty acids. Higher proportions of unsaturated fatty acids are generally found in the cells of younger bacterial cultures. With ageing, these substances, by acquiring methylene bridges, are transformed into more stable cyclopropane-ring-containing forms. The methylene donor in the process is S-adenosyl-methionine. Parallel with the saturation of fatty acids the resistance of older bacterial cells is enhanced. It may be assumed that in treponemes the enzyme system necessary for transmethylation is lacking. If correlated with our knowledge concerning the structure of the membrane of living cells, these analytical data give a key to the understanding of some biological properties of treponemes.

Soluble mycelia, constituting the frame-work of the membrane, can be formed from phospholipids containing either unsaturated fatty acids or fatty acids stabilized by cyclopropane rings. Beside saturated fatty acids, unsaturated ones and cyclopropane-ring components are also needed for the appropriate function of the bacterial cell membrane. A bacterial cell membrane containing high amounts of cis-unsaturated fatty acids is more readily permeable and thus renders the cell increasingly vulnerable against external influences. Saturation of double bonds by cyclopropane ring enhances the rigidity and decreases the permeability of cell membranes. This is probably the biochemical defence mechanism of ageing cultures with high transmethylating activity. Bacterial genera such as treponemata and streptococci unable to carry out this biochemical function, are more sensitive against environmental influences. This may explain why streptococci and treponemata cannot be made resistant to certain antibiotics. Our investigations, which have so far revealed a high complexity of the phospholipid composition of treponemes, indicate the significant role of these cell components in the processes of cell metabolism.

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STUDIES ON THE INITIAL PHASES OF POLIOVIRUS REPRODUCTION CYCLE

By

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Summary. Type 1 poliovirus (strain Mahoney) and a permanent monkey kidney cell line (PMK III/1) were used throughout. In Parker's No. 199 medium this system produced full virus yields whether or not bovine albumin had been added. To obtain full virus yield in Hanks' balanced salt solution the presence of bovine albumin was indispensable and its omission resulted in a 90 per cent or even greater reduction of the amount of virus produced.

Presence of bovine albumin in Hanks' balanced salt solution was required only during the first 30 minutes of the cycle, while its addition after 30 to 60 minutes caused 50 and 80 per cent reduction of the yield, respectively. Bovine albumin added in the second hour of the cycle or later did not increase the yield above that obtained in Hanks' balanced salt solution alone.

Absence or delayed addition (2 hours or later) of bovine albumin caused a 30 to 40 minutes retardation of the cycle's onset in Hanks' balanced salt solution.

Replication rate was practically identical in all cases, duplication time being approximately 10 minutes.

Bovine albumin seems to be a factor involved in the initial mechanisms of viral infection (penetration or eclipse). Its probable role in the "virtual eclipse" of adsorbed virions is discussed.

A closer approach to the possible mechanism of bovine albumin action was attempted by studying the effect of cysteine and glutathione and of K-linoleate on the initial phase of viral cycle.

It has been demonstrated that for the replication of poliovirus the intracellular metabolite and co-factor pool is made use of rather than that available in the environment [1]. Nevertheless, poor yields of virus were usually obtained in cells maintained after the infection in a simple, physiological, balanced salt solution containing glucose as sole source of energy [2, 3]. To clarify the possible reasons of these seemingly contradictory observations, comparative studies were performed in a complete medium (Parker's No. 199) and a simple balanced salt solution (Hanks').

Materials and methods

Virus. The Mahoney prototype strain of type 1 poliovirus was obtained from the collection of the Diagnostic Laboratory of this Department.

Cells. The permanent line of monkey kidney cells PMK III/1 isolated by Ruzicska [4] in this laboratory was used throughout. The line was maintained in a medium containing 40 per cent Parker's 199 medium (P 199), 45 per cent Hanks' balanced salt solution (HBS), 5 per cent lactalbumin hydrolysate (5 per cent solution) and 10 per cent calf serum. Forty-eight hours prior to use, each culture was washed and its nutrient fluid replaced by the following medium: 40 per cent P 199, 51 per cent HBS, 5 per cent of 5 per cent solution of lactal-

bumin hydrolysate, 2 per cent calf serum and 2 per cent of a 10 per cent solution of bovine albumin.

Cell line PMK III/1 has been known to exhibit a satisfactory susceptibility to infection with poliovirus [5].

Virus assay. The principles of DULBECCO and VOGT's plaque method [6] were applied for all quantitative virus assays. Monolayers were grown in 6 cm Petri dishes in 5 per cent CO_2 atmosphere under 8 ml of the medium used for the replacement of the culturing fluid in flasks. The initial concentration of cells was 1.5×10^6 per dish in 8 ml of medium. Confluent monolayers were obtained in 3 days. Monolayers were rinsed with HBS and virus was allowed to adsorb for 30 minutes at 37° C. Infected monolayers were overlayed without previous rinsing. The overlay consisted of 0.9 per cent purified [6] Difco Bacto agar (Difco, Detroit, Michigan, USA) in P 199 + 0.2 per cent BA. Reincubation was performed at 37° C in 5 per cent CO_2 atmosphere for 3 or 4 days. Plaques were counted after staining with an overlay containing 0.02 per cent neutral red (v/o "Soyuzkhimexport", Moscow, USSR) in a 0.9 per cent agar solution.

One-step growth experiment. The principles of LWOFF and LWOFF's method [7] were adapted to our purposes. Two litre Roux flask cultures of PMK III/1 cells were used 48 hours after the medium had been replaced. Cells were suspended by versenization (0.1 per cent versene in Parker's balanced salt solution, PBS) and washed 3 times in HBS. After the second washing, cells were resuspended in a measured volume and counted. The third washing was performed in siliconized centrifuge tubes on a definite number (usually 8×10^6) of cells required for the actual experiment.

Infection of cells was performed by adding an appropriate amount of undiluted stock virus suspension [8×10^7 plaque forming units (PFU) per ml] so as to obtain an input multiplicity of one. The sedimented cells were suspended directly in the stock virus by means of a siliconized pipette. Adsorption was allowed for 10 minutes at room temperature and for an additional 5 minutes at 37°C under continuous stirring. Adsorption was stopped by a rapid 100fold dilution of the mixture in HBS at room temperature. Unadsorbed virus was removed by two rapid washings in HBS at room temperature.

The sedimented infected cells were suspended in HBS so as to obtain 1×10^6 cells per ml. One ml of this suspension was added to 9 ml of the appropriate medium at 37°C. This final cell suspension was prepared in 100 ml siliconized Jena-glass Erlenmayer flasks provided with tightly fitting glass caps. The atmosphere in the flasks contained 5 per cent CO_2 in air. Flasks were immersed into a water bath at 37° C and agitated by means of a rotary shaker (60 r. p. m.).

The whole procedure from the start of adsorption until completion of the final cell suspension in the Erlenmayer flasks was regularly performed in 30 minutes.

Samples taken from the flasks at appropriate points of time were immediately frozen and stored in that state until further processed. The atmosphere of the flasks was readjusted after each opening by flushing with CO_2-air mixture.

Prior to titration the samples underwent 5 subsequent cycles of rapid freezing and thawing to disrupt cells. Thus all titres given in this paper represent total virus contents of the actual system.

Chemicals. Inorganic salts and glucose were products of Reanal (Budapest, Hungary). All components of P 199 medium were preparations of NBC (Cleveland, Ohio, USA), K-linoleate was a product of SERVA (Heidelberg, Germany).

Bovine albumin was kindly supplied by Dr. E. NOVÁK (National Blood Bank and Transfusion Centre, Budapest) to whom authors are pleased to express their thanks. The same lot of electrophoretically homogeneous bovine albumin (BA) prepared by Cohn fractionation was used throughout. A stock solution of 10 per cent BA was prepared in saline and filtered through a glass filter (Jena G—5). The stock solution was stored at +4°C until used.

Experimental

One-step growth curves of poliovirus in P 199 and HBS, both with and without the addition of a 0.2 per cent final concentration of BA at 0 time are shown in Fig. 1.

Growth curves obtained in the presence of BA both in 199 and HBS were identical. In plain P 199 a slight (about 5—6 min.), while in plain HBS a re-

markable (about 40—45 min.) delay was observed at the cycle's onset. The slope of the straight parts of the curves was practically identical, showing the similarity of the replication rate in all media tested.

The influence on the final virus yield of different concentrations of BA added at 0 time to suspensions of infected cells in HBS is shown in Fig. 2.

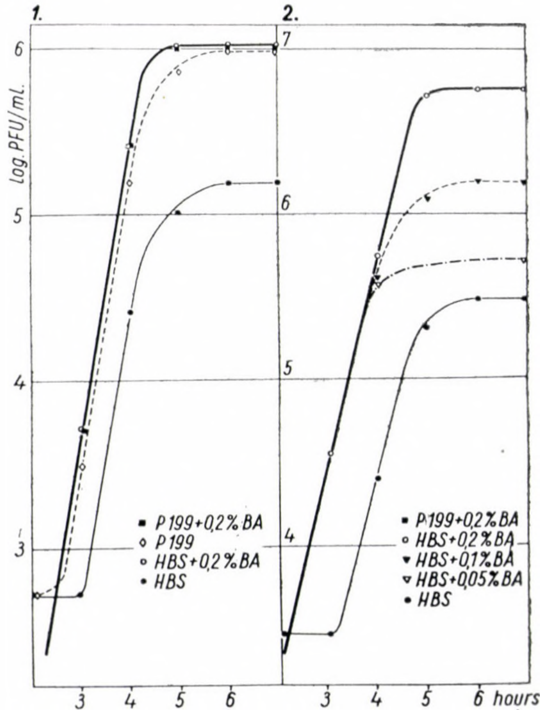

Fig. 1. Poliovirus growth in PMK III/1 cells supplied with different media

Fig. 2. Effect of different BA concentrations on poliovirus growth

It appeared that 0.2 per cent BA was the critical concentration required for a full yield. The replication rate was practically identical at any concentration of BA as well as in its absence. Thus BA did not seem to have any influence on the intracellular events involved in virus replication.

The presence of 0.2 per cent final concentration of BA in the nutrient fluid of poliovirus-infected cells for different periods of time was found to influence the final virus yield, as shown in Figs. 3/A and 3/B.

As shown in Fig. 3/A infected cells suspended in HBS produced full yields of virus if BA had been added at 0 time. Addition of BA at the 30th and 60th minutes of the one-step growth experiment resulted in yields reduced by 50 and 80 per cent, respectively. BA failed significantly to influence the final

yield if added at the 2nd hour or later. Replication rates were identical in all systems.

Presence of BA during the first 30 minutes of the one-step growth experiment ensured a full final yield (see Fig. 3/B). The moderate delay of about 10 minutes is supposed to have been the result of manipulations involved

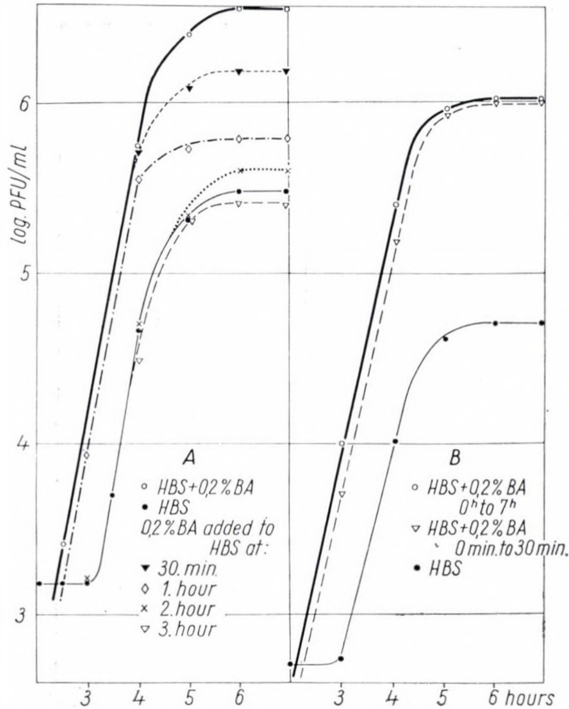

Fig. 3. Poliovirus growth as influenced by the addition of BA at different points of time (A) or by its presence in the initial 30 minutes (B)

in the removal of BA. This was done by centrifuging the cells for 2 minutes at 1000 r. p. m. and returning the sediment into an Erlenmayer flask with BA-free HBS. Both centrifugation and resuspension were performed at 37°C, using siliconized glassware.

The amounts of infectious virus demonstrable during the first 3 hours of the experiments with different media are given in Fig. 4.

In the absence of BA, the amount of infectious virus was essentially unchanged in both HBS and P 199. Addition of 0.2 per cent BA at 0 time resulted in a rapid "virtual" eclipse in both HBS and P 199. Final yields were identical in all media but plain HBS, the latter allowing for a 10 per cent yield only (see Fig. 1).

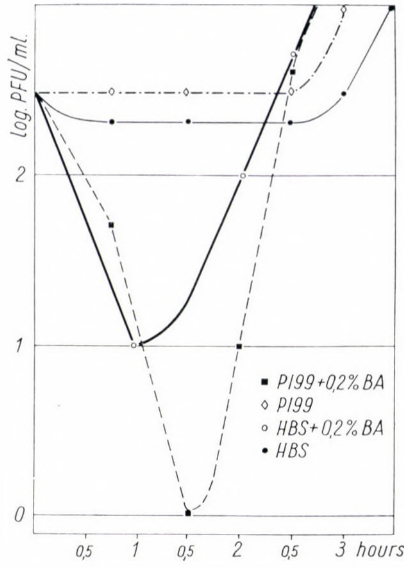

Fig. 4. "Virtual eclipse" of poliovirus in the presence of BA

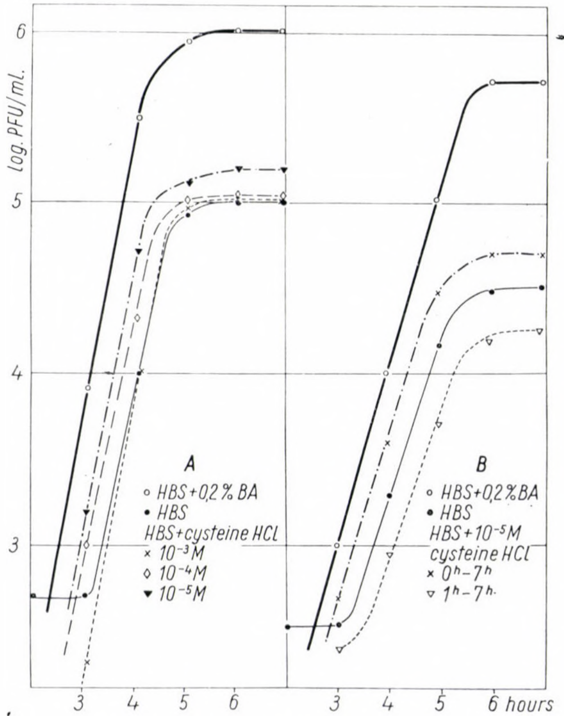

Fig. 5. Influence on poliovirus growth of cysteine added at different concentrations (A) and at different points of time (B)

The identity of growth curves in P 199 and HBS containing 0.2 per cent BA suggested the presence of some common active agents in both P 199 and BA. In this respect we have performed some preliminary assays with cysteine, glutathione and methionine, SH radicals having been supposed to be involved.

The effects of the addition of cysteine at different concentrations at 0 time are shown in Fig. 5A.

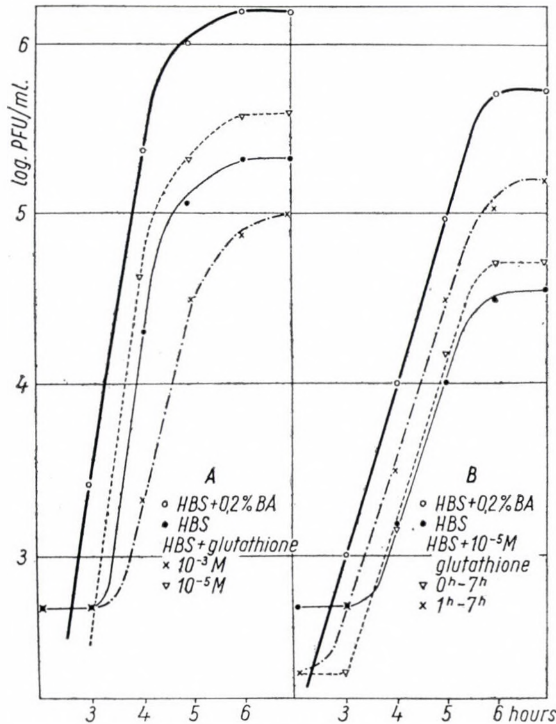

Fig. 6. Influence on poliovirus growth of glutathione at different concentrations (A), and at different points of time (B)

Cysteine at low concentrations (10^{-6}, 10^{-5}, or 10^{-4} M) reduced the lag period without significantly affecting the final yield. A 10^{-3} M concentration of cysteine failed to reduce the lag period, while allowed for a final yield identical with that obtained in plain HBS.

These experiments suggested that cysteine had a role in the early phase of the cycle. Therefore, the effect of delayed cysteine (10^{-5} M) addition was also studied. As shown in Fig. 5B, addition of cysteine after one hour resulted in a markedly delayed onset and in a moderately reduced yield of the viral cycle, as compared to that in plain HBS. Thus cysteine appeared to enhance some initial and to inhibit some late events of the viral cycle's initial phase.

Glutathione added at 0 time at 10^{-6} or 10^{-5} concentrations increased the final yield by about 10 per cent, without affecting the lag phase. At 10^{-3} *M* concentration added at 0 time, it caused both a retardation of onset (about 30 minutes) and a slight decrease (about 7 per cent) of the final yield (see Fig. 6A).

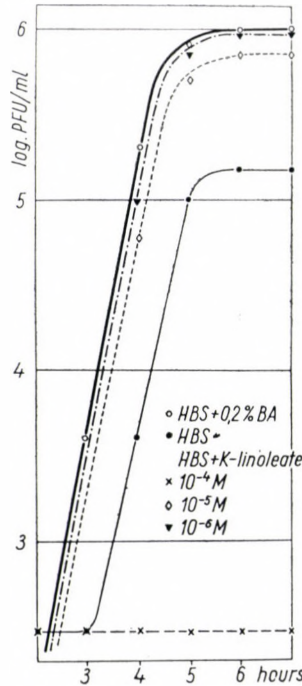

Fig. 7. Influence of K-linoleate at different concentrations on poliovirus growth

Glutathione (10^{-5} *M*) if added after 1 hour, reduced the lag by about 25—30 minutes and increased the final yield by about 25 per cent as shown in Fig. 6A.

Thus glutathione appeared to inhibit some events in the first 1 hour of the viral cycle and to enhance some other processes occurring after the first hour of incubation.

Methionine, carrying a methylated sulfur atom instead of an SH group failed to have any effect on the viral cycle when added at 0 time in the concentration range of 10^{-3} to 10^{-6} *M*. This observation was considered to furnish indirect evidence of the necessary presence of SH radical rather than another radical with substituted sulphur atom.

P 199 contains 15 μg/ml of Tween 80, while BA carries as standard contaminants certain fatty acids, including linoleic, linolenic and arachidonic

acids, etc. Thus, a further possible group of common factors in P 199 and BA is that of substances with detergent activity. Up to now only a single preliminary experiment was carried out with K-linoleate. This compound carries two unsaturated bonds and is one of the so-called "essential fatty acids". Result of the experiment is shown in Fig. 7.

K-linoleate was definitely inhibitory in 10^{-4} *M* final concentration. In lower concentrations, however (10^{-5}, 10^{-6} and 10^{-7} *M*), K-linoleate in plain HBS allowed for a final yield essentially identical with that obtained in HBS containing 0.2 per cent BA.

Discussion

These experiments have shown that PMK III/1 cells suspended in any of the media tested were able to support the replication of type 1 poliovirus (Mahoney). Time of onset, rate and final yield of virus replication were identical in P 199 as well as in HBS, if BA was present. A remarkable difference was, however, observed in the time of onset and the final yield of virus replication, if BA was omitted. In plain HBS not only an about 30 minutes' delay of onset, but also a 90 per cent reduction of yield was demonstrable. Plain P 199 allowed for a cycle practically identical with that in BA-containing P 199, except for a slight delay (about 5—7 minutes) of the onset.

These observations suggest that at least two different factors are influencing the early phase of viral cycle in the system studied. Out of them BA represents one, while a component, certain components or a given combination of components in P 199 represent another. The active principle(s) in BA and P 199 may be identical or different. Anyway, the lack of the active principle(s) results in delayed onset and reduced yield of the viral cycle as observed in plain HBS. The delay of onset may reflect some disturbance in the early phase of infection (penetration or eclipse) or in the mechanisms triggering virus replication in due course. Replication rates being practically identical in all media tested, reduction of yield may be brought about by a reduction of the number of successfully infected cells or by the production of a reduced number of virions by all or most infected individual cells.

Experimental evidence obtained with the system maintained in HBS and supplemented with BA at different phases of the viral cycle has shown that BA was fully active only if present during the initial 30 minutes. This observation suggests that the delay of onset in the absence of BA may be caused by impaired penetration or eclipse rather than by some disturbance in the mechanisms triggering replication.

The intermediary metabolite and co-factor pool available for the cells maintained in HBS must have been very similar, if not identical, irrespective of

whether BA was present. Nevertheless, delayed addition of BA improved poorly or not at all the final virus yield as compared to the full activity of the same substance when present during the first 30 minutes. Thus, it is questionable whether in the absence of BA the reduced number of virions produced by the individual infected cells would have caused the reduction of the final yield. We may, therefore, state that all indirect evidence available suggests the involvement of BA in the initial phase(s) of viral infection (penetration or eclipse).

No quantitative study has been conducted to determine the absolute number of successfully infected cells, thus not even estimates were available concerning the number of virions initiating a complete cycle with full final yield. It was, however, observed that in the absence of BA there was no change in the number of demonstrable virions in the system from 0 time until the onset of replication in P 199 as well as in HBS. In any case, infected cells suspended in plain P 199 produced a final virus yield equal to that obtained in the same system supplemented with BA. Thus the number of successfully infected cells must have been identical in both. Therefore the remarkable decrease in the number of infectious virions observed during the first 2 1/2 hours of incubation in the presence of BA in both P 199 and HBS did not seem to represent a "true eclipse". For the explanation of the phenomenon we accept the suggestion of FENWICK and COOPER [8] that this "virtual eclipse" represents "inactivation" or "rejection" of virions rather than their actual penetration into the cells. It seems to be of interest that "virtual eclipse" took place only in the presence of BA. This observation pointed to the possibility that BA may play at least two different roles in the initial phases of viral infection. First, it facilitates "true eclipse", secondly it is involved also in the phenomenon called "virtual eclipse".

In plain P 199 the absence of "virtual eclipse" and the indirect evidence for the presence of an unimpaired "true eclipse" suggested the possibility that the factor(s) responsible for the latter may be some active radical(s) or contaminant(s) in BA rather than the whole protein molecule itself.

Considering the possible common components of P 199 and BA, the involvement of -SH radicals or of contaminants with detergent activity appeared to be most probable. The experiments have shown that both cysteine and glutathione were able to reduce the lag phase, in other words, to facilitate "true eclipse", although apparently through different mechanisms. Nevertheless, none of these substances was able to increase the number of successfully infected cells as reflected by the unchanged final yields. These observations point to the active, though secondary role of -SH radicals in the initial phases of viral infection. The complete lack of a similar effect of methionine was considered an indirect evidence of the necessity of the presence of an unsubstituted -SH radical.

Out of the substances with detergent activity we have tested up to now only K-linoleate, a regular contaminant of BA preparations [9]. This substance appeared to have an effect essentially identical with that of BA (see Fig. 7).

This observation seems to be of interest, but nothing more can be stated until the completion of studies in progress in this laboratory on further biologically active fatty acids and on Tween 80, the detergent incorporated into P 199.

Results available at present suggest the similarity of the "infection enhancing" factors in P 199 and BA. These factors seem to play an important role in the true eclipse of adsorbed virions. In addition, BA as a protein molecule appears to be involved somehow in the phenomenon of "virtual eclipse", too.

Further, more detailed studies on the nature and mechanism of action of "infection enhancing" factors are in progress.

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A STATEMENT CONCERNING THE USE OF NAMES FOR ANTIBIOTICS

(Received January 31, 1966)

The medical field has need of certain attributes in drug names which may not be met by the name first applied to a drug. Although a drug name first applied by its discoverer may meet all the requirements needed for the scientific community, it may be unsuitable for general use in pharmacy and medicine.

The rapid progress now being made in medicine applies new requirements, some of which are noted here. These include a distinctive name, readily recognized, yielding itself to quick recall and easy pronunciation. The name must not be easily confused with another drug or chemical name in oral or written communication by accidental deletion of a letter or figure or because of it close similarity in sound or spelling. There should be only one commonly used name for a drug for use in medical communication.

In the United States, non-proprietary names for use by the medical community are proposed for adoption to the U.S. Adopted Names Council, successor to the A.M.A.-USP Nomenclature Committee. Before final adoption the names may be submitted to the World Health Organization, British Pharmacopoeia Commission, French Codex Commission, the Nordic Pharmacopoeia Council, as well as to the United States Pharmacopeia, the National Formulary and the Food and Drug Administration. "Inreasing emphasis is on the worldwide adoption of the same name for each therapeutic substance in view of the manifest advantages it offers to better communication and world trade." The USAN Council has drawn up a set of guiding principles for coining U.S. Adopted Names for drugs. These are published in United States Adopted Names (1965).

In the past, sometimes a USAN name has been the same as the name originally applied to the substance, at other times, following the guiding principles a change was required in the name for a substance. When this change involves a well known substance it becomes distressing to the scientific community.

As one of the major scientific groups concerned with various aspects of antibiotics, especially with the first published accounts containing new antibiotic names, the American Society for Microbiology is attempting to minimize the number of these name changes, operating through its Committee on Nomenclature of Antibiotics. Dr. J. B. JEROME, Secretary of the USAN Council, is cooperating with this ASM Committee by submitting proposed antibiotic names to an ASM referee for comment, before adoption by the USAN Council. The president of the ASM has appointed the chairman of the Committee on Nomenclature of Antibiotics (or a designated alternate) to act as referee for the ASM.

The Committee on Nomenclature of Antibiotics believes that antibiotic name changes will be few if, in coining new names, scientists will follow the guiding principles for USAN as published. The Committee has, after study, adopted a series of recommendations which were approved by a general meeting held in Washington, D.C. on October 20th, 1965 by participants in the Fifth Interscience Conference on Antimicrobial Agents and Chemotherapy and the IVth International Congress of Chemotherapy as follows:

The Committee affirms the fundamental right of the scientist first isolating an antibiotic to give a suitable name (non-proprietary) to the substance isolated. If that name meets the necessary criteria, the scientific community has strong interests in the continuity of that name. The name should conform with the guiding principles for coining U.S. Adopted Names for drugs.*

* Unites States Adopted Name (USAN); United State Pharmacopoeial Convention Inc., New York, N.Y. (April, 1965).

It is recommended that the following general guiding principles be used by scientists in devising names for new antibiotics:

1. Cognizance should be given to the fact that antibiotics are often chemically related members of a series or "family". A name should be chosen which yields a root (or suffix or prefix) which can be modified to show that the variants are members of a related series.

2. A name should be chosen which is euphonious.

3. The chosen name should be based on the chemical structure of the compound if the chemical structure has been established or there is strong evidence for a structure.

4. When the investigator has little or no knowledge of the chemical structure of the new antibiotic the following principles should be used as a guide:

a. The genus (or family or order) epithet of the producing organism should be given first consideration as a source of the root for the new name.

i. It is recommended that the suffix "mycin" be limited for application to products derived from organisms belonging to the order Actinomycetales.

ii. If the genus, family and order epithet of the producing organism are preempted as sources of the root (or suffix or prefix) for the name, the investigator may consider the use of the species (or subspecies or variety or form) epithet of the organism.

b. Failing to find in 4a a suitable source for a name, it may be based on some property of the antibiotic which has scientific merit such as spectrum of antibiotic activity or mode of action. It is not believed that the geographic area of origin gives a meaningful name.

c. If for some reason a name cannot be given to a new antibiotic following the above principles a code designation may be given—the code to begin with a letter or letters, to assist in index alphabetization, followed by one or more digits. It is highly desirable that about the time a compound under investigation moves into clinical evaluation, a name rather than a code designation be made available.

We invite and urge your participation in this endeavor. If any questions, reservations, or suggestions occur to you, please give us the benefit of your thoughts. If you find our recommendations acceptable, please join with us in (1) publishing them in your journal and (2) incorporating them in your editorial practice.

Selman A. Waksman Chairman,
Nomenclature Committee of the American Society of Microbiology

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THE SIGNIFICANCE OF SEROLOGICAL TESTS IN CONTROLLING THE SUCCESS OF SMALLPOX REVACCINATION

By

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(Received October 7, 1965)

Summary. Adults who had been vaccinated in infancy and revaccinated at 6—10 years of age were revaccinated against smallpox. Correlations among the following four factors were analyzed by statistical methods: (1) take; (2) age of vaccinees; (3) prevaccination haemagglutination-inhibiting (HI) and neutralizing antibodies; (4) HI and neutralizing antibody responses.

No correlation was found between prevaccination HI or neutralizing antibodies and take. Only the age of vaccinees, reflecting the time that had elapsed since revaccination in childhood, showed an appreciable positive correlation with take.

There was no correlation between HI and neutralizing antibody titres in the prevaccination serum samples of subjects with subsequent take, whereas a positive correlation was demonstrable in this respect for those who could not be revaccinated successfully.

After successful revaccination (take) the neutralizing antibody titres increased significantly, irrespective of the prevaccination titre, and a well-defined positive correlation developed between HI and neutralizing antibodies. A neutralization titre of 1 : 128 or an HI titre of 1 : 32 may be considered indicative of successful revaccination.

After failure to take the neutralizing antibody titres increased significantly, but to a lesser degree than after take; in contrast to the successful revaccination, the antibody responses showed a pronounced negative correlation with the prevaccination neutralization titres. Moreover, the failure of take was followed by disappearance of the prevaccination correlation between HI and neutralizing antibodies, since in this case there was no or a very poor HI response. According to this last observation, HI antibody response is a more reliable serological indicator of take than a rise in the neutralizing antibody.

The success of primary smallpox vaccination is obvious if it is accompanied by the typical dermal lesion. The lesion following revaccination is, however, variable. It is unclear which types of lesion are and which are not indicative of an immunity satisfactory from the epidemiological point of view. There are some contradictions as regards the serologically demonstrable antibody responses accompanying the different types of dermal lesion. The type of skin reaction is only informative of the pre-revaccination immune status if the interval between the previous and actual vaccination was short.

To estimate the immunological status, the haemagglutination-inhibition (HI) test and/or the neutralization test are usually applied. According to our present knowledge the latter test measures the most specific antibody [9], whereas an HI antibody response shows a correlation with the virus multiplication inside the organism [16]. There are significant differences between HI and neutralizing antibodies as regards their appearance, peak and persistence.

We have attempted to elucidate the protective value of "unsuccessful" revaccinations (no take), *i.e.*, of those without pustule formation, and of

"successful" revaccinations (take), by statistical analysis of the antibody responses.

Serological studies in connection with revaccination of hospital staff have already been undertaken [1], estimating the HI antibody responses. In the present work the neutralizing antibodies were titrated mainly in the same serum samples.

The aim was to establish (i) whether there is any interrelation between pre-revaccination neutralizing antibody titres and the type of dermal reaction in subjects vaccinated several years before; (ii) the quantitative neutralizing antibody responses to revaccinations accompanied by different lesions; (iii) the possible correlations between (a) the age of vaccinees (which reflects the time that had elapsed since their compulsory revaccination), (b) the pre-vaccination neutralizing and HI antibody titres and (c) the quantitative antibody responses to revaccination.

Materials and methods

Revaccination. Hospital staff of both sexes, from 16 to 61 years of age, were revaccinated in 1963. They had received the compulsory primary vaccination and revaccination in infancy and between 6 and 10 years of age, respectively. The bovine dermovaccine of Hungarian produce, used for the present revaccinations, contained 7×10^6 pock-forming units (PFU) per ml, determined on the chorio-allantoic membrane. The skin of the arm was scarified in the form of two double crosses or, after several failures to take, two 5 + 5 cross-hatchings.

Control of skin lesions. The results were read seven days after revaccination. Vaccinations followed by pustule formation (primary type or vaccinoid) were only accepted as "take". The others (immediate or no reaction) were considered as "no take". The latter were repeated after seven days, when the reaction was inspected, in a few cases several times at weekly intervals.

Serum samples. Blood was taken immediately before the first revaccination trial and three weeks later, *i.e.*, in case of further trials two weeks after the second one and one week after the third one. Neutralizing antibodies were only titrated in the serum samples of those who had shown a take after the first trial and of those in whom at least two revaccinations had failed. Sera were inactivated at 56 °C for 30 minutes and stored in the frozen state.

Neutralization tests. Virus suspension was prepared according to BOULTER's [2] method, with some modifications. The commercial dermovaccine was subjected to two consecutive passages on the chorio-allantoic membrane of 12-day-old chicken embryos. The second-passage membranes showing confluent lesions were thoroughly rinsed with saline and ground with quartz sand, then saline was added to make a 20 per cent suspension and this was centrifuged at 1000 r. p. m. for 5 minutes in a refrigerated Phiwe centrifuge model "Linde". The supernatant was thoroughly agitated with glass pearls for about 10 minutes and centrifuged at +4 °C at 10 000 r. p. m. for 25 minutes.

The sediment was resuspended in sterile skimmed milk, 2 ml per membrane. The suspension was distributed in ampoules, frozen in CO_2-ice-alcohol mixture and stored at —20 °C. A new ampoule was opened for every titration. At the start of the study the suspension contained 1.2×10^6 PFU per ml; 9 months later, when the last tests were performed, 6.3×10^5 PFU per ml. The working dilution used in the neutralization tests contained 40—80 PFU per 0.1 ml.

From each of the sera to be tested a twofold dilution series was prepared starting with a 1 : 4 dilution in 0.4 ml saline containing 50 μg of streptomycin and 100 units of penicillin per ml. To each dilution an equal volume of viral suspension was added. The "virus control" tube contained 0.4 ml virus + 0.4 ml 1 : 4 diluted inactivated normal rabbit serum. The pooled serum of successfully revaccinated subjects (positive control) was re-titrated simultaneously with every titration.

The mixtures were kept at 37 °C in the incubator for an hour and at least 4, 11-day-old chick embryos were inoculated with each mixture on the chorio-allantoic membrane, 0.1 ml

per membrane. The eggs were prepared as recommended by NADEJE *et al.* [3]. The titre of serum was expressed in the highest serum dilution reducing the pock count by 60 per cent [4]. The pooled positive serum was titrated 17 times and its titre was found to be 1 : 128 or 1 : 256 in every instance. Thus, the serum titres needed no correction.

In statistical analysis the criterion of the take was the same as given above. The data of only those subjects were included whose paired sera had been titrated for both HI and neutralizing antibodies. Instead of titre in dilution, the ordinal numeral of dilution steps served for the basis of statistical analysis. Thus, <1 : 4 was substituted by 0, 1 : 4 by 1, 1 : 8 by 2 *etc.*, forming a logarithmic scale.

Results

(I) Serological tests

(1) ***Correlation between pre-vaccination neutralization titres and success of revaccination.*** The neutralization test was performed in 94 sera. The upper two diagrams of Fig. 1 show the distribution of the vaccinees by prevaccination titre. The first diagram illustrates the 47 subjects who had been revaccinated with success at the first trial, whereas revaccination of the 47 subjects of the second diagram failed to take at two or three trials. The distribution of the

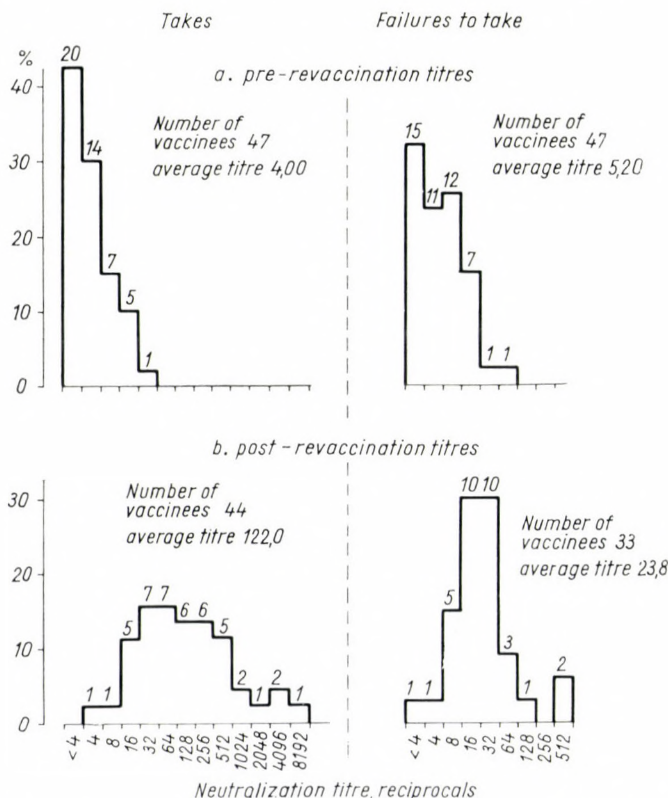

Fig. 1. Distribution of vaccinees by pre- and post-revaccination neutralization titres

two groups is nearly identical. The slight difference in the geometric means of basal titres in favour of the subjects with no take (5.2—4.0) proved to be non-significant statistically ($t_{[92]} = 1.57$, $P > 10$ per cent).

It seems to be of importance that at least 10 years had elapsed between the last vaccination and the actual revaccination of the subjects under study. The results clearly show that after such a long postvaccination period the titre of the circulating neutralizing antibodies fails to determine the result of revaccination. According to our previous studies [1] the same is valid for the prevaccination HI titre.

(2) *Neutralizing-antibody responses to successful and unsuccessful vaccinations.* The diagrams in the lower part of Fig. 1 summarize the postvaccination neutralizing antibody titres. Of the 77 sera 44 were taken from successfully revaccinated subjects, the remaining 33 originated from persons whose revaccination was unsuccessful in spite of two or three trials.

After take the titres conspicuously shifted to higher values. The geometric mean of postvaccination titres (1 : 122) significantly surpassed the pre-revaccination level ($t_{[89]} = 1.9$, $P < 0.1$ per cent) and the curve became strikingly flat, indicating a wide scattering.

The geometric mean of the neutralization titres of subjects with no take was also elevated (1 : 23.8) $t_{[78]} = 6.72$, $P < 0.1$ per cent). This value was, however, significantly lower than the geometric mean of the postvaccination titres obtained after take ($t_{[75]} = 4.70$, $P < 0.1$ per cent). Besides, the diagram is not flat, the scattering being relatively small.

(II) Statistical analysis

For further statistical analysis the data of the 64 subjects whose pre- and postvaccination HI and neutralization titres were available, were only used. We sought correlations between the following factors: (1) Success of revaccination; (2) age of vaccinees; (3) pre-revaccination HI and neutralizing antibodies; (4) postvaccination rises in titres.

The statistical analysis was performed from the following aspects:

(A) Correlation between the pre-revaccination antibody status and success of revaccination:

(a) significance of pre-revaccination HI and neutralizing antibodies;

(b) significance of age distribution of vaccinees;

(c) correlation between pre-revaccination HI and neutralizing antibodies.

(B) Correlation between success of vaccination and the subsequent serological status:

(a) postvaccination changes in the correlation between HI and neutralizing antibodies;

Table I

Age, pre-revaccination titres and antibody responses of vaccinees

Age of vaccinees years		Prevaccination titres in steps of dilution				Antibody responses in steps of dilution			
		HI		neutralization		HI		neutralization	
x_2		x_3		x_4		x_5		x_6	
16	18	1	1	3	1	0	3	0	8
17	19	0	2	1	2	0	1	—1	3
17	21	2	2	2	0	0	3	1	7
18	21	0	0	0	1	1	3	4	7
19	23	0	1	2	3	3	3	1	2
19	26	0	2	0	1	0	2	6	2
20	28	0	0	3	0	0	2	1	0
20	30	0	0	0	1	0	2	3	0
20	31	1	1	1	4	1	5	7	7
21	31	0	0	0	0	1	4	3	6
21	31	2	0	2	1	0	3	2	3
21	32	1	0	2	0	0	2	2	6
21	34	1	1	2	1	0	4	2	11
22	36	1	0	3	1	0	3	1	6
22	36	0	2	0	3	2	2	3	1
23	38	2	2	2	0	0	1	0	4
25	39	1	0	2	1	1	4	3	5
25	40	1	2	0	1	1	3	4	6
26	40	0	2	0	1	2	1	5	2
27	42	3	0	3	0	0	5	0	5
27	42	3	0	1	0	0	3	3	8
28	42	2	1	4	0	0	3	0	4
30	47	0	0	1	0	0	4	2	10
31	47	2	0	1	0	0	2	3	3
32	51	0	0	1	2	0	4	0	5
37	52	0	0	1	0	0	3	2	7
39	54	0	0	0	1	0	4	2	7
40	57	0	3	2	2	0	1	6	4
44	58	1	1	2	0	1	3	0	5
47	60	2	3	5	2	0	3	0	6
50	61	1	0	2	3	0	6	0	6
52		0		0		0		2	
61		4		3		1		0	
Total 938	1184	31	26	51	32	14	92	67	162

Note: No takes: ($x_1=0$) Takes: ($x_1=1$)

Under each heading the data of unsuccessfully and successfully revaccinated subects are listed in the left and right column, respectively.

Table II

Comparison of averages for successfully and unsuccessfully revaccinated subjects

Designation	No take	Take	Difference	$t_{[62]}$	P, %
Average age $\bar{x}_2$	28.42	38.19	9.77	3.62	< 1
Average prevaccination antibody levels*					
HI $\bar{x}_3$	0.94	0.84	−0.10	0.38	>70
neutralization $\bar{x}_4$	1.54	1.03	−0.51	1.69	> 5
Average antibody response*					
HI $\bar{x}_5$	0.42	2.96	2.54	10.09	< 0.1
neutralization $\bar{x}_6$	2.03	5.23	3.20	5.65	< 0.1

* Expressed in steps of dilution.

(b) correlation between pre-revaccination titres and the degree of antibody responses;

(c) correlation between the degree of antibody responses and the success of revaccination.

(C) Quadruple correlation among success of vaccination, age of vaccinees and pre-revaccination HI and neutralizing antibodies.

(D) Quadruple correlation among success of vaccination, age of vaccinees, HI and neutralizing-antibody responses.

Of the 64 subjects 31 developed pustules, 33 did not. The data (age, prevaccination titres, antibody responses) for both of these groups are summarized in Tables I and II.

(A) *Correlation between pre-revaccination status and success of vaccination.*

(a) *Significance of pre-revaccination HI and neutralization titres.* There was no statistically appreciable difference between takes and no takes in respect of the pre-revaccination serological titres though the averages were somewhat lower before take than before no take.

(b) *Significance of the age of subjects to be revaccinated.* There was a striking difference in average age between the two groups, the age of the groups with take having been nearly 10 years higher than that of the group with no take. Since all the vaccinees had received their compulsory primary and revaccination in infancy and childhood, respectively, their age was closely related to the interval between the last vaccination and the actual revaccination. The results suggest that the vaccinated organism has a residual or potential immunity which declines with age; it may still be present but cannot be detected by serological methods.

(c) *Correlation between pre-revaccination HI and neutralizing antibodies.* A significant correlation was demonstrated between HI and neutralizing anti-

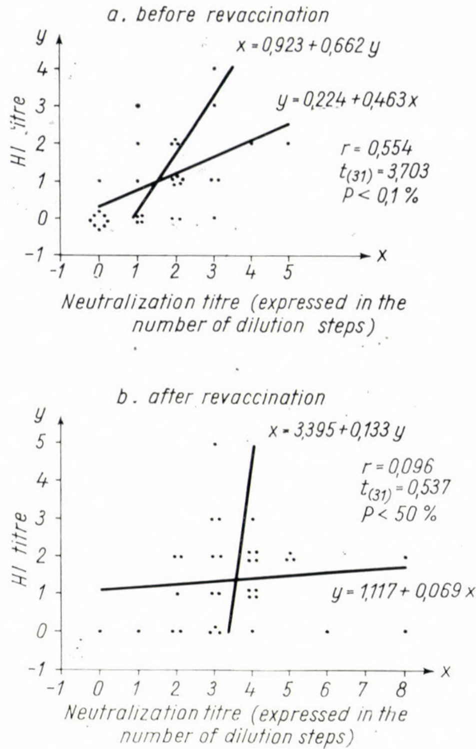

Fig. 2. Correlations between neutralizing and HI antibodies. (A) Group of no take

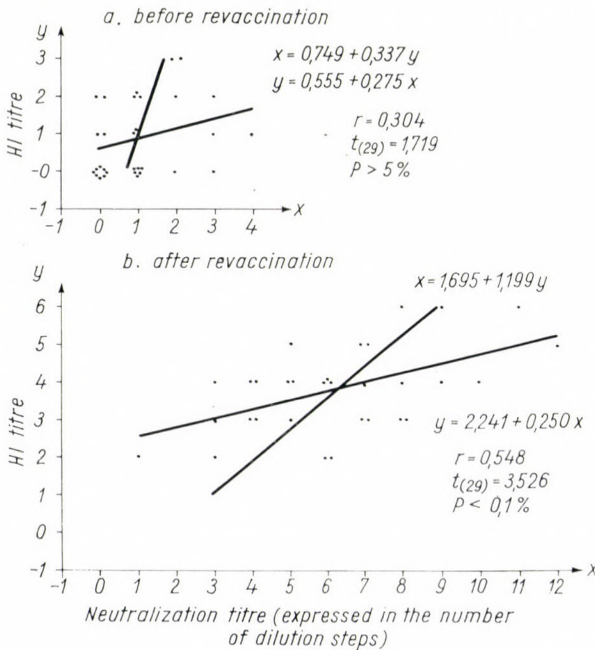

Fig. 3. Correlations between neutralizing and HI antibodies. (B) Group of take

bodies in the "no take" group (Fig. 2) (correlation coefficient, $r = 0.554$). In the "take" group the correlation ($r = 0.304$) (Fig. 3) was not appreciable statistically.

It should be remembered that from the prevaccination titres of an individual one cannot conclude to the result of a subsequent vaccination. The correlation coefficient for the two kinds of antibodies represents the only,

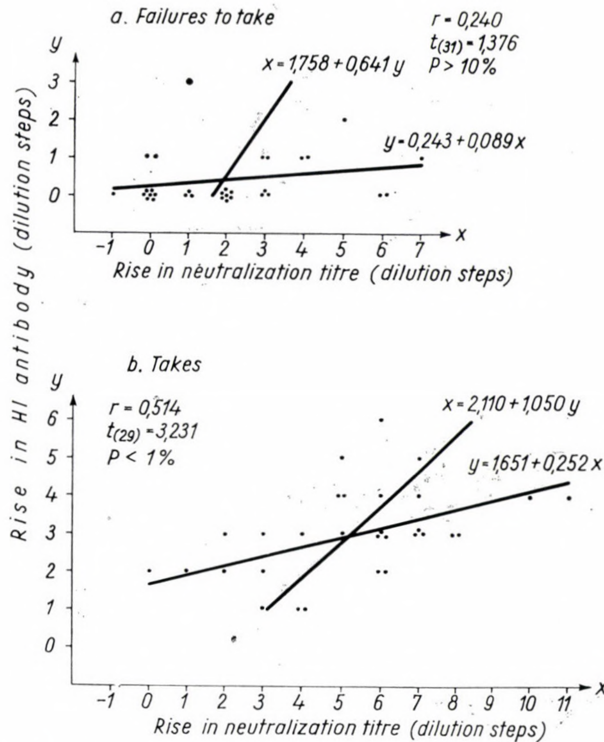

Fig. 4. Correlation between neutralizing and HI antibody responses

and still uncertain, basis for estimating the probability of take for the members of a group.

Since to our best knowledge a similar analysis has never been published, we cannot tell whether such a difference in the correlation coefficient was demonstrable in other populations (*e.g.* in a homogeneous age group), under different conditions.

(B) *Correlation between success of revaccination and postvaccination serological status.*

(a) *Postvaccination changes in the correlation between HI and neutralizing antibodies.* The correlation between HI and neutralizing antibodies showed

a characteristic change depending on the result of revaccination. After no take the well-defined prevaccination correlation disappeared (Fig. 2). After take, on the other hand, the non-significant correlation turned into a well-defined one (Fig. 3). This peculiar change was fully explained by the correlation between the quantitative changes in the two kinds of antibody (Fig. 4).

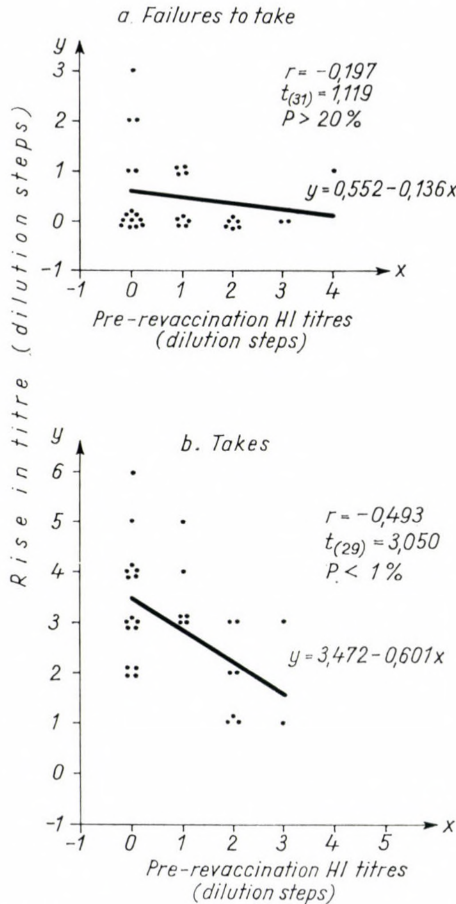

Fig. 5. Correlation between HI antibody response and pre-revaccination HI titre

After no take there was no appreciable correlation between the increases in HI and neutralizing antibodies, while take was followed by approximately parallel increases; the correlation coefficient, 0.514, was highly significant. The incoordinated change in the two kinds of titre led to disappearance of the correlation between HI and neutralizing antibodies after revaccination (Fig. 2). In the successfully vaccinated group (Fig. 3), on the other hand, the nearly parallel increase accounted for the close postvaccination correlation.

(b) *Correlation between pre-revaccination titres and antibody responses.* The comparison of these data was instructive. In the group of no take the HI antibody response was poor and showed no correlation with the prevaccination titres (Fig. 5). In contrast, takes were accompanied by considerably stronger HI responses exhibiting a negative correlation with prevaccination titres.

An inverse situation developed in the case of the neutralizing antibodies (Fig. 6). Failure to take was associated with a highly significant negative correlation between prevaccination titres and antibody responses, *i.e.*, the lowest

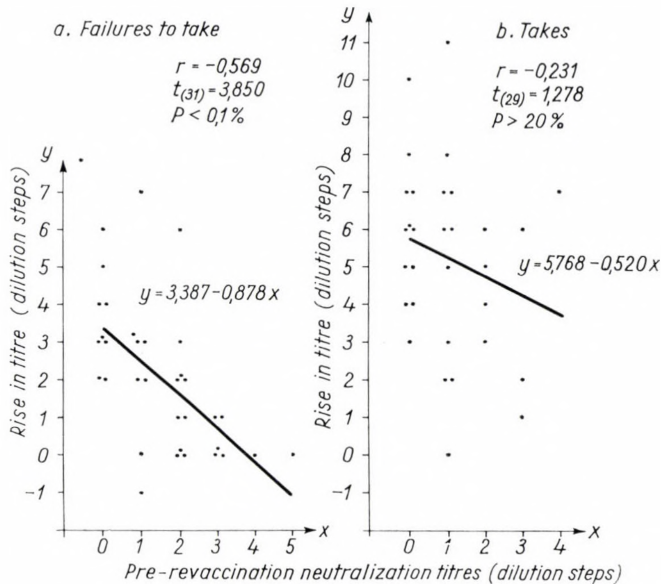

Fig. 6. Correlation between neutralizing antibody response and pre-revaccination antibody titre

neutralization titres increased to the greatest degree. Takes, though followed by greater neutralizing antibody responses than failures to take (Fig. 6), exhibited no appreciable correlation and the corresponding regression line was less steep. Accordingly, takes were followed by great increases in neutralizing antibodies which, however, were independent of the initial titres.

(c) *Correlation between success of revaccination and antibody responses.* Revaccination, whether successful or unsuccessful, was followed by a significant increase in mean HI and neutralization titres. Failure to take elicited a more pronounced increase in neutralization titre whereas after take the rise in HI antibodies was more significant (Table III). Furthermore, the average antibody responses in both the neutralization and HI tests were significant, irrespective of the success of revaccination (Table II).

(C) *Quadruple correlation among success of revaccination, age of vaccinees and pre-revaccination HI, and neutralizing antibodies.*

Table III

Evaluation of serological responses

Designation	HI	Neutralization
	Average antibody response	
	x_5	x_6
Unsuccessful revaccination		
Average rise in antibody*	0.42	2.03
$t_{[32]}$	3.24	5.81
P, %	<1	<0.1
Successful revaccination		
Average rise in antibody*	2.96	5.23
$t_{[36]}$	13.50	11.64
P, %	<0.1	<0.1

* Expressed in steps of dilution.

The aim was to elucidate the possible statistical interrelation between the success of vaccination (as indicated by the skin lesion) and the other variables under study. Though the skin reaction is a typical quantal response, it may nevertheless be rendered quantitative by substituting a negative response by zero and a positive response by the unity (+1), and determining the partial correlation coefficients. This is necessary to avoid overlappings.

Table IV shows the correlations among success of revaccination, age, and prevaccination titres. There was a well-defined, significant positive correlation between the success of revaccination and age; the correlation persisted when the effects of prevaccination HI or neutralization titre or of both of these titres had been eliminated from the determination of the partial correlation coefficients. In contrast, there was no correlation between prevaccination HI or neutralizing antibodies and the success of revaccination. If we suppose that the appearance of the skin lesion is dependent on the immunological status of the organism, this is not indicated by the prevaccination titres; it is however, highly influenced by the age of the vaccinees, which reflects the interval between the compulsory revaccination and the actual revaccination.

(D) *Quadruple correlation among success of revaccination, age of vaccinees and antibody responses.* Having obtained no appreciable relationship between prevaccination titres and the success of revaccination, these data have been neglected. According to Table V, the correlation between age and the success of revaccination was well-defined when only the changes in the neutralization titre were eliminated, but it dropped below statistical significance after the effects of the changes in HI titre or those in both titres had been eliminated.

Thus, it may be stated that, retrospectively, the success of vaccination is more closely related to the rise in HI titre than to age.

Accordingly, in a given population, applying the same vaccine with a standard technique, the percentage of take can be predicted if the age of

Table IV

Correlations between success of revaccination (skin lesion), age of vaccinees and pre-revaccination HI, and neutralization antibodies

Analyzed elements	Basal correlations	
1. Skin lesion	r_{12} 0.383	r_{23} 0.086
2. Age of vaccinees	r_{13} −0.049	r_{24} 0.010
3. Pre-revaccination HI titre	r_{14} −0.210	r_{34} 0.447
4. Pre-revaccination neutralizing titre		

Designation of relationship	Eliminated element	Correlation coefficient		Indices of significance		
		designation	value	degree of freedom	t	P, %
Skin lesion–age	—	r_{12}	0.383	62	3.26	< 1
	HI titre	$r_{12\cdot3}$	0.389	61	3.30	< 1
	Neutralization titre	$r_{12\cdot4}$	0.394	61	3.35	< 1
	HI and neutralization titre	$r_{12\cdot34}$	0.391	60	3.29	< 1
Skin lesion–HI titre	—	r_{13}	−0.049	62	0.38	>70
	Age	$r_{13\cdot2}$	−0.089	61	0.69	>40
	Neutralization titre	$r_{13\cdot4}$	0.052	61	0.40	>60
	Age and neutralization titre	$r_{13\cdot24}$	0.018	60	0.14	>80
Skin lesion–neutralization titre	—	r_{14}	−0.210	62	1.69	> 5
	Age	$r_{14\cdot2}$	−0.232	61	1.86	> 5
	HI titre	$r_{14\cdot3}$	−0.211	61	1.68	> 5
	Age and HI titre	$r_{14\cdot23}$	−0.215	60	1.71	> 5

vaccinees informing about the time elapsed since the compulsory revaccination is known. However, knowledge of the postvaccination HI antibody response is a better indicator of positive skin reaction than is the age of the vaccinees.

This observation has been confirmed by an analysis of the correlation of the success of revaccination and the neutralizing antibody response. In this case the basal correlation was highly significant and this significance was not reduced by elimination of the effect of age. However, it dropped far below the level of significance after the effect of HI antibody responses had been eliminated, and remained at a similarly low level after the effects of age and HI antibody response were both eliminated.

Table V

Correlations between success of revaccination, age of vaccinees and HI and neutralizing antibody responses

Analyzed elements	Basal correlations	
1. Skin lesion	r_{12} 0.383	r_{25} 0.371
2. Age of vaccinees	r_{15} 0.788	r_{26} 0.197
5. HI antibody response	r_{16} 0.583	r_{56} 0.668
6. Neutralizing antibody response		

Designation of relationship	Eliminated element	Correlation coefficient		Indices of significance		
		designation	value	degree of freedom	t	P, %
Skin lesion–age	—	r_{12}	0.383	62	3.26	1
	HI antibody response	$r_{12 \cdot 5}$	0.158	61	1.25	20
	Neutralizing antibody response	$r_{12 \cdot 6}$	0.337	61	2.79	1
	HI neutralizing antibody response	$r_{12 \cdot 56}$	0.169	60	1.33	10
Skin lesion–HI antibody response	—	r_{15}	0.788	62	10.09	0.1
	Age	$r_{15 \cdot 2}$	0.753	61	8.74	0.1
	Neutralizing antibody response	$r_{15 \cdot 6}$	0.659	61	6.85	0.1
	Age and neutralizing antibody response	$r_{15 \cdot 26}$	0.617	60	6.08	0.1
Skin lesion–neutralizing antibody response	—	r_{16}	0.583	62	5.66	0.1
	Age	r_{16}	0.561	61	5.29	0.1
	HI antibody response	$r_{16 \cdot 5}$	0.124	61	0.98	30
	Age and HI antibody response	$r_{16 \cdot 25}$	0.138	60	1.08	20

Discussion

Acceptance of local pustule formation as an indicator of successful revaccination with vaccinia virus is based on the principle that take indicates the breakthrough of the actual immune level whereas no take indicates a higher immune level, which cannot be overcome with the given dose of virus.

Furthermore, in a given population the size of inoculum will be in direct relation to the percentage of take [5]. Thus, raising the virus titre of the vaccine will increase the security of protection but reduce the possibility of noticing fine differences in the immunological status on the basis of the result

of revaccination. Obviously, the low virus content of the vaccine applied in the present studies has made it possible to find a well-defined correlation between the time elapsed since the last vaccination and the success of revaccination. The low virus content of the vaccine confirmed also the statement that there is no significant correlation between the antibody level in the pre-revaccination serum and the result of revaccination.

Immunity in general, and immunity following smallpox vaccination in particular, is a complex phenomenon. It has serologically measurable indicators which are most reliable soon after vaccination, but then gradually disappear. After the serologically measurable antibodies have disappeared, there still persists a potential immunity which manifests itself in mobilization of protective mechanisms against an antigenic stimulus. The pre-revaccination serological titres provide no information as to the potential and residual immunity of the subjects to be vaccinated. The fact that older subjects were revaccinated with more success suggests that this type of immunity also declines with the progress of time. Naturally, there are great individual differences in this respect since numerous young subjects develop a positive reaction and *vice versa.*

It is also of statistical significance that there exists a positive correlation between pre-revaccination HI and neutralization titres in the group of subjects who failed to develop an appreciable skin lesion, whereas a similar correlation was not demonstrable in the group with positive skin reaction. It might serve as a mechanical explanation that the neutralizing antibodies are more persistent than the HI ones, therefore the presence of both antibodies may be considered to indicate an immunologically more active status. This view is supported by the fact that the pre-revaccination HI and neutralizing antibodies of the subjects who failed to develop a positive skin reaction showed a correlation similar to that exhibited by the postvaccination antibodies of successfully revaccinated subjects.

The serological consequences of unsuccessful revaccinations have been discussed by numerous authors. After immediate or no response PINCUS and FLICK [6], EPP [12] and MCCARTHY *et al.* [7], observed rises in the HI titre, whereas HOLIK and SZATHMÁRY [8], CUTCHINS *et al.* [9], ELISBERG *et al.* [4], FINGER and MŐBERT [10] and ourselves found no or hardly any rise.

Rises in the neutralizing antibodies were demonstrated by all the authors having investigated the neutralizing-antibody response of vaccinees developing local pustules; the only differences were in the percentage of positive responses [6, 7, 11, 12, 13].

We have found qualitative differences between the serological responses given by vaccinees developing a positive skin reaction and those failing to do so.

(a) After several revaccination attempts, the neutralizing antibodies increased to a certain level in most cases, and the increase showed a definite negative correlation with the prevaccination neutralizing antibody.

After take, on the other hand, the titres, probably highly depending on virus multiplication and the individual reaction, showed a substantial rise, irrespective of their prevaccination levels.

(b) After no take only the neutralization titre increased in a biologically appreciable degree. The minimal rise in HI titres proved to be significant statistically, but its biological significance could not be appreciated.

It is well-known that an increase in vaccinia antihaemagglutinin is indicative of viral multiplication in the organism. It seems likely that the unsuccessful vaccinations with the vaccine and technique applied by us were not followed by substantial multiplication of virus. The rises in the neutralizing antibody titre of vaccinees giving no HI antibody response might be explained by supposing that in the course of repeated revaccination attempts a considerable amount of protective antigen had been introduced, which then brought about a serological response in the presence of potential immunity. A similar conclusion has been drawn by CUTCHINS *et al.* [9], and this view is also supported by the observation of KÜHN and ROHDE [14] that revaccination with the Herrlich vaccine was followed by a rise in complement-fixing antibodies.

In practice, a neutralization titre higher than 1 : 128 is suggestive of take; such high titres have not been observed either before revaccination or after failure to take. However, only 17 of the 44 successfully revaccinated subjects attained that level. Consequently, the postvaccination titre in itself fails to give a definite answer to the question whether or not the revaccination of a given subject was successful. It should be noted in this respect that the interval between taking the first and second blood sample was uniformly three weeks. In case of failure to take, several trials were performed during this period. The serological consequences of these trials on the dynamics of serological responses are unknown.

Examination of paired sera fails to give much more reliable information on the character of the vaccination reaction. In this respect only 32-fold or greater rises in neutralization titre may be considered significant. Still, such an increase occurred in three cases out of the 32 failures to take, whereas out of the 31 takes only 16 exhibited a 32-fold or greater rise in the neutralization titre.

The present data have shown that the HI antibody response is the most reliable serological indicator of successful revaccination; a serum titre of 1 : 32 or higher 3 weeks after revaccination, or an 8-fold or greater rise in titre may be considered as indicative of take. As mentioned previously [1], 21 of the 31 takes were followed by an 8-fold or greater increase in titre, which was observed only exceptionally after unsuccessful revaccinations (in a single case out of 32).

The present neutralization tests were performed in order to throw light upon the interrelation between the neutralizing antibody, which is considered

the most specific and most persistent of the antibodies, and local dermal sensitivity. However, the interrelation with the prevaccination titre as well as with the antibody response proved to be loose.

The present studies have shown the lack of an interrelation between neutralization antibody and local skin immunity (according to DIXON's classification [17]). This view is supported by the studies of KAPLAN *et al.* [15], who succeeded in producing skin lesions in subjects having high-titre neutralizing antibodies.

The present data also suggest that the introduced protective antigen may induce some neutralizing antibody response even without a considerable multiplication of virus, but an immunity manifesting itself in all factors requires adequate viral multiplication.

The HI antibody response has proved to be a more appropriate serological indicator of take than a rise in neutralizing antibodies. Presumably, the introduced HA antigen is not enough to induce a detectable HI antibody response. It is therefore likely that shortly after vaccination the HI antibody is in correlation with the anti-invasion and anti-dissemination factors. Later, however, these correlations disappear because of the disappearance of HI antibody.

It may be concluded that neither the antihaemagglutinin nor the neutralizing antibody is identical with the main determinant of the implantation of vaccinia virus in the scarified skin. It is uncertain whether the true determinant of resistance to revaccination (and also to smallpox) is measurable by any serological test. To elucidate this question, the possible role of further antigens or allergens should be clarified.

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STUDY ON THE EFFECT OF FLAVONOIDS AND RELATED SUBSTANCES

I. THE EFFECT OF QUERCETIN ON DIFFERENT VIRUSES

By

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(Received October 27, 1965)

Summary. The effect of quercetin on different viruses has been studied. *Herpesvirus hominis, Herpesvirus suis,* type 3 parainfluenza virus and the Sindbis virus were found to be sensitive to quercetin. The sensitivity of type 1 poliovirus was moderate, while types 2 and 3 of poliovirus and types 3 and 4 of adenovirus were completely resistant. Quercetin, being active only against extracellular virus, was considered virucidal. The effect of morin on *Herpesvirus suis* was identical with that of quercetin, while rutin was practically ineffective.

In a previous paper we have shown the antiviral effect of aqueous dry oak and beech leaf extracts and of commercial tannic acid [1]. Condensed tannic acid, being a flavonoid polymer [2], seemed to be of interest in view of the possible antiviral effect of certain flavonoid monomers. Catechin, the precursor of tannic acid, is a quercetin derivative [3], thus we examined the effect of quercetin on viruses. A short report has already been published on the results [4], of which the present paper gives a more detailed survey.

Materials and methods

Tissue cultures and media. For HeLa tube cultures we used GEY's solution with 5 per cent inactivated rabbit serum and 0.25 per cent lactalbumine hydrolysate.

For plaque assay, cell suspensions of trypsinized 11 days old chick embryos were used. Petri dishes of 50 mm diameter were seeded with 2.5×10^7 cells. The nutrient fluid consisted of GEY's solution, containing 4 per cent pH 7.6 Tris buffer, 5 per cent calf serum and 0.25 per cent lactalbumine hydrolysate.

Viruses. A strain of *Herpesvirus hominis* isolated from conjunctival rinsing fluid in HeLa cells was used. Further strains tested were poliovirus type 1 (Mahoney), type 2 (MEF 1) and type 3 (Saukett), and a type 3 parainfluenza virus isolated in this laboratory [5]. All viruses were maintained in HeLa cell cultures. *Herpesvirus suis* was isolated from pig brain. The Sindbis virus was kindly supplied by *Dr.* I. GRESSER (Boston, U.S.A.). Both viruses were cultured on chick embryo cells.

Virus assays. The titre of *Herpesvirus suis* and Sindbis virus was determined on chick embryo fibroblast monolayers by the plaque technique. The other viruses were titrated in HeLa cell culture tubes. The methods used were described previously [6, 1].

*Preparation of flavonol solutions.** Quercetin (MP 315—317 °C), morin (MP 294—298 °C) and rutin (MP 198 °C) were dissolved in saline at a slightly alkaline pH adjusted by 0.2 *M*

* The authors are indebted to *Dr.* M. GÁBOR (Institute of Pharmacodynamics, University Medical School, Szeged) for quercetin and to Koch Light Laboratories, England, for morin and rutin.

NaOH so as to obtain final concentrations of 300 μg/ml. The pH of the final solutions varied from 7.4 to 7.6.

Antiviral effect. Virus suspensions obtained in HeLa cell cultures and having a titre of $10^{3.5}$ to $10^{4.5}$ $TCID_{50}$/0.1 ml were mixed with an equal amount of flavonol solution. The mixtures were usually allowed to stand at room temperature for 2 hours and inoculated undiluted or in tenfold dilution into three HeLa tubes each. As controls, virus-saline mixtures were used.

In experiments with *Herpesvirus suis* and Sindbis virus equal amounts of flavonol solution, respectively saline, were mixed to virus suspensions of 10^{3} to $10^{3.5}$ PFU/ml. After appropriate incubation, 0.2 ml samples were inoculated on chicken embryo fibroblast monolayers. After adsorption for 2 hours at 36 °C, the infected cultures were overlaid.

Results

The effect of quercetin on the different viruses is presented in Table I.

As shown in Table I, *Herpesvirus hominis* and type 3 parainfluenza virus were highly sensitive, type 1 poliovirus moderately sensitive, whereas all the other viruses tested were resistant, to the effect of quercetin. The inac-

Table I

*Effect of 300 μg/ml quercetin solution on infectivity of different viruses**

Virus	Log $TCID_{50}$/0.1 ml in HeLa cells		Log inhibition
	Control	Quercetin-treated**	
Herpesvirus hominis	4.24	0.50	3.74
Poliovirus type 1	4.24	2.74	1.50
Poliovirus type 2	4.50	4.50	0.00
Poliovirus type 3	3.74	4.24	—0.50
Parainfluenza virus type 3	3.50	0.74	2.76
Adenovirus type 3	4.50	3.74	0.76
Adenovirus type 4	4.50	5.00	—0.50

* Viruses were grown in HeLa cells.

** Incubated for 2 hours at room temperature.

tivating effect of quercetin on *Herpesvirus hominis* proved to be constant. In experiments repeated 13 times under identical conditions, it reduced the infective titre of $10^{3.5}$ — $10^{4.24}$ $TCID_{50}$/ml virus by 2 logs in two cases and by 3.74 logs in 4 cases. In the remaining cases, the decrease of titre varied between the above two values. As shown by the data of five identical experiments, quercetin caused a 0 to 1 log titre decrease of type 2 poliovirus. In five identical experiments the titre reduction caused by quercetin in type 1 poliovirus varied within the limits of 0.75 to 2 log. In the controls titre variations did not exceed 0 to 0.75 log.

Herpesvirus hominis exhibited a marked sensitivity to quercetin. The effect on type 1 poliovirus was considered moderate, while against type 2 poliovirus it was negligible.

Results concerning the influence of the time of incubation on the antiviral effect of quercetin are presented in Fig. 1.

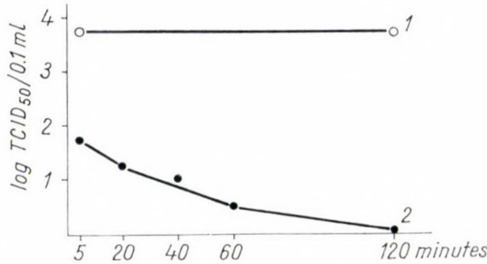

Fig. 1. Effect of 300 μg/ml quercetin on *Herpesvirus hominis* after different times of exposure. 1: Control; 2: Treated

The virus inactivating effect of quercetin increased with the duration of incubation. Complete inactivation of $10^{3.74}$ $TCID_{50}$ *Herpesvirus hominis* took two hours.

The action on *Herpesvirus hominis* of quercetin solution at different concentrations is shown in Table II. Virus—quercetin, resp. virus—saline mixtures were incubated for two hours at room temperature.

Table II

*Effect of quercetin at different concentrations on Herpesvirus hominis**

Quercetin concentration, μg/ml	Log $TCID_{50}$/0.1 ml in HeLa cells
0	3.74
25	2.50
50	1.50
100	0.66
150	0.50
300	0.00

* Incubated for 2 hours at room temperature.

Inactivation by quercetin increased parallel to the concentration, full inactivation being attained at 300 μg/ml concentration.

The effect of quercetin on *Herpesvirus suis* and the effects of morin and rutin at various concentrations were also examined. Results are shown in Fig. 2.

The antiviral effect of quercetin and morin was practically identical, rutin being relatively less active.

The inactivating effect of heparin on *Herpesvirus hominis* is known to be inhibited by protamine sulphate [7]. Attempts were made to inactivate the action of quercetin by the same substance. Identical volumes of 300 μg/ml quercetin were mixed with protamine sulphate solutions of different concentrations. The precipitate formed was removed by centrifugation for 1 hour

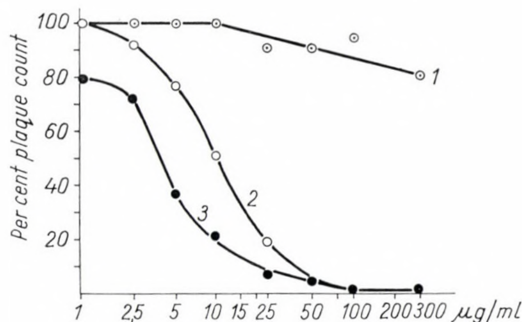

Fig. 2. Inactivation of *Herpesvirus suis* by rutin, morin and quercetin at different concentrations. 1: rutin; 2: morin; 3: quercetin

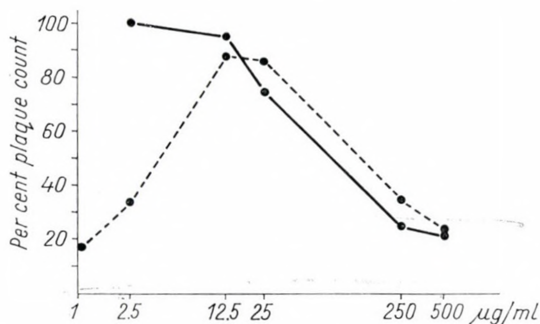

Fig. 3. Effect of 150 μg/ml quercetin on *Herpesvirus suis* in the presence of protamine sulphate at different concentrations. — Protamine; - - - Protamine + Quercetin

at 2500 r. p. m. To 0.5 ml of the respective supernatants, identical volumes of *Herpesvirus suis* of 10^3 PFU/ml were added. The mixtures were incubated at room temperature for 2 hours and inoculated in 0.2 ml volume each on chick embryo fibroblast monolayers. Following adsorption for 2 hours at 36 °C, the infected cultures were overlaid. The virus inactivating effect of protamine sulphate was checked in mixtures containing saline instead of quercetin. Results are shown in Fig. 3.

The virus inactivating effect of quercetin gradually declined with increasing the concentration of protamine sulphate from 1 to 25 μg. At higher

concentration protamine sulphate had no neutralizing effect since it began to reduce the plaque count.

The effect of quercetin on Sindbis virus is shown in Fig. 4.

The sensitivity of Sindbis virus to quercetin was similar to that of *Herpesvirus suis* (see Fig. 2).

Next the activity of quercetin on virus growth was studied in infected cultures. HeLa cultures were infected with 10^{-1} to 10^{-6} dilutions of *Herpesvirus hominis*. Adsorption was allowed to take place for 2 hours at 36 °C. Subsequently, the cultures were washed 3 times with Hanks' solution. To the

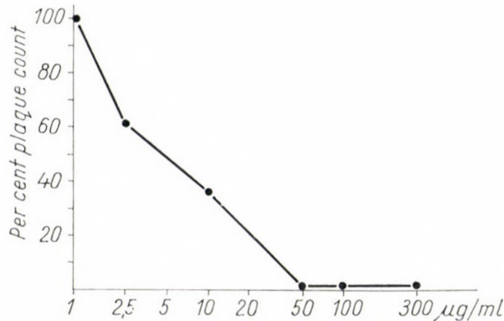

Fig. 4. Effect of quercetin at different concentrations on Sindbis virus

infected cultures a nutrient medium containing 30 µg/ml quercetin was added. Controls were set up with saline instead of quercetin. On the 6th day of incubation, no difference was detectable in the $TCID_{50}$ of control and quercetin-treated cultures. Thus the addition of quercetin after the infection had no effect whatever. Similar results were obtained with *Herpesvirus suis*. With this virus, the studies were performed on chick embryo fibroblast monolayers overlaid with a medium containing 30 or 120 µg/ml quercetin. Plaque counts were identical in both quercetin-treated and untreated cultures.

Discussion

It has long been known that certain polyanions reduce the infective titres of some viruses [9, 10, 11, 12]. The virucidal effect of tannic acid observed by us [1] was interpreted by its polyanionic character. On the basis of our experiments similarities may be supposed in the action of monomeric quercetin and the polyanionic tannic acid. The sensitivity of a variety of viruses was very similar to both substances. The action of the materials proved to be virucidal and tended to increase with the time of incubation [1]. The antiviral effects of both tannin and quercetin are inhibited by protamine sulphate [8]. Hence the virucidal activity of tannic acid seems to be due not so much to its polyanionic nature as to its structural units resembling the quercetin molecule.

Protamine sulphate is known to reduce the infective titre of certain viruses [13, 14]. In our hands, protamine sulphate at concentrations higher than 25 μg/ml definitely inhibited plaque formation by *Herpesvirus suis*.

The antiviral effect of certain flavonoids and related substances was studied in animal experiments by CUTTING *et al.* [15, 16, 17]. A prophylactic effect was observed against rabies, neurovaccinia and ectromelia virus infections in mice treated with quercitrin or quercetin by the oral route. None of these substances proved to be protective when fed after the virus infection. This protective action of flavonoids might be ascribed to the virucidal effect observed in our experiments. CUTTING *et al.* [16] found that rutin was ineffective; this is in accordance with our present data (Fig. 2).

The highest sensitivity to tannic acid and quercetin was exhibited by enveloped viruses, *viz.* herpesviruses, type 3 of parainfluenza virus and Sindbis virus. Viruses lacking an envelope like polio- and adenoviruses, were moderately sensitive or even resistant. Further studies are required to elucidate whether the antiviral effect really depends on the presence of the envelope and to detect whether or not the different strains of the different virus types exhibit similar sensitivities.

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AGE-INCIDENCE OF HAEMAGGLUTINATION-INHIBITING ANTIBODIES TO REOVIRUS TYPES 1, 2 AND 3

By

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(Received November 4, 1965)

Summary. The haemagglutination-inhibiting (HI) antibodies to the three reovirus types were titrated in serum samples collected from 1982, mostly healthy, persons living in Hungary. Antibodies to types 1, 2 and 3 were found in 73, 74 and 81 per cent of the samples, respectively. The percentage of seropositive sera appears to grow till the fourth decade of life in the case of types 1 and 2; in the case of type 3 it reaches its highest level between 3 and 5 years of age.

Serological response of 273 hospitalized acute respiratory cases was compared with that of 114 cases of viral hepatitis. No appreciable difference could be demonstrated. In 22 cases under 10 years of age clinically diagnosed as "viral infection" the incidence of antibody response to reovirus types 1 and 3 was relatively high (3 and 5, respectively).

The HI test was found to be equivalent to the neutralization test in detecting reovirus antibodies. A close correlation exists between HI and neutralization titres.

The term reovirus was introduced by Sabin in 1959 for the designation of the viruses classified earlier into echovirus type 10 and some related viruses [1]. Since then the morphology [2, 3] and antigenic structure of reoviruses, the existence of three serotypes [1, 4] and of several subtypes within type 2 [5], the chemical nature of the reovirus haemagglutinin, the relationship between infective virus and haemagglutinin production [6—8] and the influence of different circumstances on the multiplication of reoviruses in various tissue cultures [9—11] have been clarified. Reoviruses have been isolated from healthy children [12, 13], outbreaks of different diseases [1, 14—18], several species of domestic animals [19—21], laboratory animals [1, 22, 23] and uninoculated monkey-kidney cell cultures [24—26].

The wide dissemination of reoviruses has been demonstrated by serological surveys [1, 4, 14—17, 27—32]. Further, the existence of serological cross reactions among the three reovirus types [1, 4, 30] and among strains of human and lower animal origin [19—20] have been evidenced.

To elucidate the possible pathogenicity of reoviruses, volunteers were infected, but the results of these experiments were not unequivocal. On the other hand, isolation of reoviruses from ill-defined minor illnesses, including respiratory, enteric and exanthematic cases, and serological evidence of reovirus infection in connection with such cases have often been reported [12, 14, 15, 23, 35—37]. The so-called HEV virus, which causes jaundice associated with encephalitis in mice has also been proved to be a reovirus [36, 37].

All these data supplied little information on the human pathogenicity of reoviruses, isolation of which is seldom successful although a high percentage of the population appears to be positive serologically [14, 15, 17, 18, 27, 30, 32].

To obtain some information on the immunological status of the population of Hungary, we examined serum samples from subjects from two months to 70 years of age for haemagglutination-inhibiting (HI) antibodies to the three serotypes of reoviruses. In addition, paired sera were collected from patients suffering from different diseases to obtain some information on the pathogenetic role of reoviruses; in 50 serum pairs both neutralizing and HI antibodies, in the others only the HI antibodies were titrated.

Materials and methods

Sera. Group I: A total of 1982 serum samples taken partly for syphilis screening tests from apparently healthy subjects, partly from subjects suffering from various acute diseases.

Group II: 433 serum pairs from hospitalized patients. Of these 273 suffered from respiratory diseases, in 46 cases the clinical diagnosis was "viral infection" with fever, upper respiratory and enteric symptoms sometimes accompanied by atypical exanthems. Paired sera of 114 patients with viral hepatitis served as control.

The sera were kept at —20 °C until tested.

Serological methods. The reovirus type strains used for preparation of HI antigens were kindly supplied by *Dr.* K. ŽAČEK (Prague). The designation of the strains was: type 1 VR 230 101476, type 2 VR 231 201477 and type 3 VR 232 201479. Antigens were produced in primary monkey-kidney cell cultures and in RUZICSKA's [38] permanent cell line No. III/1 of monkey-kidney origin. Stationary cultures were used. The maintenance fluid was PARKER's medium No. 199 throughout. After complete destruction of cells the cultures were frozen and thawed successively three times. Haemagglutination (HA) and HI titrations were carried out according to TAKÁTSY's microtitrator method [39]. The HA titre of the viral preparation was determined in every case when HI titration was performed and 4 HA units of virus were added to each serum dilution. To remove nonspecific inhibitors and haemagglutinins, the sera were absorbed with kaolin and human 0 erythrocytes [4]. Subsequently a twofold dilution series was prepared from 1 : 8 to 1 : 256. The virus-serum mixtures were allowed to stand at room temperature for one hour, whereafter a 1 per cent suspension of human 0 erythrocytes was added. After incubation at room temperature for 60—90 minutes the results were read.

Neutralization (N) tests were performed with type strains propagated in primary monkey-kidney cell cultures; aliquots of virus containing 100 CPD_{50}/0.1 ml were mixed each with an equal volume of appropriately diluted heat-inactivated serum. The mixtures were kept at +4 °C for 12 hours. Three tube cultures of permanent monkey-kidney cells were inoculated with each of the mixtures, 0.1 ml virus per culture.

Results

In Group I 73, 74 and 81 per cent of the sera contained HI antibodies in a titre 1 : 8 or higher to reovirus type 1, 2 and 3, respectively (Table I).

The percentages of sera positive to types 1 and 2 ran parallel in the different age groups, rising with age. In the age group from 6 to 10 years 74 and 72 per cent, respectively, of the sera contained HI antibodies. With type 3, on the other hand, the same level was reached much sooner; 74 per cent of

Table I

Incidence of HI antibodies to reovirus types 1, 2 and 3 in the sera of subjects of different age

Age group	No. of sera tested	Sera with titres ≥1 : 8 to					
		Reo 1		Reo 2		Reo 3	
		No.	%	No.	%	No.	%
2— 5 months	69	29	42	28	41	38	56
6—11 ,,	111	44	39	39	35	84	76
1 year	88	43	49	43	49	55	63
2 years	82	40	49	37	45	61	74
3— 5 ,,	141	88	62	83	59	107	76
6—10 ,,	162	120	74	117	72	130	80
11—20 ,,	182	145	80	145	80	151	83
21—30 ,,	278	226	81	236	85	246	88
31—40 ,,	236	204	87	215	91	211	90
41—50 ,,	197	162	82	164	83	165	84
51—60 ,,	234	189	80	196	84	196	84
61—70 ,,	131	99	76	98	75	112	86
>71 ,,	71	60	85	59	83	53	75
Total	1982	1449	73	1460	74	1609	81

the two-year-old children already had detectable HI antibodies to reovirus type 3.

The distribution of different HI antibody titres to the three reovirus types are presented in Figs 1 to 3.

In the incidence of high (1 : 128 and 1 : 256) titres there was no appreciable difference among the three types; in the incidence of medium titres fluctuation was more pronounced and the excess type-3-positive sera in early childhood had low or medium titres.

Since the above results, in accordance with literary data, suggest that the first reovirus infections usually occur in early childhood, paired sera from ill children (Group II) were examined to obtain some information on the pathogenetic role of reoviruses (Table II).

In the patients with respiratory disease (273 cases under 10 years of age) 4-fold or greater rises in titre were found in 3.3, 3.0 and 4.7 per cent against types 1, 2 and 3, respectively.

In the group with the clinical diagnosis of "viral infection" (22 cases) under 10 years of age the respective percentages were 13.6, 4.5 and 22.7 per cent. None of the 24 patients over 10 years of age showed a rise in titre.

As to the antibody titres to reoviruses in hospitalized patients suffering from a non-respiratory disease, paired sera of 114 patients with viral hepatitis

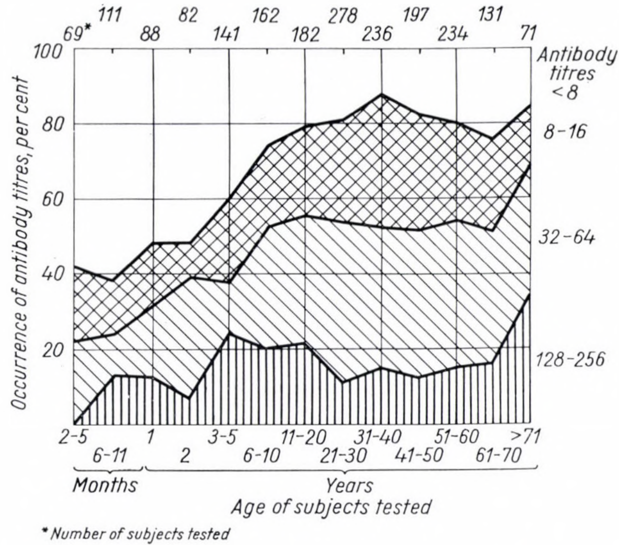

Fig. 1. Antibody titres against reovirus type 1 by age

Fig. 2. Antibody titres against reovirus type 2 by age

were examined. Rises in titre suggestive of simultaneous reovirus infection were mainly demonstrated in the paired sera of patients over 10 years of age.

In accordance with literary data, heterotypic immune responses were frequent. Most of the cases giving a well-defined immune response to reovirus type 1 or 2 showed an antibody response to both, others only to one, of the

Fig. 3. Antibody titres against reovirus type 3 by age

heterologous types. Infections attributable to reovirus type 3 were, however, not accompanied by heterotypic responses.

The relationship between the HI and neutralization titres was examined in 50 serum pairs. For this purpose sera completely negative in the HI reaction, those only inhibiting reovirus type 3 and paired sera giving an antibody response, were selected. In deciding whether a serum was or was not positive, the two reactions appear to be equivalent. The 11 HI-negative serum pairs were found to be negative in the neutralization test, too, whereas the 39 HI-positive sera also contained demonstrable neutralizing antibodies. The titre values either equalled or there was a two- or fourfold difference in titre in favour of either of the HI or the neutralization titre.

Table II

Antibody response in 433 hospitalized patients

Clinical diagnosis	Age, year	No. of serum pairs	≧4-fold rise in HI titre to					
			Reo 1		Reo 2		Reo 3	
			No.	%	No.	%	No.	%
Respiratory infection	0—10	273	9	3.3	8	3.0	13	4.7
"Viral infection"	0—10	22	3	13.6	1	4.5	5	22.7
	>10	24	—		—		—	
Viral hepatitis	0—10	56	1	1.7	3	5.3	5	8.9
	11—20	38	4	10.5	2	5.2	5	13.4
	21—60	20	3	15.0	3	15.0	3	15.0

Discussion

It has been shown by numerous authors [1, 4, 14—16, 27—31] that the incidence of HI antibodies to reoviruses is generally high but percentage data have only been published in two reports. El Rai and Evans [17] found antibodies to all three types in one quarter of children's sera and in one half of the young adult sera tested. Schmidt *et al.* [32] demonstrated HI antibodies to reovirus type 1 in 48.8 per cent, to type 2 in 87 per cent. They did not examine type 3 antibodies.

In contrast to the latter authors, we found type 1 and type 2 antibodies at about the same percentage (73 and 74 per cent). The incidence of type 3 antibodies was somewhat higher (81 per cent). The reason for the higher percentage appears to be clear: this includes the most heterotypic reactions. There was no difference in the incidence of high titres most indicative of homologous infection.

As to the age incidence of reovirus antibodies, Lerner *et al.* [27] compared the titres of maternal and neonatal sera. They found equal titres in 80 per cent of the cases. According to our studies the maternal antibodies soon disappear, but the high incidence of antibodies (especially to reovirus type 3) even in high titres, during the second half year of life suggests that infection with one or more reovirus types is common in infancy. The occurrence of high titres in all age groups points to the long persistence of antibody titres, which may be raised at intervals by reinfections. We did not examine serum dilutions higher than 1 : 256. Schmidt *et al.* found type 1 antibodies up to 1 : 320 to 1 : 640 dilution and type 2 antibodies up to 1 : 320 to 1 : 1280 in 2.9 and 21.9 per cent, respectively. Presumably some of the sera tested by us also had titres higher than 1 : 256.

The low incidence of antibody response in the sera of patients with acute respiratory disease speaks against the significance of reoviruses as respiratory pathogens. It may be of interest that a relatively high percentage of sera obtained from cases with the clinical diagnosis "viral infection" gave an antibody response to one or more reovirus types. Further investigation of such clinically ill-defined cases might be justified.

Rises in the HI titre, indicative of simultaneous reovirus infections, were displayed by a high proportion of hospitalized hepatitis cases over 10 years of age. It is unlikely that these rises should have been in relation to the actual illness of the patients. It is more probable that inapparent reovirus infections occurred during the long periods of hospitalization. Since such infections may also occur in patients hospitalized with respiratory diseases of different origin, the serological response to reoviruses of hospitalized patients should be interpreted with great precaution.

The present investigations have shown that in the serological diagnosis of reovirus infection the HI test is nearly equivalent to the neutralization test.

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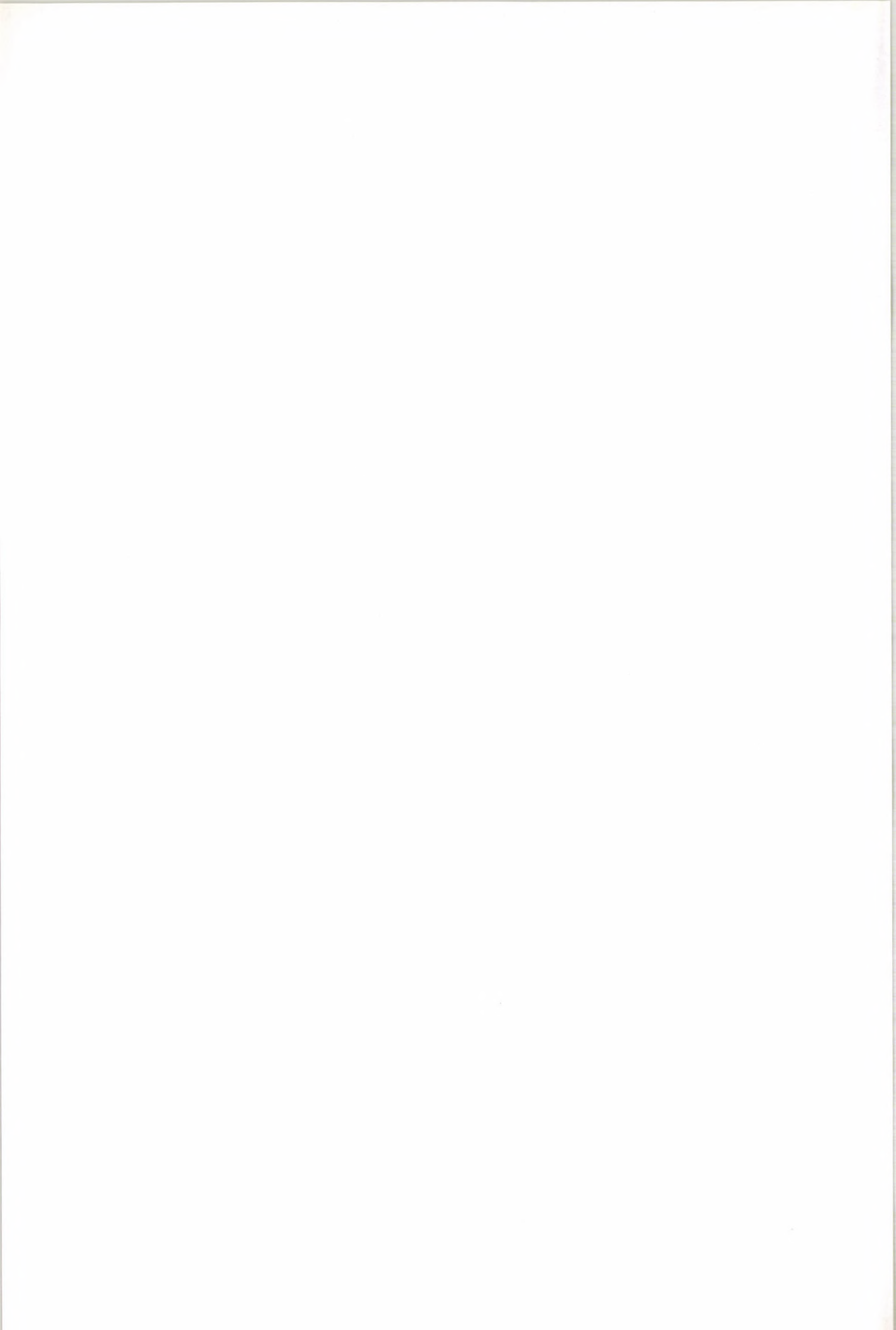

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SIMULTANEOUS DETERMINATION OF ERYTHRO- AND LEUKOCYTOTROPINES IN VIVO

By

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Summary. A new method has been elaborated for the simultaneous determination of erythro- and leukocytotropines *in vivo*. Mixtures consisting of 0.5 ml serum dilution to be tested, 0.25 ml of 10 per erythrocyte suspension of conforming blood group and 0.25 ml of a 20 000 per cu. mm. leukocyte suspension each are administered intraperitoneally to 3 mice. After 3 hours, peritoneal exudate smears are examined for the occurrence and percentage of erythro- and leukophagocytosis. Phagocytosis observed in over 3 per cent of mouse leukocytes indicates a positive reaction. In a series of 25 cases suffering from immunohaematological disorders, erythrophagocytosis could be detected in 14 cases, leukophagocytosis in 9, and simultaneous erythro-leukophagocytosis in 9 instances. Parallel tests *in vitro* disclosed the presence of antibodies against erythrocytes in 12, against leukocytes in 5, and against both erythrocytes and leukocytes in 4 cases. Testing of 20 control subjects yielded uniformly negative results. The method is suitable for the detection of heterocytotropines, complete and incomplete autoantibodies, both as a direct and as an indirect reaction.

In the field of immunohaematological diseases an important role has been attributed to the substances reacting with the own erythrocytes, leukocytes and thrombocytes of the organism and with factors active in blood clotting. Autoantibodies may develop against several haematological systems, resulting in cytopenia of variable form and intensity. The most frequent manifestation is anaemia accompanied by moderate or marked leukopenia and often also by thrombopenia.

A whole battery of *in vitro* tests has been developed for the detection of cytopenia-inducing autoantibodies, including autoagglutinin determination, cold agglutination, direct and indirect antiglobulin reaction, mono- and biphasic cold haemolysin tests for erythrocytes, as well as leukocyte agglutination, leukocyte phagocytosis *in vitro*, the leukocyte complement consumption test of CHUDOMEL, JEZKOVA and LIBANSKY [5], leukocytolysis, *etc.* These methods, however, often lack adequate sensitivity. As demonstrated in our previous studies [1, 25], even in cases, where *in vitro* tests failed to demonstrate anti-Rh (anti-D) antibodies in mother's milk, urine and infants' sera simultaneous cytotropine determination *in vivo* was occasionally still successful. The purpose of the investigations described below was to work out a method suitable for the simultaneous detection of erythro- and leukocytotropines *in vivo*.

Materials and methods

Fresh human sera or serum dilutions to be tested are mixed with three times washed human erythrocytes of conforming blood group, and with human leukocytes as follows. 0.5 ml serum + 0.25 ml 10 per cent erythrocyte suspension + 0.25 ml of a 20 000 per cu. mm. leukocyte suspension. All washings and dilutions are made with physiological saline. Leukocytes are isolated and concentrated by differential centrifugation according to MÜLLER [18]. The reaction mixtures are set up in triplicate and administered intraperitoneally to three mice in each group. After the lapse of 3 hours, the animals are killed with ether. Smears made from the peritoneal exudate are dried in air, stained with MAY-GRÜNWALD—GIEMSA solution and examined for the occurrence of erythro- and leukophagocytosis. About 400 to 1000 cells are counted in each specimen and the results are expressed in the percentage of mouse leukocytes exhibiting phagocytosis. The mean value of the three counts made in individual mice exceeding 3 per cent is regarded as the border-line of positivity both for erythro- and leuko-phagocytosis.

Results

A total of 25 patients suffering from various haematological disorders and 20 healthy control subjects were tested for the presence of cytotropines, partly *in vivo* as described above, partly by different methods *in vitro* as listed in the introduction of this paper. Included were 5 patients with haemolytic anaemia, 4 with immune agranulocytosis, 14 with pancytopenia and one patient each with porphyria cutanea tarda and cold agglutinin disease, respectively. Results are summarized in Table I.

Table I

Occurrence of antibodies against erythrocytes and leukocytes, as demonstrated in vivo and in vitro in 25 patients with various immunohaematological disorders and 20 control subjects

Cases tested	Erythro-phago-cytosis *in vivo*	Antibodies against erythrocytes de-monstrated *in vitro*	Leuko-phago-cytosis *in vivo*	Antibodies against leukocytes de-monstrated *in vitro*	Simultane-ous erythro- and leuko-phagocytosis *in vivo*	Antibodies against both erythrocytes and leuko-cytes de-monstrated *in vitro*
Haemolytic anaemia (5 cases)	5	5	2	1	2	1
Immunoagranulocytosis (4 cases)	2	2	2	2	2	2
Pancytopenia (14 cases)	5	3	3	2	3	1
Porphyria cutanea tarda (1 case)	1	1	1	—	1	—
Cold agglutinin disease (1 case)	1	1	1	—	1	—
Healthy controls (20 cases)	—	—	—	—	—	—

As demonstrated in Table I, the method *in vivo* yielded positive results in 14 cases for erythrophagocytosis and in 9 cases for leukophagocytosis. The simultaneous occurrence of erythro- and leukophagocytosis could be established

in 9 cases. On the other hand, by means of the different tests *in vitro* antibodies against erythrocytes were found in 12 sera, against leukocytes in 5 sera, while antibodies against both erythrocytes and leukocytes could be demonstrated

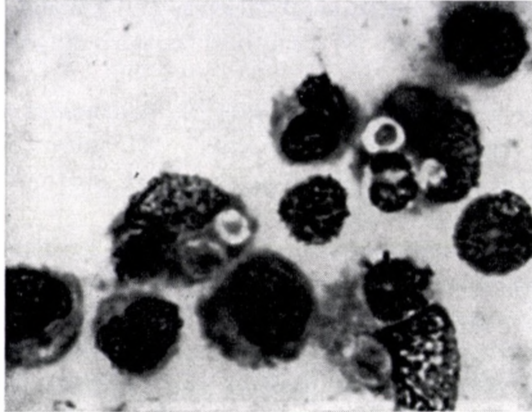

Fig. 1. Simultaneous erythro- and leukophagocytosis

in 4 cases only. Blood sera taken from the control subjects yielded uniformly negative results, both *in vivo* and *in vitro*. An example for simultaneous erythro- and leukophagocytosis is illustrated in Fig. 1, visualizing the partial destruction of phagocyted cells.

Discussion

Phagocytosis of different cells may occur along two lines, by means of non-specific and by specific mechanisms. Aged cells or cells damaged by known or unrecognized injuries are eliminated by what may be called normal phagocytosis. This process may sometimes grow to considerable dimensions. LANGHANS was the first to describe the rapid penetration of leukocytes into haemorrhages, resulting in phagocytosis and digestion of erythrocytes. Large-scale phagocytosis of erythrocytes in the cerebrospinal fluid was observed by BALÁZS [3]. VAJDA and SZÜCS reported on a case of suppurative otitis with marked leukophagocytosis in the CSF even in the absence of antibodies against leukocytes, after the penetration of pus into the cerebral ventricles [24]. Such an intensive normal cytophagocytosis is evoked partly by the "increased offer", *i.e.* the great number of cellular elements to be phagocyted, partly by the action of different exudation-promoting substances, such as leukotaxin [14], leukocytosis-promoting factor [15], exudin [16], C-reactive protein [23], *etc.*

If antibody is produced against blood cells, as it is the case in paroxysmal haemoglobinuria or after incompatible blood transfusions, then the erythrocytes sensitized by the corresponding antibodies are subjected to phagocytosis

like specific antigen-antibody complexes. This process is called immunophagocytosis, and the antibodies promoting phagocytosis are named opsonins.

According to their target cells, origin and mode of action, the opsonins may be grouped as follows.

Opsonins directed against erythrocytes. (1) Natural isohaemopsonins; (2) immunoisohaemopsonins; (3) acquired autohaemopsonins; (4) heterohaemopsonins.

Opsonins directed against leukocytes. I. Antibodies reactive with the cell surface. (1) Natural isoleukoopsonins; (2) immunoisoleukoopsonins; (3) autoleukoopsonins; (4) heteroleukoopsonins. II. Antibodies reactive with cell nuclei (L. E.-factor, antinuclear factors).

Isohemopsonins in human serum were found by SCHIFF [21], RUBINSTEIN [20] and numerous other authors. BACKHAUSZ and VAJDA developed a method for the demonstration *in vivo* of the haemopsonic activity of anti-Rh (anti-D) sera. This titration *in vivo* yielded usually titres, higher than those obtained in tests *in vitro*, and was positive in some cases judged negative on the basis of methods *in vitro*. A further improvement was achieved by rendering the test suitable for the simultaneous determination of erythro- and leukoopsonins, as demonstrated in this paper.

Blood transfusion, organ transplantation [2] and pregnancy are known to result occasionally in isoopsonin production. Since no such episodes could be established in the history of the patients included in the present series, the positive erythro- and leukophagocytosis observed should be attributed to the presence of autohaemopsonins and autoleukoopsonins in the patients' sera.

Our observations on the simultaneous occurrence of erythro- and leukophagocytosis are in accordance with the previous findings of FISHER [10a], EVANS and DUANE [10], DAUSSET *et al.* [6, 7, 8], MOESCHLIN [17], GASSER and HOLLÄNDER [11], BERNARD *et al.* [4], STEFFEN *et al.* [22], MÜLLER and WEINREICH [19], LOGHEM *et al.* [9] and HERMANN [13], demonstrating the co-existence of leukopenia and thrombopenia in haemolytic anaemia, and describing the simultaneous production of antibodies against the three main cell types during pancytopenia.

Sensitization of blood cells may also take place *in vivo*, as indicated by the positive result of the direct cytotropin reaction performed in 3 cases (one patient each with haemolytic anaemia, porphyria cutanea tarda and cold agglutinin disease) out of the 9 exhibiting a positive indirect cytotropine test, thus, suggesting that autoantibodies must have been bound *in vivo* to erythrocytes and leukocytes.

Phagocytosis of blood cells during different autoimmune conditions could be verified to occur in the organism itself. Thus, large-scale phagocytosis of blood cells of all types by mature and immature megakaryocytes in the sternal marrow obtained from a patient with immunothrombopenia and *E. coli*

infection was described by GORECZKY and VAJDA [12]. The mechanism of the latter phenomenon has not been elucidated. It was, however, noted that sensitized cells added to rat bone marrow in short-term cultures were phagocyted by the megakaryocytes [12a].

The cytotropine reaction *in vivo* as described in this paper, is suitable for the demonstration of both complete and incomplete autoantibodies. It also yielded uniformly positive results with hetero-immune sera against blood cells developed in rabbits.

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EFFECT ON INFLUENZA VIRUS OF A MODIFIED FRANCIS INHIBITOR AND ITS ACETONE-SOLUBLE FRACTION

I. STUDIES IN ROLLER DRUM, AND IN DE-EMBRYONATED EGGS

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Summary. Comparative studies were performed on Francis inhibitor (K_0), a modified Francis inhibitor (*8m*) prepared in this laboratory and their acetone-soluble fractions (K_0ac and *8ac*).

A 2 per cent solution of *8ac* exhibited a remarkable virucidal effect *in vitro* as compared to the other three substances lacking this effect.

In roller drum, by measuring the inhibition of virus multiplication by haemagglutination at different concentrations of the various inhibitors and evaluating the results statistically, we have made the following observations. The regression of inhibition measured as the geometric mean of haemagglutination titres as referred to the dose of the inhibitor derivatives yielded a first order function at intermediary doses.

Using identical doses of virus, the effectiveness of the most active *8ac* preparation exceeded 39 (32 to 44) times that of the original Francis inhibitor as referred to the respective 50 per cent inhibitory doses (E_{50}).

Using an identical strain of virus, the order of activity of the individual materials was *8ac*, *8m*, K_0ac, K_0. The activity depended on the characters of the given strain and, though not always significantly, on its dose expressed in ID_{50}.

As to the development of haemagglutinating activity in de-embryonated eggs, the activity of *8ac* depended on the time of its addition to the system and on the dose of virus actually used. The earlier the addition of identical concentrations of *8ac*, the better the inhibition. Similarly, the lower the initial virus dose, the more pronounced the inhibitory effect.

No notable effect was exerted by *8ac* on the production of vaccinia virus HAN.

As already reported [1, 2, 3], we have coupled by diazotization an alkylized aniline derivative with the Francis inhibitor obtained from human serum. The affinity of this modified inhibitor was unaltered to active virions of influenza A-0, A-1, A-2 and B type strains. Nevertheless, the release of bound virions took longer than with the unmodified Francis inhibitor.

It was supposed that the modified inhibitors more firmly coupling with the influenza virus may inhibit the attachment of the virus to sensitive cells, thus inhibiting virus growth. The results obtained have justified this assumption.

The interest in nonspecific inhibitors constituting a firm complex with virions has increased since such substances have been encountered in different natural sources against enteroviruses [4, 5] and the A-2 influenza virus [6]. The possible practical application of these materials for the prevention of viral infections has also been considered.

The present study reports on studies performed in roller drum and de-embryonated eggs with influenza virus and a modified Francis inhibitor and its acetone soluble fraction.

Materials and methods

Production of crude Francis inhibitor. Principles of the method have been published [1].

Preliminary test. A series of M/15 phosphate buffer mixed in a ratio 3 : 1 to the human serum was studied. 15 × 100 mm tubes were used. The series thus obtained was put into a boiling water bath for 20 minutes. After cooling in running tap water, each sample was centrifuged for 15 minutes at 3000 r. p. m. The increase of the haemagglutination inhibition (HAI) activity was determined by TAKÁTSY's micromethod [7], using four HA units of heat inactivated Lee strain of influenza B virus and 2 per cent suspension of chicken or human erythrocytes.

Preparation. The combination resulting in the greatest titre increase was used for the preparation of larger amounts of supernatants (from 5 to 15 litre human serum) in 500 ml individual batches. The batches were boiled in stainless steel containers. The internal temperature of the material exceeded 90 °C. The supernatant obtained after centrifugation of the boiled material served as starting material for further preparation.

Modification of the Francis inhibitor. Determination of the dry material. Batches of 100 ml of the starting material were dialysed against saline at +4 °C for 3 days usually against saline. The material was then clarified by centrifugation at 6000 r. p. m. for 20 minutes in a MOM Superfug apparatus. (This procedure is termed clarifying centrifugation in the following.) The supernatant was treated with 10 volumes of acetone and the precipitate collected by filtration through a filter paper of known weight. The material was then dried to constant weight. The acetone precipitable dry material content varied from 2 to 8 mg/ml.

Preparation of diazonium salts from the inhibitor. An amount of diethyl-p-phenylenediamine (Merck, analytic purity) corresponding to one quarter of the dry weight of the inhibitor to be modified was transformed to diazonium chloride at 0 °C. An appropriate amount of alkalinized starting material was then mixed with the above substance under constant stirring. The amount of *N* NaOH used for alkalinization was calculated so as to obtain a pH of 8 after the addition of diazonium chloride. The pH was adjusted to 7.2—7.4 after half an hour. The supernatant obtained after 6 days of dialysis at 4 °C and clarifying centrifugation was the substance designated as substance 8 in our previous paper.

Preparation of "8 modified" (8m). Material 8 was added under continuous stirring to 4 volumes of acetone at —15 °C. The precipitate was sedimented by centrifugation at 3000 r. p. m. for 10 minutes and resuspended in saline of one tenth of the volume of starting material. After three days dialysis and clarifying centrifugation for 10 minutes, the supernatant was frozen and lyophilized. The amorphous material deep violet in colour thus obtained was designated as "*8m*". Substance *8m* was readily dissolved in saline at 1 to 2 per cent concentration if previously finely ground in an agate mortar.

Preparation of "8 acetone soluble" (8ac). The supernatant obtained after sedimentation of the acetone treated *8m* was freed from acetone by heating in a water bath and vacuum distillation. The soluble material thus obtained was dialysed against distilled water for 3 days and centrifuged for clarification. The supernatant obtained was frozen and lyophilized. Substance *8ac* was sticky, deep violet in colour and had a fatty appearance. It dissolved readily in saline at 1 to 2 per cent concentration during grinding in an agate mortar.

Similar methods were used except the diazotization for the preparation of control K_0 and control K_0ac corresponding to *8m* and *8ac*, respectively.

Roller drum technique. This has been described [1]. In the present study, however, the amount of inhibitor added was referred to dry weight instead of HAI-titre, since the lyophilized materials were satisfactorily soluble. This appeared to be an advantage, as the modification of the inhibitor was found to alter its HAI-activity. Decrease to about one half or one eighth of HAI-activity was demonstrable in *8m* as compared to K_0. Substance *8ac* exhibited a further titre decrease by 1/2 to 1/4. No difference was observed in the HAI-activity of K_0ac and *8ac*.

Statistical analysis. In the roller drum experiments, eight parallels were prepared simultaneously. Quantitation of virus replication was expressed in base 2 logarithms of HA titres of the individual tubes. The inhibitory effect of the individual derivatives on virus replication was measured by regression analysis, taking the concentration of the individual derivatives for the independent variable (x). For analysis, the individual concentrations were numbered

in sequence (20 mg/ml = 8; 10 mg/ml = 7; etc.). The dependent variable (y) was the logarithmic power (to base 2) of HA titres.

The regression coefficient (b) was calculated and its significant divergence from 0 demonstrated in every case by STUDENT's *t*-test. The F-test performed parallel to the analysis of variance has shown the first order function of regression at appropriate concentrations. The quotient of the error and the square of variance of regression has shown the existence of regression in a significant way ($P < 5$ per cent). The squares of variance of the individual classes approached or exceeded that of the deviation. The reason for this was the wide scattering of HA titres.

The inhibitor concentration causing a 50 per cent decrease of HA titre (E_{50} = 50 per cent efficient concentration) was referred to the appropriate virus control and expressed in terms of average base 2 logarithms of HA titres. The fiducial limits of the individual E_{50} values were calculated, taking the deviations into account.

The significance of the difference between two E_{50} values in each given case were calculated by STUDENT's double *t*-test at the $P = 0.1$ per cent level.

The control titres were different in the different experiments. To make simultaneous presentation of several experiments possible, the results obtained were expressed in per cents of the control value.

Studies in de-embryonated eggs. Fourteen to 16 days old embryonated eggs were de-embryonated according to BERNKOPF [9]. In the area of the air sac, the shell membrane, together with the chorioallantoic membrane (CAM), was cut into three segments. The edge of each segment was attached to the egg-shell by means of a drop of paraffin. The embryo was then removed. The CAM was rinsed three times in nutrient medium [8], the eggs were closed by means of UV-sterilized synthetic caps sealed to the egg shell by means of paraffin and then rotated in plastic containers [8]. De-embryonated eggs thus obtained were then inoculated with the required amount of virus in 3 ml volume, injected through a 4—6 mm diameter hole on the synthetic cap. After 30 minutes of adsorption, the membranes were rinsed 3 times with 8 ml of nutrient medium each. Finally, the eggs were filled with 10 ml of appropriate nutrient medium with or without the inhibitor. For HA-titration, samples were taken with TAKÁTSY loops of 0.025 ml. The resulting loss of volume was not readjusted. For presentation, the arithmetic means of HA titres per group were used.

Vaccinia virus. The strain used for vaccine production in Hungary was adapted to CAM. The adapted strain induced the production of both HAN and infectious virions in the epithelial cells of the allantoic sac. For haemagglutinin titration, a 0.5 per cent suspension of 3-times washed cock erythrocytes was used after a preliminary test. The diluent was HANKS' balanced salt solution.

Results

Virucidal effects of Francis inhibitor derivatives. From each derivative, 20—10—2.5 mg/ml solutions were prepared in buffered glucosol containing 1 per cent egg white. Equal volumes of the solutions were mixed with freshly harvested allantoic fluid from eggs infected with strain Bp. 4/49 of type A-1 influenza virus. The control virus was diluted in the diluent. All samples were incubated at room temperature and at appropriate intervals 0.1 ml samples were taken. Each sample was immediately diluted tenfold and starting from this, a series of $10^{0.5}$-fold dilutions was prepared. Infectivity titrations were made by HORVÁTH's roller drum method [8], starting with a $10^{-1.5}$ dilution. In Table I are presented the obtained $ID_{50}/0.1$ ml values (according to REED and MUENCH).

No virucidal effect was observed after incubation for 1 hour of the virus in the presence of 20 mg/ml of K_0, *8m* or K_0ac. Substance *8ac* was, however, virucidal. The infectious titre of the virus exhibited a $10^{1.85}$-fold decrease and a significant decrease in titre was observable at the 10 mg/ml dose level.

Table I

Inactivation in vitro of Bp. 4/49 strain of influenza A_1 virus

Time of incubation (hours)	Virus control log ID_{50}/0.1 ml	Virus infectivity (log ID_{50}/0.1 ml) after incubation with					
		K_0 20 mg/ml	*8m* 20 mg/ml	K_0ac 20 mg/ml	*8ac* 20 mg/ml	*8ac* 10 mg/ml	*8ac* 2.5 mg/ml
0	7.00	ND	ND	ND	ND	ND	ND
0.5	6.50	ND	ND	ND	ND	6.05	6.15
1	6.05	6.10	6.00	6.00	4.20	5.70	6.00
2	5.85	ND	ND	ND	ND	ND	5.65
6	5.75	5.21	5.20	5.10	2.00	5.15	5.65

ND = not done.

On incubation for 6 hours, a $10^{0.5}$-fold decrease of infectivity was observed with K_0, *8m* and K_0ac as well as with 10 mg/ml *8ac*, as compared to the control. This value, though significant, was negligible in respect of virucidal effect. In contrast, the $10^{3.75}$-fold titre decrease observed in the presence of 20 mg/ml *8ac* represented a remarkable virucidal effect. The 2.5 mg/ml dose level of *8ac* failed to exert a virucidal effect.

The growth inhibitory effect of Francis inhibitor derivatives on A-1 influenza virus as related to the doses applied. Different concentrations ranging from 2.44 μg/ml to 20 mg/ml of K_0ac, *8m* and *8ac*, were tested against 10^5 ID_{50} doses of Bp. 4/49 strain of A-1 influenza virus.

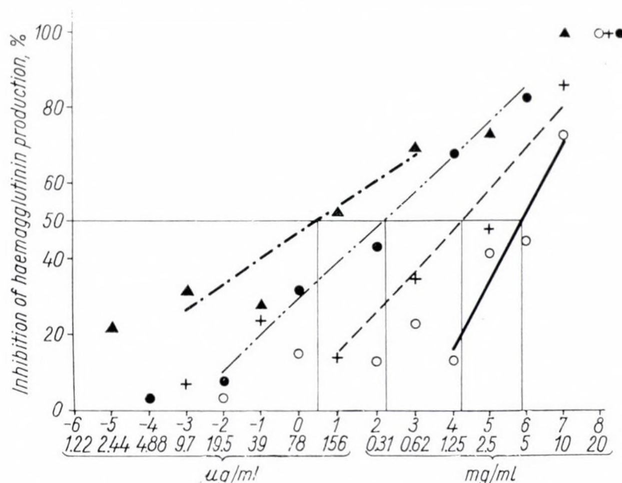

Fig. 1. Inhibition of influenza virus replication as related to the dose of inhibitor, tested in roller drum. Virus strain, Bp. 4/49. Virus dose, 10^5 ID_{50}/0.1 ml.

K_0 ○———○ ($y = -0.4156x + 3.1812$; $t_{[2]} = 5.691$; $P < 1\%$)
K_0ac +———+ ($y = -0.5468x + 4.7968$; $t_{[2]} = 6.44$; $P < 1\%$)
8m ●.-.-..-.-.● ($y = -0.919x + 7.820$; $t_{[2]} = 5.40$; $P < 2\%$)
8ac ▲- . -▲ ($y = -0.3156x + 2.4843$; $t_{[2]} = 3.52$; $P < 5\%$)

The percentual inhibition by the individual derivatives as referred to the control is presented in Fig. 1. The computed values are shown by the designations specified in the captions for Fig. 1. The characteristic equations of regression are presented adjacent to the plots obtained by geometric presentation of the computed values. The first members of the equations are the regression coefficients (b), the second ones the pure members. Fig. 1 also presents the related t and P per cent values showing the significance of the deviation from 0 of the regression coefficient.

The grade of inhibition decreased proportionally to the concentration of the different inhibitors. Each dose response curve comprises a linear part. The linear part covers a greater or a smaller number of doses according to its slope. At extremely high or low doses, the linear correlation is no longer demonstrable. In Fig. 1, only the linear relations are presented as curves and all the values obtained are shown as individual points. The perpendicular line drawn from the point representing 50 per cent inhibition gives the E_{50} concentration of the respective inhibitor. The E_{50} values expressed as mg/ml are given in Table II, together with the fiducial limits of the actual measurements.

The four lines in Fig. 1 run separately and exhibit a certain divergence. By simple inspection, the following order of activity is apparent: $K_0 < K_0ac < 8m < 8ac$.

As calculated by the double t-test, the difference between all the four E_{50} values was significant at the 0.1 per cent level. Taking the effectiveness of K_0 as unit, that of the derivatives was, K_0, 1; K_0ac, 2.82 (2.33—3.24); *8m*, 11.89 (9.9—14.88); *8ac*, 38.66 (32.2—44.3).

Deviation of the increase in efficiency was calculated — at the 1 per cent level — from the total deviation of all compared values. Owing to gain in grade of liberty, the deviation of values was lower than the fiducial limits of individually calculated E_{50} values.

The results indicate a higher efficiency of fractions obtained from the supernatant after acetone fractionation (K_0ac, *8ac*) as compared to those in the starting material (K_0, *8m*). The twelvefold increase in efficiency, arisen between K_0 and *8m* in consequence of modification, increased further 3 times with the acetone soluble *8ac*. Substances K_0 and *8m*, similarly to the Francis inhibitor, were precipitated at the actual concentration of acetone, while *8ac* exhibited characters different from those of the classical Francis inhibitor.

Inhibition of growth of different influenza virus inocula in roller drum as referred to the doses of 8ac applied. Substance *8ac*, having been found the most active in the preliminary test, was further studied within the dose range of 1.22 μg/ml to 10 mg/ml against 10^6, 10^5 and 10^4 ID_{50} inoculum doses of Bp. 4/49 strain of type A-1 influenza virus. The results obtained are presented in Fig. 2 and Table II. Three roughly parallel straight lines showed that on decreasing the inoculum, the relative effectiveness of *8ac* increases. A hundredfold decrease

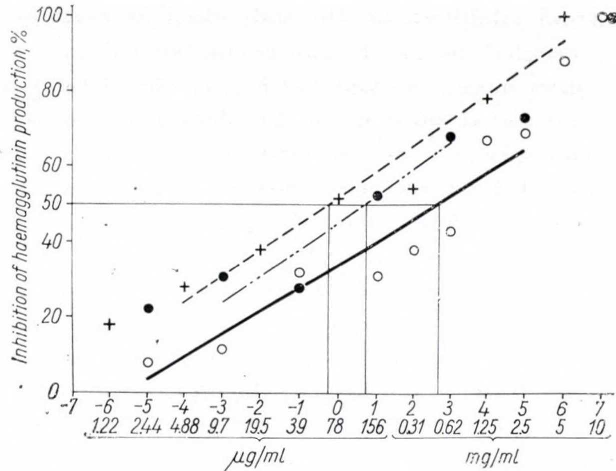

Fig. 2. Inhibition of different doses of influenza virus inoculum by different doses of *8ac* as measured by the roller drum technique. Virus strain, Bp. 4/49. Virus doses:

10^6 ID_{50}/0.1 ml ○——○ ($y = -0.2735x + 2.9786$; $t_{[6]} = 7.25$; $P < 0.1\%$)
10^5 ID_{50}/0.1 ml ●- . . -● ($y = -0.3156x + 2.4843$; $t_{[2]} = 3.52$; $P < 5\%$)
10^4 ID_{50}/0.1 ml + - - - + ($y = -0.3374x + 2.02$; $t_{[4]} = 7.27$; $P < 1\%$)

of the inoculum dose (10^6 to 10^4 ID_{50}) resulted in a 7.52-fold increase of the inhibitor's activity. This was significant at the 0.1 per cent level. The increase of activity between 10^6 and 10^5 ID_{50} doses (E_{50}: 0.54 and 0.12 mg respectively) was significant only at the 5 per cent level, while no significant activity increase was demonstrable between 10^5 to 10^4 ID_{50} doses.

Table II

Fifty per cent inhibitory doses of Francis inhibitor derivatives in roller drum

Derivative	Virus strain	Virus dose (ID_{50})	E_{50}/ml (mg)	Fiducial limits (mg/ml)
K_0	Bp. 4/49 A-1	10^5	4.64	2.71—7.57
8m	Bp. 4/49 A-1	10^5	0.39	0.12—1.27
K_0ac	Bp. 4/49 A-1	10^5	1.64	0.68—3.98
8ac	Bp. 4/49 A-1	10^6	0.54	0.10—2.81
8ac	Bp. 4/49 A-1	10^5	0.12	0.04—0.40
8ac	Bp. 4/49 A-1	10^4	0.0698	0.0149—0.149
8ac	PR8 A-0	10^4	2.07	1.107—8.47
8ac	Singapore 1/57 A-2	10^4	1.80	0.74—6.68
8ac	Lee B	10^4	0.0006	0.00003—0.612
8m	Bp. 4/49 A-1	10^6	1.2	0.01—2.73
8m	Bp. 4/49 A-1	10^4	0.258	0.121—0.48

Inhibition of growth of different influenzavirus inocula in roller drum as referred to the doses of 8m applied. Substance *8m* was tested in the dose range from 2.44 μg/ml to 20 mg/ml, against three different doses of Bp. 4/49 strain of type A-1 influenza virus. As shown in Table II, the increase in activity of the less active *8m* substance was but 4.65-fold when the dose of inoculum had been reduced 100-fold. This value was still significant. The tenfold decrease of the virus dose from 10^6 to 10^5 ID_{50} resulted in a 3.07-fold increase in inhibitor activity. This value was also significant. Nevertheless, further lowering of the inoculum dose caused no significant increase of inhibition.

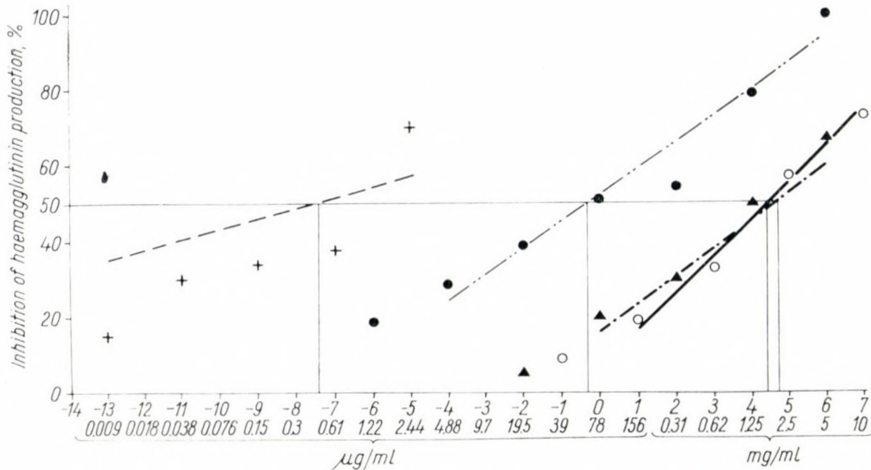

Fig. 3. Inhibition of growth of different influenza viruses by different doses of *8ac* in roller drum. Virus strains:

Singapore ○——○ ($y = -0.507x + 0.992$; $t_{[2]} = 11.71$; $P < 1\%$)
PR8 ▲--.--▲ ($y = -0.3937x + 4.525$; $t_{[2]} = 4.72$; $P < 5\%$)
Bp. 4/49 ●- . . -● ($y = -0.3374x + 2.02$; $t_{[4]} = 7.27$; $P < 1\%$)
Lee +- - - -+ ($y = -1.0017x + 2.35$; $t_{[3]} = 25.55$; $P < 0.1\%$)

Dose-dependent inhibitory action of 8ac on different virus strains. Next the effect of *8ac* on 4 different strains of influenza virus was examined.

Different concentrations of the inhibitor ranging from 0.009 μg/ml to 10 mg/ml were added to 10^4 ID_{50} doses of the respective virus. The strains used were Lee (B), Bp. 4/49 (A-1), non-avid strain Singapore 1/57 (A-2), and PR8 (A-0). A decrease in sensitivity was found in accordance with the above sequence. The difference in sensitivity of the Singapore and PR8 strains was not significant, in contrast to the others. The high sensitivity of strain Lee was remarkable. It seemed that other, less old, laboratory strains may exhibit a lesser sensitivity. The differences in sensitivity probably reflected certain particularities of the individual strains and the numerical values obtained might be valid but for the roller drum method used in this laboratory. The absolute grade of inhibition must be different with each method of testing.

Inhibitory effect of 20 mg/ml 8ac on different inocula of strain PR8. Strain PR8 exhibited the highest resistance against our most active preparation. Nevertheless, even this strain was totally inhibited at the maximal 20 mg/ml concentration of *8ac*. Should this action have been the result of tissue impairment, it would not have been influenced by the change of the inoculum's concentration.

Inocula ranging from 10^1 to 10^8 ID_{50} were added each to four ampoules containing control medium and *8ac*. After 72 hours incubation in a roller drum, the haemagglutinating activity in each ampoule was determined. Calculation of the results was performed as previously.

As shown in Table III, 20 mg/ml doses of *8ac* caused 100 per cent inhibition of inocula up to 10^3 ID_{50}. A further increase of the dose of inoculum resulted in the lowering of inhibition as revealed by an increase in HA titres.

Table III

Inhibitory action of uniform 20 mg/ml doses of 8ac on different doses of inoculum of PR8 strain

Virus dose/ampoule ID_{50}	Control		*8ac*		
	HA titre in 4 ampoules	$\log_2$ HA, average	HA titre in 4 ampoules	$\log_2$ HA, average	Per cent inhibition
10^1	0,0,32,32	2.5	0,0,0,0	0	100
10^2	0,32,32,48	3.875	0,0,0,0	0	100
10^3	32,64,64,64	5.75	0,0,0,0	0	100
10^4	32,32,64,64	5.5	2,4,6,8	2.375	57
10^5	32,64,64,64	5.75	2,8,16,16	3	48
10^6	32,32,64,64	5.5	4,12,16,24	3.5	36
10^7	32,32,64,64	5.5	4,8,16,32	3.5	36
10^8	32,64,64,64	5.75	8,16,16,24	3.875	33

Examinations in de-embryonated eggs. Experiment No. 1 (Fig. 4). Fifteen de-embryonated eggs were inoculated each with a 6×10^8 ID_{50} dose of Bp. 4/49 strain. After 30 minutes of adsorption and three subsequent rinsings, two groups received nutrient medium and one group nutrient medium containing 20 mg/ml *8ac*. One of the groups with nutrient medium was subjected after 5 hours to rinsing followed by the substitution of the medium with a new one containing 20 mg/ml *8ac*.

After 18 hours of incubation, the HA-titre of the fluid phase of the control and of the two inhibited systems showed a remarkable difference (48 against 12 and 16). After 24 hours, the HA-titre of the control increased to 64, while those of the inhibited systems varied between 8 and 12. Then all groups were subjected to three subsequent rinsings with fresh nutrient medium

and all de-embryonated eggs were further incubated in the absence of inhibitor. The haemagglutination of the control group increased rapidly by the 26th, 40th and 44th hours. The rate of increase of haemagglutinating particles in the previously *8ac*-inhibited system lagged behind, though with time it gradu-

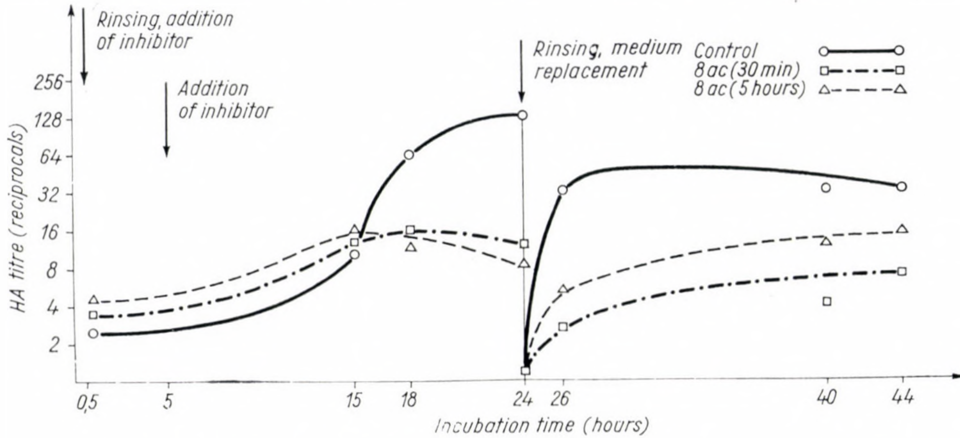

Fig. 4. Growth of strain Bp. 4/49 A-1 influenza virus in de-embryonated eggs

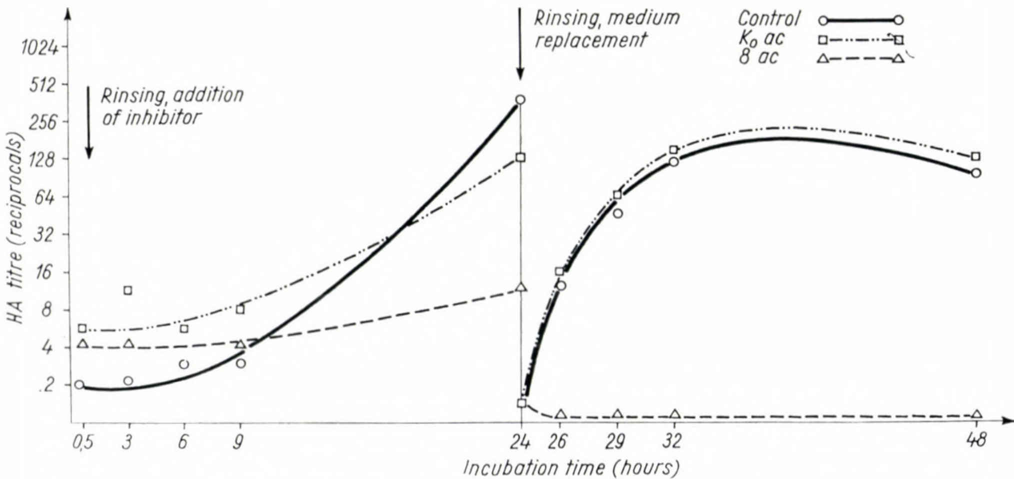

Fig. 5. Growth of strain PR8 in de-embryonated eggs

ally approximated the control values. Regeneration of the group inhibited by *8ac* in the 5th hour was more rapid than of the group inhibited in the 30th minute.

Upon addition of the inhibitor after the completion of adsorption, the grade of inhibition was found to be related to the time of addition.

Experiment No. 2 (Fig. 5). A group of 15 de-embryonated eggs was inoculated with 3×10^7 ID_{50} inoculum of PR8 virus. Thus, the inoculum was

remarkably less and the virus more resistant than in the first experiment. After allowed to adsorb for 30 minutes and rinsed 3 times, 20 mg/ml of *8ac*, K_0ac and pure nutrient fluid, respectively, were inoculated into 5 eggs each. The HA titre of nutrient media was determined 3, 6, 9 and 24 hours later. Then after 3 rinsings the nutrient medium was exchanged. HA titres of the nutrient media were again determined 26, 29, 32 and 48 hours later.

The haemagglutination titre of the control increased from the 9th to the 24th hour to 384 as compared to the 128 and 12 with K_0ac and *8ac*, respectively. After rinsing and replacement of the medium, virus growth was parallel

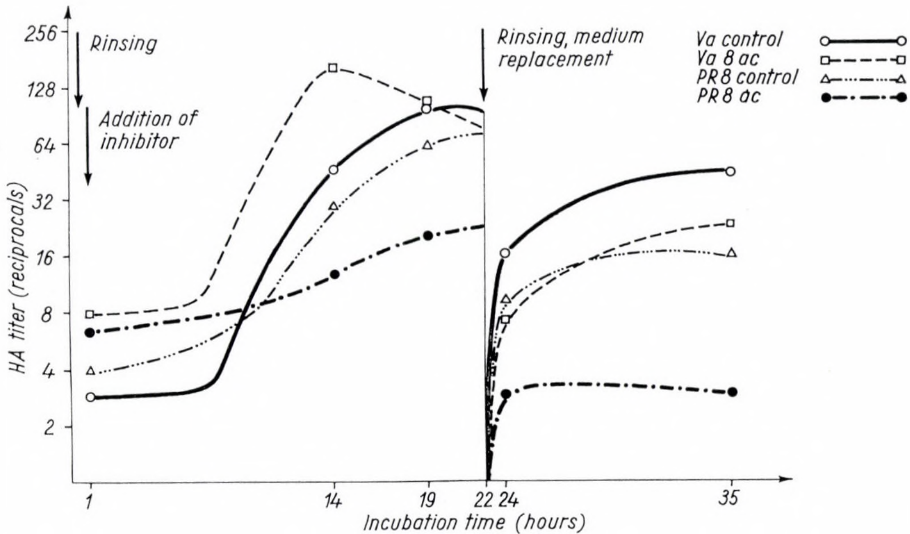

Fig. 6. Effect of *8ac* on the growth of influenza (PR8) and vaccinia (Va) viruses in de-embryonated eggs

in the K_0ac and control groups during the next 24 hours. The system treated with *8ac* appeared to be "cured", as no increase in HA-titre could be detected. It should, however, be remembered that a remarkable amount of virions may be present even in the absence of HA-activity.

Experiment No. 3 (Fig. 6). In this experiment, a higher dose of PR8 virus was used (3×10^8 ID_{50}) and a group infected with 6×10^7 PFU of vaccinia virus was included. Adsorption was allowed to take place for one hour. After three subsequent rinsings, the respective groups received nutrient medium with or without 20 mg/ml *8ac*. The HA titres in this experiment were determined with a 0.5 per cent suspension of cock erythrocytes sensitive also to vaccinia haemagglutinin.

In all the above experiments, the initial HA titre was always higher in the groups containing the inhibitor than in the controls. The situation was the same in this experiment. In the 14th hour, the HA titre of vaccinia-virus-

infected and *8ac*-treated eggs exceeded remarkably that of the control eggs. In the 19th hour, however, their titres were identical. In the experiment with PR8, by the 19th hour the mean titre of the control group was 64 as compared to the 20 exhibited by the treated group. In the 22nd hour, the eggs were rinsed with nutrient and the medium was replaced. By the 35th hour of incubation, the HA-titre of vaccinia-infected eggs was 48 and 24 in the control and *8ac*-treated groups, respectively. With virus PR8, the control exhibited an HA-titre of 16, while the *8ac*-treated group one of 3.

The allantoic membrane is not optimal for the production of HAN, which is not identical with the vaccinia virion. Nevertheless, the amount of HAN produced by the control group was comparable to that produced in the presence of *8ac*. In the 35th hour, the HA titre of the vaccinia virus control group exceeded that of the *8ac*-treated one, but the difference was not more than by which the latter exceeded the former in the 14th hour. Thus we may state that, under identical conditions, the production of vaccinia HAN was not remarkably inhibited as compared to the marked inhibition of PR8 virus growth.

Discussion

Francis inhibitor is a mucopolypeptide. The coupling of the diazonium salt with the cyclic amino acid of its peptide part and the resulting alteration of the macromolecule's behaviour is an interesting fact. The molecule forming a firm complex with the virion will inhibit the replication of influenza virus when administered simultaneously with the infection as revealed by the roller drum method, this sensitive indicator of growth inhibition. The acetone soluble fraction is a more active inhibitor of replication, in spite of its insignificant HAI activity. Thus, this fraction may be similar to the Francis inhibitor though being less hetero- disperse and consisting of smaller molecules. The procedure of preparation, however, suggests the possibility that this fraction may be a substance containing a peptide-saccharide-lipid component present in human serum and not corresponding to Francis inhibitor. According to our unpublished data, both *8ac* and K_0ac contain neuraminic acid. Nevertheless, in spite of their high lipid content they are different from the known gangliosides [10] inhibiting the haemagglutination and replication of influenza virus; the HAI activity of *8ac* and K_0ac are namely very low. This makes it improbable that *8ac* should compete with the receptor substance for the virions. Its virucidal effect might be related with its high solubility in lipids, facilitating its penetration into the envelope of influenza virus. Nevertheless, its virucidal activity does not explain its increased growth inhibitory action.

The inhibition of growth was found to depend on the dose of inhibitor and to a lesser degree on the dose of inoculum, as demonstrated by HORVÁTH's

roller drum method making use of surviving chorioallantoic membrane pieces maintained in ampoules.

In the three experiments performed in de-embryonated eggs, the inhibitor was added after the completion of adsorption. Thus, under these conditions, the activity of Francis inhibitor (K_0, *8m*) was negligible. The acetone soluble fraction (*8ac*), however, exhibited a remarkable activity. During the eclipse phase, the medium's HA titre exceeded that of the control not only with *8ac*, but also with K_0ac, influenza and vaccinia virus. The remarkable growth inhibitory activity of *8ac* was suspended on the inhibitor's removal when high doses of inoculum were used. In systems infected with low inocula, however, the inhibitor eliminated the haemagglutinating particles and thus reduced the virus concentration below 10^6 ID_{50}. The later the *8ac* had been added to the nutrient fluid of the infected de-embryonated egg, the more rapid was the regeneration of virus production. Substance K_0ac exhibited an inhibition exceeding the expected one, nevertheless, the regeneration of virus multiplication was more rapid than with *8ac*.

In further analyses of the inhibitory action on intracellular virus replication, one must not restrict the study to the medium's HA titre; the changes in the infective titre both intracellularly and after the release of virions have also to be considered.

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STUDIES ON THE INFLUENCE OF ENDOGENOUS REGULATORY FACTORS ON THE GROWTH OF HERPES SIMPLEX VIRUS

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Summary. The growth inhibitory action of heparin on Herpes simplex virus in human embryonic fibroblast and HeLa cells was enhanced by certain biogenic amines and hormones (histamine, adrenaline, serotonin, cortisone, hydrocortisone, ACTH). The inhibitory action of heparin was demonstrable also in rabbits infected with herpesvirus by the subcutaneous route. The inhibition manifested itself with a decrease of cutaneous lesions and the local growth of virus.

According to the recent survey by Lwoff [1], there are several factors influencing the growth of viruses, such as the temperature, interferon and the pH of the medium, among others. Other authors attributed similar effects to certain mucopolysaccharides [2, 3], stress factors [4, 5, 6], and different hormones [7].

Heparin has been found by several authors to inhibit the growth of Herpes simplex virus [2, 8—12]. Nevertheless, data are scanty as to the simultaneous action of heparin and other substances.

In earlier studies we have observed a summation of effects on Herpes simplex virus in a system containing heparin and interferon [13]. The aim of the present study was to examine *in vitro* as well as *in vivo* the effect of some biogenic amines and hormones, *viz.* histamine, adrenaline and serotonin, cortisone, hydrocortisone and ACTH, administered alone or in different combinations with heparin.

Materials and methods

Nutrient medium. Primary human embryonic fibroblast cultures were prepared in Parker's medium No. 199 containing 20 per cent calf serum. For maintenance and dilutions the same medium was used with 5 per cent calf serum. HeLa cells were grown in the same medium with 10 per cent calf serum reduced for maintenance to 5 per cent.

Cell cultures. Cultures of human embryonic fibroblasts were obtained by trypsinization of 2—3 months old embryos. Primary cultures were grown in Roux flasks. Secondary cultures were obtained by trypsinization of the primary cultures on the 3rd or 4th day of cultivation. HeLa cells were grown in 1 litre Roux flasks and used for the preparation of secondary tube cultures after 2—3 days cultivation.

Viruses. In the experiments *in vitro*, a strain of Herpes simplex virus isolated from human material and maintained in HeLa cell cultures was used [14]. In animal experiments we used a freshly isolated strain of Herpes simplex virus.

Determination of focus count reduction. Tube-cultures were infected with 0.1 ml virus suspension containing 100 PFU and after 48 hours' incubation the foci formed were counted. The differences in the number of foci as referred to the control cultures were expressed as per cent focus count reduction calculated from the mean for 4 to 6 tubes. Mean deviation of the method was ±20 per cent.

Animal experiments. The method described by FORCE *et al.* [15] was adapted to our purposes. Into the dorsal skin of rabbits 30 IU of hyaluronidase in 0.2 ml volume were injected. The virus and the test material containing fluid were then inoculated into the same spot in a total volume of 0.5 ml. In general, the infectious titre of the virus was 10^4 $TCID_{50}$ per 0.1 ml, as determined in human fibroblast tissue culture. According to our preliminary experiments, heparin did not inhibit the activity of hyaluronidase *in vivo*, in the rabbit's skin. On the fourth day following the infection, the diameter of the cutaneous lesion was measured and the lesion was excised. The excised lesions were ground in 10 volumes by wet weight of nutrient fluid and centrifuged. The virus content of the cell-free supernatant was determined by infecting 3 to 4 parallel tubes with 0.1 ml each of a tenfold serial dilution. Results are given in terms of the differences between the $TCID_{50}$ values of skin specimens from treated and those from untreated animals, taking the mean values obtained with two identically treated skin specimens.

Chemicals. Heparin, adrenaline, hydrocortisone, ACTH were products of the G. Richter Chemical and Pharmaceutical Works, Budapest. Histamine dihydrochloride (Peremin) was obtained from the Chinoin Pharmaceutical and Chemical Works, Budapest. Cortisone acetate (Adresone) and hyaluronidase (Hyasone) were products of NV Organon-OSS, Holland. Creatine sulphate (Serotonin) was a product of Reanal, Budapest.

Results

Tables I and II show the growth of Herpes simplex virus in HeLa and human fibroblast cell cultures, respectively, in the presence of histamine, adrenaline, or serotonin, with or without the simultaneous presence of heparin.

Table I

Effect of heparin and certain biogenic amines on the multiplication of Herpes simplex virus in HeLa cell cultures

Biogenic amine (μg/ml)	Per cent focus count reduction	
	without heparin	with heparin (0.5 μg/ml)
—	0	61
Histamine, 25	36	85
Histamine, 12.5	30	82
Adrenaline, 25	71	90
Adrenaline, 12.5	46	80
Serotonin, 25	32	78
Serotonin, 12.5	21	71

Each biogenic amine was found to inhibit virus growth. Inhibition was most intensive with adrenaline and serotonin in HeLa and Human fibroblast cultures, respectively. Inhibition was enhanced by heparin.

In Table III are shown the effects of certain hormones on the virus infecion in HeLa cells in the presence as well as in the absence of heparin.

Table II

Effect of heparin and certain biogenic amines on the multiplication of Herpes simplex virus in human secondary fibroblast cell cultures

Biogenic amine (μg/ml)	Per cent focus count reduction			
	without heparin	with heparin		
		1.0 μg/ml	2.0 μg/ml	4.0 μg/ml
—	0	18	53	77
Histamine, 25	19	53	68	84
Adrenaline, 25	24	56	84	97
Serotonin, 25	55	63	90	99

Table III

Effect of certain hormones on the multiplication of Herpes simplex virus in HeLa cell culture

Hormone	Per cent focus count reduction	
	without heparin	with heparin (0.5 μg/ml)
—	0	61
Cortisone acetate, 160 μg/ml	75	98
Cortisone acetate, 40 μg/ml	50	96
Hydrocortisone acetate 160 μg/ml	75	99
Hydrocortisone acetate 40 μg/ml	40	96
ACTH, 0.5 IU/ml	66	93
ACTH, 0.25 IU/ml	56	91

Table IV

Simultaneous effect of heparin and certain biogenic amines on the multiplication of Herpes simplex virus in cell culture

Composition of mixture (μg/ml)	Per cent focus count reduction in	
	HeLa	human embryonic fibroblast
	cell culture	
Heparin, 0.5 + Histamine, 25 + Adrenaline, 25 + Serotonin, 25	98	75
Heparin, 0.5 + Histamine, 25 + Serotonin, 25	80	81
Heparin, 0.25 + Histamine, 12.5 + Adrenaline, 12.5 + Serotonin, 12.5	88	33
Heparin, 0.25 + Histamine, 12.5 + Serotonin, 12.5	63	48

Table V

Effect of heparin and certain biogenic amines on cutaneous Herpes simplex virus infection of rabbits*

Substance tested (μg/ml)	Diameter of cutaneous lesion (cm)	Decrease in virus multiplication** (log $TCID_{50}$)
—	4	—
Heparin, 50	2.5	0.5
Heparin, 400	0	0.75
Heparin, 800	0	1.0
Heparin, 1200	0	1.4
Heparin, 50 + Histamine, 250 + Serotonin, 250 + Adrenaline, 25	0.5	0.8
Heparin, 1200 + Histamine, 250 + Serotonin, 250 + Adrenaline, 25	0	1.5

* The data represent means for ten experiments.

** Titre of the controls: 10^6 $TCID_{50}$/ml.

Cortisone, hydrocortisone and ACTH were inhibitory. In combination with heparin, inhibition was enhanced.

The inhibitory effect of heparin added to various mixtures of biogenic amines on virus growth in HeLa and human fibroblast cells is shown in Table IV.

The simultaneous administration of heparin and biogenic amines was found to exert a remarkable inhibitory action. None of the materials tested were toxic under the applied experimental conditions.

The action of heparin and biogenic amines on cutaneous Herpes simplex virus infection of rabbits is shown in Table V.

As revealed by Table V, the inhibitory action of heparin alone as well as in combination with biogenic amines, was demonstrable also *in vivo* as an inhibition of the development of cutaneous lesions.

Discussion

According to the presented results, the growth of Herpes simplex virus was inhibited by heparin *in vivo*. Other authors [16, 17] have shown the similar activity of heparin *in vivo* against the viruses of fibroma and myxoma. In agreement with several authors [2, 3, 10, 18] we have supposed that heparin was one of the factors of the organism's protective mechanisms against virus infection. Heparin exerts its effect probably through an extracellular mechanism; the substance being a polysaccharide of polyanionic character, it binds

onto receptors of opposite charge on the virion's surface, thus inhibiting the latter's adsorption to susceptible cells [2]. According to Voss [3] and Higginbotham [19], other tissular mucopolysaccharides of polyanionic character might play a similar role. Changes in the concentration of tissular heparin may have some influence on the extracellular spreading of herpesvirus, on the development of persistent infection, or on the manifestation of recurrent infections. In this respect, however, beside the heparin also other factors of the organism should be considered, particularly the biogenic amines and, in general, the hormones. In his recent monograph, Vaheri [2] has justly put the question whether or not the biogenic amines are able to release the inhibitory action of heparin on virus growth in the organism. Further, the working hypothesis of Higginbotham [19] should also be considered a noteworthy approach. He supposed that the anti-viral effect of tissular mucopolysaccharides might be reduced by factors carrying an opposite charge, *e.g.* ACTH.

Similar considerations have prompted us to perform studies on some important biogenic amines and certain hormones.

It was found that histamine, adrenaline and serotonin as well as cortisone, hydrocortisone and ACTH, inhibited the growth of Herpes simplex virus. These substances, though being cationic, did not inhibit the action of the polyanionic heparin but addition of the latter rather enhanced the activity of the said amines and hormones. The above basic substances are supposed to form labile readily dissociating complexes with heparin under the experimental conditions tested, thus there is a fair possibility of a summation of the individual activities.

On the basis of these results we suppose (without, however, claiming a general validity of the idea) that under the given conditions the biogenic amines and the hormones may represent protective factors of the organism against viral infections and may support the similar activities of heparin and interfeıon. As a result of the stimulus presented by viral infection, the heparin and biogenic amine content of mast cells is released at the site of inflammation and the general hormonal preventive reactions of the organism are also making appearance. The factors mobilized in the course of this process at a given phase may be regarded as members of a protective system. According to our assumption, heparin, interferon, biogenic amines, and the hormones in general, represent a cooperative protective system against viral infections. Results obtained in the present experiments did not contradict this supposition.

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TYPE DISTRIBUTION OF STREPTOCOCCUS PYOGENES STRAINS IN THE YEARS 1964—1965

ACTIVITIES WITHIN AN INTERNATIONAL SURVEY OF THE DEPARTMENT OF BACTERIOLOGY, NATIONAL INSTITUTE OF PUBLIC HEALTH, BUDAPEST

By

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Summary. Between June 1, 1964, and June 1, 1965, in the course of a survey organized by the International Committee on Streptococci and Pneumococci, 395 *Str. pyogenes* strains had been isolated in 11 different collecting laboratories and sent to our laboratory for serological typing. The strains fell into 26 different types; 0.8 per cent of the strains could not be typed either by agglutination or precipitation. Most prevalent strains belonged to complexes *5, 11, 12 . . .* and *3, 13,* B_{3264} and to types *3, 12, 1* and *6.* The results agreed with known data in that, excepting nephritis, there was no definite association between the different streptococcal diseases and the serotype of the causative agent. Five per cent of the strains were sent to Prague for checking; those giving discrepant results at repeated typing were forwarded to Genova for final control. All isolated strains were sensitive to penicillin; 5.3 per cent of them were resistant to tetracycline.

We have been participating in an international *Str. pyogenes* type distribution survey proposed by Professor R. E. O. WILLIAMS, Chairman, International Subcommittee on Streptococci and Pneumococci, and Dr. M. T. PARKER, Chief, Streptococcus and Staphylococcus Reference Laboratory, London. Countries participating in the survey were Britain, Canada, Czechoslovakia, Denmark, the German Democratic Republic, Hungary, Israel, Italy, Japan, the Netherlands, the United Soviet Socialist Republics and the United States of America.* The aims of the international cooperation were (1) to examine whether there are major differences in the type distribution of *Str. pyogenes* in different parts of the world; (2) to promote contact between laboratories dealing with this problem and to improve the reproducibility of typing methods and results. Each participating central laboratory had been asked to type 300 to 500 strains during the one year survey. In order to obtain a representative collection of the prevalent types, at least 5 widely scattered collecting laboratories were to be selected in each country. Strains isolated from scarlet fever, tonsillitis and other streptococcal infections were to be sent monthly or half-yearly to the central typing laboratory. Streptococci isolated from carriers were excluded from the survey. Typing was performed by agglutination and precipitation. Each participating central laboratory was

*According to the List of Participants (March 1, 1964).

asked to send 5 per cent of the examined strains (every 20th culture) to a central laboratory situated in another country, which had been appointed to check the typing results. Thus we had to send 5 per cent of our strains to Prague, Czechoslovakia, and we received 5 per cent of the material of an Italian typing laboratory (Genova). Strains giving discrepant results were sent to Italy for final examination. The results were forwarded on different report forms.

The survey was commenced on June 1, 1964, and closed on June 1, 1965.

Materials and methods

Streptococcal strains were collected in 11 different laboratories of the following institutes: Department of Bacteriology, National Institute of Public Health, Budapest; 8 different Regional Public Health and Infectious Disease Laboratories; Department of Microbiology, University Medical School, Debrecen; László Central Hospital for Infectious Diseases, Budapest. Fig. 1 indicates the geographical distribution of the collecting laboratories.

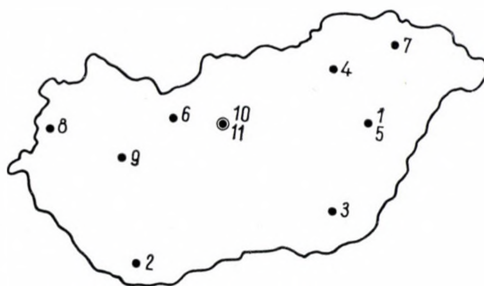

Fig. 1. Geographical distribution of collecting laboratories. 1 = Institute of Microbiology, Debrecen; 2 — 9: Regional Public Health and Infectious Disease Laboratories; 2 = Pécs; 3 = Békéscsaba; 4 = Miskolc; 5 = Debrecen; 6 = Tatabánya; 7 = Nyíregyháza; 8 = Szombathely; 9 = Veszprém; 10 = László Central Hospital, Budapest; 11 = National Institute of Public Health, Budapest

In order to distinguish A group strains from other beta haemolytic streptococci, the collecting laboratories were supplied with filter paper strips impregnated with bacitracin. All laboratories were asked to send monthly at least one *Str. pyogenes* strain from each group of diseases (scarlet fever, tonsillitis and other streptococcal infections), so that at least 30 strains could be submitted to typing. The cultures were sent on Loeffler serum or blood agar slants. All strains were checked for purity and bacitracin sensitivity. Serological grouping was performed with Lancefield extracts (which were also needed for *M* precipitation); in doubtful cases group determination was carried out also with Fuller extracts. Typing was performed by precipitation in anti-M and by agglutination in anti-T sera as described in previous papers [1, 2, 3]. The following sets of sera were used. *M* sera: *1, 2, 3, 5, 6, 8, 11, 12, 13, 14, 15, 17, 19, 22, 24, 25, 26, 29, 35, 36, 44, 46, 48* (total, 23); *R* sera: *3, 28* (total, 2); *T* sera: *1, 2, 3, 4, 5, 6, 8, 9, 11, 12, 13, 14, 17, 18, 19, 22, 23, 25, 27, 28, 44, 47,* Imp_{19}, B_{3264} (total, 24).

Antibiotic sensitivity of the strains was tested by use of penicillin and tetracycline paper discs.

In the order of arrival in our laboratory, every 20th strain was transferred to two Dorset slants then lyophilized in 5 ampoules. These strains were checked also with anti-T sera obtained from Britain and Czechoslovakia. Strains received for checking were similarly examined. The results were reported to London monthly.

Results

During the one-year survey period, 395 *Str. pyogenes* strains were collected and typed. Especially in summer, a difficulty was expected in obtaining the required number of cultures. However, with the exception of July when only 24 strains were collected, the number of strains received per month exceeded 30. Although not all laboratories succeeded each month in isolating at least one strain from all 3 disease groups, during the survey 101 strains were collected from scarlet fever, 150 strains from tonsillitis and 144 strains from other streptococcal diseases. The distribution of strains among the 3 disease groups varied from laboratory to laboratory. Thus among strains received from Laboratories 1 and 7 those originating from other streptococcal diseases predominated. In contrast, in Laboratory 5 these strains were encountered the least frequently. The low number of scarlet fever strains was striking in the material of Laboratories 9 and 11. Less effective culturing in scarlet fever was probably due to penicillin treatment which is often commenced prior to taking throat swabs for bacteriological examination. In view of the small number of collected strains, no conclusion can be drawn as to the association between monthly or regional distribution of strains and disease groups.

In the material of most collecting laboratories the complexes *3, 13,* B_{3264} and *5, 11, 12 . . .* predominated. In Laboratory 8 (Szombathely) type *3,* in Laboratory 4 (Miskolc) type *19* strains were relatively frequent (18 out of 48 and 9 out of 48 strains, respectively).

Type distribution of the 395 strains is presented in Table I, which indicates the number of strains determined by *T* agglutination, by *M*(*R*) precipitation and by both methods. By use of these techniques 99.2 per cent of the strains were determined serologically and only 3 strains (0.8 per cent) remained untypable. If each complex is regarded as one separate type, the 395 strains fell into 26 serotypes. By *M*(*R*) precipitation 46.3 per cent of the strains were typed. Only precipitation was used for the determination of types 19 and 36. By agglutination 89.5 per cent of the examined strains could be determined; on the basis of agglutination, types 2, 9, 18 and 22 were regarded as separate types.

Types occurring at a frequency higher than 5 per cent were arbitrarily regarded as prevalent organisms. Among these, the members of complex *5, 11, 12 . . .* were the commonest (19 per cent); streptococci belonging to complex *3, 13,* B_{3264} (17 per cent) and to types *3* (10 per cent), *12, 28* (7 per cent) and *1, 6* (5 per cent) were next in order. The prevalent types comprised 70 per cent of all strains (Table II).

Analysis of the association between streptococcal diseases and serotypes is presented in Table III.

It is seen that the prevalent types were most common in all disease groups. One third of type 1 strains and more than two thirds of type 19 strains

Table I

Type distribution of Str. pyogenes strains isolated between June 1, 1964 and June 1, 1965

Type	No. of strains determined by antigens			Total		Type	No. of strains determined by antigens			Total	
	M	*T*	*M + T*	No.	%		*M*	*T*	*M + T*	No.	%
1		9	12	21	5.3	*15, 17, 19...*		2		2	0.5
2		4		4	1.0	*17*			1	1	0.3
3M(3R)			38*	39	9.9	*18*		1		1	0.3
3R			2**	2	0.5	*19*	15			15	3.8
3, 13, B_{3264}		67		67	16.1	*22*		6	1	7	1.8
4, 24, 26...		15		15	3.8	*24*	1			1	0.3
5	7		7	14	3.5	*28R*			27**	27	6.8
5, 11, 12...		74		74	18.7	*29*	5		1	6	1.5
6	2	2	16	20	5.0	*36*	2			2	0.5
8			2	2	0.5	*46*			1	1	0.3
8, 25, Imp_{19}		17		17	4.3	*48*			1	1	0.3
9		3		3	0.8	Total typable	38	209	145	392	99.2
12			27	27	6.8	Untypable				3	0.8
14	2	9	6	17	4.3	Total				395	100.0
15	3		3	6	1.5						

* 6 strains precipitated also in serum *3R*.

** *R* antigen.

Table II

Percentage distribution of prevalent types in 1964—1965

Type	No. of strains	Per cent
5, 11, 12, 27, 44	74	19
3, 13, B_{3264}	67	17
3	41	10
12	27	7
28	27	7
1	21	5
6	20	5
Prevalent types, total	277	70
Other types	118	30
Total	395	100

Table III

Distribution of Str. pyogenes types according to disease groups

Type	Scarlet fever		Tonsillitis*		Other streptococcal infections**		Total	
	No.	%	No.	%	No.	%	No.	%
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
3, 13, B_{3264}	6	5.9	30	20.0	31	21.5	67	17.0
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...	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
12	8	7.9	14	9.3	5	3.5	27	6.8
14	5	4.9	4	2.7	8	5.6	17	4.3
15	—	—	4	2.7	2	1.4	6	1.5
15, 17, 19...	—	—	2	1.3	—	—	2	0.5
17	1	1.0	—	—	—	—	1	0.3
18	—	—	—	—	2	1.4	2	0.5
19	12	11.9	3	2.0	—	—	15	3.8
22	5	4.9	—	—	1	0.7	6	1.5
24	—	—	—	—	1	0.7	1	0.3
28R	3	3.0	15	10.0	9	6.2	27	6.8
29	4	4.0	1	0.7	1	0.7	2	0.5
36	1	1.0	—	—	1	0.7	2	0.5
46	—	—	1	0.7	—	—	1	0.3
48	—	—	1	0.7	—	—	1	0.3
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 and pharyngitis.

** Otitis, pyoderma, osteomyelitis, lymphadenitis, phlegmons, abscesses, burns, erysipelas, impetigo, pemphigoid, mastitis, septicaemia, rheumatic fever, nephritis.

were responsible for scarlet fever. Complex *4, 24 . . .* and type *28R* occurred frequently in tonsillitis (6.7 and 10.0 per cent, respectively). In some streptococcal infections, in addition to the prevalent types, type 1 and type 28 were frequent (for example in otitis). These data agree with known findings in that no definite association exists between streptococcal infections and the serotype of the causative agent. Nephritis, which is associated with certain types, is an exception.

Five per cent of the strains were sent for control typing to the Streptococcus Reference Laboratory, Institute of Epidemiology and Microbiology, Prague. The results of our examinations and of the repeated typings are summarized in Table IV.

Table IV

Control examination of 5 per cent of the collected strains

Designation of strains	Our results			Prague control**	Genova's re-control		
	M	*R*	*T*		*M*	*R*	*T*
20	—	—	*3, 13,* B_{3264}	*13*			
40	—	—	*5, 11, 12. . .*	*28*	—	—	*5, 11, 12, 28, 44*
60	—	—	*5, 11, 12. . .*	*28*	—	—	*5, 11, 12, 44*
80	*15*	—	*15(23)**	*23*			
100	—	—	*3, 13,* B_{3264}	*13,* B_{3264}			
120	*6*	—	*6*	*6(6M)****			
140	—	—	*5, 11, 12. . .*	*12*			
160	—	—	*5, 11, 12. . .*	*28*	—	—	*5, 11, 12, 28, 44*
180	*3*	—	*3*	*3, 13,* B_{3264}*(M3)*			
200	—	—	*22*	*22*			
220	*3*	—	*3*	*3, 13,* B_{3264}*(M3)*			
240	—	—	*3, 13,* B_{3264}	*13,* B_{3264}			
260	—	—	*3, 13,* B_{3264}	*13*			
280	—	—	*5, 11, 12. . .*	*28*	—	—	*5, 11, 12, 27, 28, 44*
300	*3*	—	*3*	*3, 13,* B_{3264}			
320	—	*28*	*28*	*4*			
340	—	*28*	*28*	*4, 28*			
360	*5*	—	*5*	*5, 27, 44(M5)*			
380	*1*	—	*1*	*1(M1)*			

* Agglutination in serum *T23*; no serum *T15* was available.

** Report forms other than the accepted were used.

*** Bracketed figures mean precipitation, others agglutination.

Four out of 19 strains gave discrepant results (strains No. 40, 60, 160 and 280). These strains according to the Prague laboratory agglutinated in serum 28, while our own examinations revealed them to be members of complex *5, 11, 12...* Neither examinations revealed any reaction in precipitating sera. Conforming to the requirements of the survey, these strains were forwarded to Genova for final checking. These results are shown in the righthand columns of Table IV.

Other differences between the first and control examination were not important. The results can be regarded as identical, save that the strain reacted in different sera within the same *T* complex (*e.g.* 20, 140, etc.), or sometimes one laboratory showed only a positive agglutination test, while the other obtained an additional positive precipitation and was therefore able not only to include the strain in a complex but also to distinguish it by serotype (*e.g.* 80, 300 etc.).

Our tasks included the checking of strains typed in the Institute of Microbiology, University Medical School, Genova. The results are shown in Table V.

Table V

Checking of strains received from Genova

Designation of strains		Result of typing					
		Genova			Budapest		
Typing laboratory (Genova)	Checking laboratory (Budapest)	*M*	*R*	*T*	*M*	*R*	*T*
8	1	—	—	*5, 12, 27, 44*	—	—	*5, 11, 12...*
18	2	*6*	—	*6*	*6*	—	—
40	3	*2*	—	*2*	*2*	—	*2*
60	4	—	—	*12*	—	—	*5, 11, 12...*
80	5	*18*	—	—	Untypable		
100	6	*12*	—	*12*	*12*	—	*12*
102	7	Untypable			—	—	*5, 11, 12...*
118	8	Untypable			—	—	*14*
120	9	*1*	—	*1*	*1*	—	*1*
117	10	Untypable			*1*	—	*1*

There was no difference between the results regarding untypable strains. Strain 80 (5) was reported as untypable by our laboratory; strains 102 (7), 118 (8) and 117 (10), in turn, could not be determined in the Genova typing centre.

Finally, although it was not regarded as a function of the survey, all strains were tested for penicillin and tetracycline sensitivity. All the 395 strains were sensitive to penicillin; 346 (87.6 per cent) were sensitive, 28 (7.1 per cent) moderately sensitive, and 21 (5.3 per cent) resistant, to tetracycline.

Discussion

As no data are available for the type distribution of streptococci in other countries in the years 1964—65, it seems advisable to compare our results with earlier findings and with data presented at the International Streptococcus Symposium, Jena, 1963.

In our opinion the results obtained with strains isolated in 11 separate collecting laboratories have given a true picture of the distribution of serotypes prevalent in Hungary. It can be concluded that two complexes (*3, 13* . . . and *5, 11* . . .) occurred relatively frequently. Because of the small number of strains, no answer can be given to the question whether certain types are really characteristic of certain regions of the country. Examinations carried out in the years 1958—1963, when strains were collected in 19 different laboratories, yielded similarly no conclusive data as to the distribution of types according to counties. Some differences were revealed when Western regions of Hungary were compared with the Budapest area (a predominance of complex *5, 11, 12* versus complex *8, 25,* Imp_{19}). Comparison of Eastern regions with the above areas showed, however, no significant difference in the incidence of prevalent types.

Our typing method has improved in efficacy as compared to earlier results yielding 7.5 per cent untypable strains in the years 1958—1963, since in the present work only 0.8 per cent of the strains remained undetermined. This was primarily due to the fact that in repeated experiments we succeeded in preparing homogeneous suspensions from many "untypable" strains and thus rendered them suitable for agglutination. Among PARKER's [4] 6279 strains 1 per cent, among KÖHLER's [5] strains 7.5 per cent and among ROTTA's [6] 1148 strains 1.3 per cent untypable cultures occurred. The percentage of our strains determined by *M* precipitation was similar to that indicated in our previous study. The 395 strains examined in the present work fell into 26 types. Types *8, 17* and *48* were first found in the course of this survey. Types *11, 13, 26, 33, 44* and *47* that had been identified in this country previously, were not encountered among the examined strains. As compared to previous examinations [2], in the years 1964—65 complexes *5, 11, 12* and *3, 13* . . . and type *3* were relatively more frequent, while types *12* and *1* were less commonly met with. KÖHLER in 1963 found complex *4, 24* in 11.6, type *12* in 16, type *22* in 9.5, and type *28* in 5.6 per cent. In May, 1962, PARKER found that the incidence of complex *5, 11, 12* . . . , type *12* and complex *3, 13* exceeded 5 per cent.

In the 1960—63 study of ROTTA the distribution of predominating types and complexes was similar to that revealed in our material.

Our data have confirmed that, with the exception of nephritis, the streptococcal diseases are not associated with particular serotypes. This was shown also by KÖHLER [5], who found that the predominant types causing scarlet fever in the German Democratic Republic varied from year to year: in 1960 type *6*, in 1961 type *14*, and in 1963—64 type *22*, were most the frequently involved. In addition to the mentioned prevalent types, ROTTA [6] found that types *30*, *9* and *33* occurred more frequently in scarlet fever than in tonsillitis. Thus it is obvious that the same streptococcal disease may be due to several types of *Str. pyogenes*.

One of the aims of the *Str. pyogenes* type distribution survey in 12 different countries has been to stimulate the contact between laboratories and to coordinate their typing methods. Four out of our 19 strains sent for checking examination to Prague gave discrepant results. These strains were then sent to Genova for further control. According to the latter laboratory the 4 strains agglutinated in the same sera in which we had obtained reaction. However, 3 of these strains agglutinated in Genova also with serum 28; thus the examination of these strains cannot be considered complete. Precipitation tests gave negative results in all three laboratories.

It should be noted that the discrepancy between two laboratories in *M* or *T* antigen determination may be due to several reasons. The laboratory carrying out the first typing may not possess all sera used in the laboratory performing the checking examination. For example, strain No. 80, which has been identified in Genova as $M18T^{-}$, was included in our report as an untypable culture, because it was devoid of a detectable *T* antigen and we had no anti-*M 18* precipitating serum. Sometimes differences in the titre of sera may cause discrepancies: for example, complex *3, 13*, B_{3264} was not unfrequently met with, while type *13* never occurred among our strains. Our serum *M 13* might have been unsuitable for the determination of type *13* strains containing small amounts of *M* antigen. Therefore on the basis of agglutination this type could be included only in the complex, as we had no absorbed anti-*T 13* agglutinating serum which did not react with strains containing antigens *3* and B_{3264}. Finally, the results may differ when some alteration affecting mainly the *M* antigen of the strain occurs between the two examinations.

Antibiotic sensitivity testing revealed that in streptococcal diseases penicillin is still the most effective drug. An increase in the incidence of tetracycline-resistant *Str. pyogenes* strains has recently been noted by some authors [7, 8]. Our pertaining observations will be reported in a subsequent paper.

The results obtained in the 12 participating countries will be summarized, evaluated and published by the International Subcommittee on Streptococci and Pneumococci. We have been glad to take part in the survey and are ready

to undertake further similar tasks since in our opinion such international collaboration may successfully improve the elaboration of uniform methods as well as the evaluation of results, and thus the fight against streptococcal infections all over the world.

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CHROMATOGRAPHY OF POLIOVIRUS STRAINS ISOLATED IN HUNGARY PRIOR TO AND AFTER THE INTRODUCTION OF LIVE POLIOVIRUS VACCINE

By

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Summary. Chromatographic behaviour of 16 type 3 poliovirus strains was examined on $Al(OH)_3$ and DEAE cellulose. Part of the strains was isolated prior, and part after, the introduction of live poliovirus vaccine. The former were eluted from a DEAE cellulose column by 0.05 to 0.25 *M* NaCl, while the latter had elution maxima similar to the reference strain (Leon $12a_1b$), being eluted at 0.035 to 0.05 *M*. Both groups of strains exhibited an essentially identical behaviour on $Al(OH)_3$ column. The relations between the chromatographic characters and the rct/40 marker have been studied as well as the possible aetiological role of the strains isolated from cases suspect for poliomyelitis during the vaccination campaign.

THOMSSEN *et al.* [1, 2, 3, 4] have published several papers dealing with the characterization of poliovirus strains by chromatography on $Al(OH)_3$ gel, resp. DEAE cellulose. Characteristic and well reproducible differences have been found among the prototype strains, the wild and the vaccinal strains. Thus, this method seemed to be practicable for the intra-typic characterization of poliovirus strains.

The Department of Virology of the National Institute of Public Health, Budapest, supplied type 3 poliovirus strains for our studies. Part of the strains were isolated in 1959, prior to the regular performance of vaccination campaigns with live attenuated poliovirus. The other strains were isolated in 1960 and later, *i.e.* following the introduction of regular vaccinations. This paper is a report on the chromatographic characterization of the above virus strains.

Materials and methods

Virus strains. The prototype strains (Mahoney, LSc 2 ab, MEF_1, P712 Ch 2ab, Saukett, Leon $12a_1b$) were maintained in this laboratory in primary rhesus monkey kidney cell cultures. The attenuated strains used in the experiment underwent at most two passages in this laboratory.

All the other strains were obtained from the Department of Virology of the National Institute of Public Health, Budapest, and all belonged to serotype 3. The individual strains were specified as follows: strain No. 158/58 had been isolated in 1958 from a child hospitalized with atypical poliomyelitis. Of the strains isolated in 1959, No. 2230 had originated from the faeces of a healthy child in a survey; Nos 2728, 2907 and 2912, from patients hospitalized with atypical signs; Nos 1918, 1922 and 1958 had originated from patients with abortive poliomyelitis, No. 1964 from a faecal sample of a patient with typical poliomyelitis. All the above strains had been isolated prior to the introduction of regular vaccinations with live attenuated polio-vaccine.

Strains isolated in 1960 and later, after the introduction of vaccination, originated from patients with a disease diagnosed clinically as poliomyelitis. Strain No. 448 was isolated late in January, 1960, whereas strains Nos 516, 840, 856 and 1198 in February of the same year. Thus, all these strains were obtained soon after vaccination with type 3 vaccine in late January. Strain No. 145 was isolated in February, 1961, soon after vaccination with type 3. The patient suffered from typical poliomyelitis. Strain R/86/1965 was isolated from a patient with aseptic meningitis developed after the vaccination campaign. All the strains specified above had undergone 3—4 monkey kidney passages by the time of examination.

Preparation of the strains for chromatography. The methods published by THOMSSEN *et al.* [2] have been followed with the modification that instead of ^{32}P assay the virus was quantitated by plaque counting. Virus material for chromatography was produced in primary rhesus monkey kidney cell cultures. After complete destruction of the cells, the virus was released by freezing and thawing. The material was centrifuged for 30 minutes at 15.000 r. p. m. at 5 °C. Part of the supernatant was further processed for adsorption experiments on $Al(OH)_3$ gel. Viruses adsorbed to $Al(OH)_3$ gel were eluted with a 0.5 *M* phosphate buffer (pH 7.5). The yield on elution was about 80 per cent [2]. The eluate was dialyzed overnight at +4 °C against a mixture of equal volumes of Hanks' solution and distilled water. This was followed by filtration through a DEAE cellulose column. Supernatants used for chromatography on DEAE cellulose were not processed except for being dialysed against 0.02 *M* phosphate buffer.

Adsorption to and elution from $Al(OH)_3$ gel. Virus material partially purified as described above was diluted 1 : 5 in 0.1 *M* pH 5.5 acetate buffer. Care was taken not to reduce the titre below 10^6 CPD_{50}/ml on dilution. Two ml aliquots of this diluted virus suspension were thoroughly mixed with 0.5 ml of $Al(OH)_3$ gel diluted 1 : 6 in distilled water. Six parallel experiments were usually performed. Adsorption was allowed to take place for 30 minutes at room temperature. This was followed by centrifugation. The supernatant was discarded. To these sediments increasing molarities of pH 7.5 phosphate buffer were added in a total volume of 3 ml each. For elution 15 minutes were allowed at room temperature. The amount of virus eluted was determined by plaque counting from the supernatant obtained after centrifugation. The amount of virus eluted with 0.32 *M* phosphate buffer was regarded as 100 per cent. Elutions of the virus at lower molarities of phosphate buffer were expressed in percentual terms. The chromatographic character of the virus was specified as the molarity of phosphate buffer required for the elution of 50 per cent of virions (ED_{50}—V).

Chromatography on DEAE cellulose [4]. DEAE cellulose (MN 2100, capacity about 0.7 meq/g) was washed five times with 0.02 *M*, pH 7.5 phosphate buffer. Columns 1 cm in diameter and 6 cm high were prepared from the pretreated adsorbent. Two ml of virus was allowed to flow through the column (fraction E_0). The column was then washed five times with 2 ml of 0.02 *M* phosphate buffer each (E_{1-5} fractions). Elution fluids contained increasing amounts of sodium chloride (from 0.025 *M* to 0.6 *M*) in 0.02 *M* phosphate buffer (E_{6-13} fractions). Each fraction was titrated for virus content and the conductivity determined and expressed in terms of sodium chloride molarity. The virus strain was characterized by the sodium chloride molarity measured in the fraction exhibiting the highest virus titre (E—$NaCl_{grad}$)

Rct/40 marker [5]. Titrations of viruses were performed in duplicate. One was incubated at 36 °C, the other at 40 °C, each for 7 days. A specific thermoregulator was used to provide constant temperatures and the possible fluctuations were registered by means of a thermograph.

Calculation of rct/40 quotient [6]. The difference of logarithms of virus titres obtained at 36 and 40 °C was divided by the same difference observed with the reference strain (Leon $12a_1b$). The quotients thus obtained were related to the rct/40 marker as follows: $\geqslant 0.75 =$ $=$ rct/40^-, between 0.3 and 0.75 = rct/40 $\pm$, $\leqslant 0.3$ = rct/40^+.

Results

Chromatographic characters of the strains examined are given in Fig. 1 in ED_{50}—V and E—$NaCl_{grad}$ values. The same data for type 1 and 2 laboratory strains have been included in the Figure together with the type 3 strains studied.

The dots represent the intersection points of lines drawn at right angles to ED_{50}—V values on the abscisse and the E—$NaCl_{grad}$ values on the ordinate. Each point was determined in at least two experiments. The virus materials

used for chromatography in the subsequent assays were obtained from two subsequent passages. As shown in Fig. 1, the ED_{50}—V values of type 3 strains isolated from patients were within the range of 0.04 to 0.08, similarly to the reference attenuated strain Leon $12a_1b$. The E—$NaCl_{grad}$ values of strains isolated in 1959 were in all cases but one shifted to higher NaCl molarities than did the reference strain. Strains isolated in 1960 and later were all but

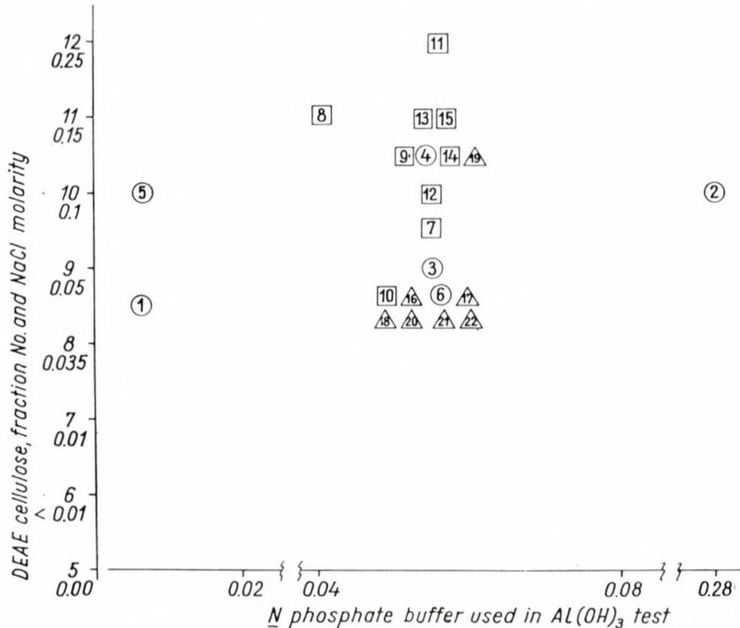

Fig. 1. Chromatographic characters of different poliovirus strains on $Al(OH)_3$ gel and DEAE cellulose column

Type 1 strains: 1 = Mahoney; 2 = LSc 2ab
Type 2 strains: 3 = MEF_1; 4 = P 712 Ch 2ab
Type 3 strains: 5 = Saukett; 6 = Leon $12a_1b$; 7 = 159/1958; 8 = 1918/1959; 9 = 1922/1959; 10 = 1958/1959; 11 = 1964/1959; 12 = 2230/1959; 13 = 2728/1959; 14 = 2907/1959; 15 = 2912/1959; 16 = 448/1960; 17 = 516/1960; 18 = 840/1960; 19 = 856/1960; 20 = 1198/1960; 21 = 145/1961; 22 = R/86/1965.

○ Prototype strains; □ strains isolated prior to; △ strains isolated after the introduction of live polio vaccine

one identical with the attenuated reference strain. It should be noted that of the assays of three subsequent passages of strain No. 1964 isolated in 1959, the last one showed two well distinguishable elution peaks in the fractions 8 and 12, although the higher peak was still present in fraction 12.

The chromatographic characters of the strains were compared with their rct/40 markers. Results are shown in Table I.

As shown in Table I, no correlation was detectable between the rct/40 marker and the chromatographic characters.

Table I

Relation of the ED_{50}–V and E–$NaCl_{grad}$ characters of type 3 poliovirus strains to their rct/40 markers

No.	Designation of strains	rct/40 quotient	ED_{50}—V	E—$NaCl_{grad}$.
1.	159/1958	0.28 +	0.04—0.08	0.05—0.1 +*
2.	1918/1959	0.46 ±	0.04—0.08	0.17 +
3.	1922/1959	0.07 +	0.04—0.08	0.10—0.17 +
4.	1958/1959	0.07 +	0.04—0.08	0.035—0.05 —
5.	1964/1959	0.14 +	0.04—0.08	0.25 +
6.	2230/1959	0.36 ±	0.04—0.08	0.10 +
7.	2728/1959	0.50 ±	0.04—0.08	0.17 +
8.	2907/1959	0.50 ±	0.04—0.08	0.10—0.17 +
9.	2912/1959	0.64 ±	0.04—0.08	0.17 +
10.	448/1960	0.28 +	0.04—0.08	0.035—0.05 —
11.	516/1960	0.50 ±	0.04—0.08	0.035—0.05 —
12.	840/1960	0.21 +	0.04—0.08	0.035—0.05 —
13.	856/1960	0.42 ±	0.04—0.08	0.1—0.17 +
14.	1198/1960	0.40 ±	0.04—0.08	0.035—0.05 —
15.	145/1961	0.14 +	0.04—0.08	0.035—0.05 —
16.	R/86/1965	0.50 ±	0.04—0.08	0.035—0.05 —
17.	Saukett	0.00 +	<0.02	0.1 +
18.	Leon $12a_1b$	1.00 —	0.04—0.08	0.035—0.05 —

* Limit values measured in different experiments

E—$NaCl_{grad}$ — = <0.05

E—$NaCl_{grad}$ + = >0.05

Discussion

Differences in the chromatographic character of the individual virus strains appeared to have resulted from certain slight differences in the fine structures of their capsides. The actual condition of the superficial active groups which determine the conditions of adsorption is largely influenced by the suspending medium. In spite of the great importance of the possible active groups on the virion's surface, there has been but one single exact analysis in the case of influenza virus [10]. Similar studies of other viruses would be highly desirable.

Precise standardization of the substances used in chromatographic and adsorption experiments is of great importance. Remarkable differences may be caused by using non-uniformly sensitized $Al(OH)_3$ gel preparations or

different batches of DEAE cellulose. According to THOMSSEN *et al.* [4], elution maxima of the prototype strains were demonstrable in the fractions containing less than 0.01 M NaCl. In our hands, the same viruses had elution maxima in fractions with 0.035 to 0.1 M NaCl, the relative differences having been exactly the same as found by the cited authors. This difference may be explained beside methodical discrepancies by the use of different batches of the adsorbent. Therefore, intra-typic differences should be considered in relative terms, the elution maxima being compared with a reference standard. Absolute values should only be considered if rigorously standardized conditions can be provided for.

The chromatographic character of the strains isolated in Hungary prior to the vaccination campaign differed markedly from that of the vaccinal strain. On the contrary, the strains isolated in 1960 and later showed characters identical with the vaccinal strain. These results suggest that the wild type 3 strains that had been in circulation in Hungary prior to the vaccination may be differentiated from the vaccinal strain by chromatography. Thus, the method appears to be useful in aetiological studies of poliomyelitis suspect cases during the vaccination campaign. Nevertheless, we do not claim to have excluded the possibility of existence of wild type 3 strains having chromatographic characters very different from, or very similar to, the vaccinal strain.

There are several explanations for the possible aetiological relation of the strains isolated since the introduction of vaccination with live virus and the diseases clinically diagnosed as poliomyelitis. These might have been diseases clinically resembling poliomyelitis, but with a different aetiology. Thus, the poliovirus strain isolated from faeces and being identical chromatographically with the vaccinal strain had not been the causative agent. It cannot, however, be excluded that these cases had been caused by some modified progeny of the type 3 vaccinal strain, particularly, as these cases occurred in the winter months soon after the vaccination with the respective strain. Increased neurovirulence or some other changes supposed to have occurred in the isolated strains, did not alter their chromatographic characters. A highly probable supposition is the causative role of a wild type 3 virus which infected the organism prior to vaccination and had been suppressed in the intestines by the vaccinal strain by the time of the clinical manifestations. It should, however, be noted that since autumn, 1960, no wild poliovirus strains have been found in circulation. An extensive survey performed late in 1960 [7] failed to reveal circulating virus and except for the vaccination period, no poliovirus was detectable either from healthy or from ill persons.

As to the $E-NaCl_{grad}$ character, the results of MASIOVA and AGOL [8] should be considered. These authors have demonstrated the dependence of the E^+ and E^- phenotype on the cystine content of the medium. Thus, chromatography in itself does not suffice for the definitive characterization of

a strain. It may be helpful, though, if considered together with the other markers. The examination of the two chromatographic parameters appeared to be a practicable tool for intra-typic differentiation of enteroviruses. ECHO prototype strains, for instance, may be further differentiated according to their E—NaCl characters [9].

Chromatography seems to be a method which, after some improvement, will serve well for the demonstration of slight differences in the surface groups of the individual virions. It also seems to offer a tool for the isolation, identification and detailed study of the minutely different virions. Thus, the perspectives of chromatographic analysis appear to be promising in both fundamental and applied virology.

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BREAKDOWN OF AMINO ACIDS BY ENTEROBACTERIACEAE

VI. BETA-ALANINE MACRO- AND MICROTEST

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Summary. Among members of the family Enterobacteriaceae, part of Serratia, Enterobacter, Arizona and Klebsiella strains caused alkalization in beta-alanine medium. The reaction can be used for the differentiation of biotypes within these genera. Alkalization is probably due to endogenous oxidation and not to the breakdown of beta-alanine.

Members of the family Enterobacteriaceae generally attack glycine [6, 7], but are inactive against the simplest beta-amino carboxylic acids.

Materials and methods

Culture media were prepared as described for glycine in references 6 and 7. As the action on beta-alanine which does not react with ninhydrin, was detected by the alkalization test, only two media were used. The buffered solution contained distilled water, 100 ml; beta-alanine, 0.20 g; KH_2PO_4, 0.10 g; K_2HPO_4, 0.05 g; freshly prepared phenol-red indicator (phenolsulphonphthalein, 0.01 g; *N* NaOH, 0.40 ml; distilled water, 4.6 ml), 2.5 ml; pH 5.6. The other (simple) medium contained the same ingredients except K_2HPO_4 at pH 5.4. Beta-alanine was omitted from media serving for the examination of endogenous oxidation. The method has been described previously [6, 7].

Results

Tubes containing the above media were seeded with 957 different Enterobacteriaceae strains using two and three loopful amounts of cultures. Table I shows the results obtained in tubes inoculated with two loopful amounts.

(1) Alkalization was observed only in Klebsiella, Enterobacter, Serratia and Arizona; a considerable part of strains belonging to these genera, however, gave negative results.

(2) Alkalization due to endogenous oxidation and beta-alanine splitting was revealed generally within the same groups of bacteria. The reaction in beta-alanine solution was given by higher number of strains and was more intensive.

(3) Positive reactions were less frequent and less intensive when the micro-test was used.

When beta-alanine in simple phosphate solution was used, or the tubes were inoculated with 3 loopful amounts of cultures, the number of alkalizing strains and the intensity of the reaction increased.

Table I

Positive alkalization reactions in beta-alanine medium and in phosphate buffer

	No. of strains	Macrotest					Microtest				
		—	(+)	+	Among positives		—	(+)	+	Among positives	
					(++)	++				(++)	++
(a) *In beta-alanine*											
Klebsiella	67	60	5	2	—	—	65	2	—	—	—
Enterobacter	34	21	8	5	2	1	30	4	—	—	—
Serratia	48	8	11	29	17	2	14	18	16	13	1
Arizona	19	13	6	—	—	—	19	—	—	—	—
(b) *In phosphate buffer*											
Klebsiella	67	62	5	—	—	—	66	1	—	—	—
Enterobacter	34	29	3	1	—	—	32	2	—	—	—
Serratia	48	28	15	5	—	—	35	13	—	—	—
Arizona	19	18	1	—	—	—	19	—	—	—	—

Key. Macrotest:
- — = No red coloration within 7 days
- (+) = Red coloration within 4—7 days
- \+ = Red coloration within 3 days
- (++) = Intensive red coloration within 4—7 days
- ++ = Intensive red coloration within 3 days

Microtest:
- — = No red coloration within 24 hours
- (+) = Red coloration within 5—24 hours
- \+ = Red coloration within 4 hours
- (++) = Intensive red coloration within 5—24 hours
- ++ = Intensive red coloration within 4 hours

Other Enterobacteriaceae (140 *E. coli*, 24 Citrobacter, 216 Salmonella, 244 Shigella, 161 Proteus-Providencia, 4 Hafnia strains) gave uniformly negative results with both micro and macro-test.

Discussion

Our findings indicate that alkalization in beta-alanine solution is suitable for differentiation, as strains reacting positively should belong to the Serratia, Enterobacter, Arizona or Klebsiella groups. Within these groups beta-alanine negative and positive biotypes can be distinguished.

Bacterial enzymes acting on beta-alanine cause either an oxidative breakdown of the amino acid or a transamination into L-alpha-alanine. ERLANDSON and RUHL [2] who examined the effect of a *Sh. flexneri* 3 culture on amino acids by the Warburg method, observed that the strain neither oxidized beta-alanine nor produced ammonia from it. MEYER and CAMERON's [5] manometric experiments with brucellae and related organisms showed that *Brucella melitensis*, *Moraxella bovis* and *Bordetella bronchiseptica* exerted a slight oxidative action on beta-alanine. HAYASHI *et al.* [4] prepared a purified transaminase from a *Pseudomonas fluorescens* culture, which catalysed the transamination between beta-alanine and pyruvic acid and thus produced malonylsemialdehyde and L-alpha-alanine. DURHAM *et al.* [1] prepared an aminotransferase-containing extract from a Flavobacterium species which, when incubated in the presence of pyruvate, beta-alanine and pyridoxal phosphate, produced L-alpha-alanine.

Under our experimental conditions transamination could be excluded because of the absence of an amino acceptor (and of pyridoxal phosphate). The alkaline product (ammonia) was the result of either an oxidative deamination of beta-alanine, or an endogenous oxidation. Generally, the same strains gave positive reactions in beta-alanine-containing and in the control amino acid-free medium. Alkalization in beta-alanine solution probably occurs only as a result of endogenous oxidation, but beta-alanine exerts a catalytic action on this process. GALE [3] assumed a similar catalytic activity to take place in connection with the oxidation of amines.

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TYPING OF BRUCELLA WITH HUNGARIAN PHAGE STRAINS

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Summary. With 6 phages isolated from natural sources and 4 phages obtained from Brucella cultures, 117 *B. abortus*, 44 *B. suis* and 26 *B. melitensis* strains have been typed. Phage suspensions at RTD and 10,000 × RTD lysed abortus strains in 94.8, suis strains in 11.4, and melitensis strains in 19.3 per cent. Only 10,000 × RTD lysed 2.6 per cent of abortus strains and 61.4 per cent of suis strains. To 10,000 × RTD abortus strains were resistant in 2.6, suis strains in 27.2, and melitensis strains in 80.7 per cent. In lytic activity, phages isolated in Hungary were similar to phages received from other countries. When the source of the examined strain is known, the key elaborated in this study allows differentiation of the 3 Brucella species. For the identification of strains with irregular phage sensitivity, the conventional typing methods should be employed.

As the conventional biochemical, biological and serological methods used for the differentiation of the 3 Brucella species are based mainly on the detection of quantitative differences, several atypical strains are encountered. Since at least 6 properties should be observed for a proper identification, the conventional method is time-consuming and laborious.

In view of the promising data concerning the identification of Brucella species by means of phages, it seemed desirable to perform classification studies with our 10 phage strains isolated in the course of a previous investigation [9]. Of the 10 phage strains 6 were isolated from the gastric contents of aborted cattle and swine foetuses, from the urine of a cow with contagious abortion, from abattoir sewage and from liquid manure of infected cattle and pigs; 3 phages were isolated from Hungarian *B. abortus* strains and 1 phage was released by a suis-like Brucella strain isolated in Roumania from a hare.

The specificity of Brucella phages has been noted by several authors [3, 6, 8, 13]. Considerable work has been devoted to elucidate the suitability of these phages for the differentiation of Brucella species [2–4, 7, 8, 12–15, 17–19]. The problem has also been dealt with at the Meeting of the Subcommittee on Taxonomy of the Genus Brucella of the International Committee on Bacteriological Nomenclature, Montreal, 1962. On the basis of several reports, the internationally accepted views on brucella-phages have been summarized by Stableforth and Jones [1] as follows.

Non-polyvalent brucella-phages lyse the classical and dye-sensitive *B. abortus* types. They act also on strains exhibiting the cultural and bio-

chemical properties of *B. abortus* and the serological properties of *B. melitensis*. *B. suis* and mediterranian *B. melitensis* strains are resistant to these phages. Some phage-sensitive melitensis-like strains including cultures isolated in England where goats and sheep are free from melitensis infection, were recovered from cows. On the other hand, strains isolated recently in Malta from cows, were resistant to these phages. Thus it can be concluded that Maltese cows acquired characteristic melitensis strains from goats. From MEYER's oxidative metabolic tests it is evident that the phages are able to lyse only those strains which yield metabolically characteristic *B. abortus* cultures. Strains isolated from cows and identified by the classical methods as *B. melitensis*, can be divided by the phage test into two groups: phage sensitive cultures with abortus, and phage resistant cultures with melitensis, metabolic pattern. In lytic activity and serological behaviour, phage "Tb" and PARNAS' phages 10/I, 24/II, 374/XXIX and 212/XV are identical.

None of the 22 *B. suis* cultures were lysed by the RTD, but with 10,000 × × RTD many of them showed characteristic lysis. In contrast, *B. melitensis* strains were resistant to undiluted phage suspensions. In the opinion of the Subcommittee, the problem of lysis by 10,000 × RTD of *B. suis* strains needs further investigation, as the reaction caused by the undiluted suspension may be confused with pseudolysis. It has been emphasized that only S or SI phase strains should be used for the phage test. Provided that such cultures are employed, the metabolic and phage-typing results are in agreement. The extensive use of phage-typing is limited by the lack of specific *B. suis* and *B. melitensis* phages. Thus it is difficult to assume that phage-typing is valuable in the definition of Brucella species. According to the Subcommittee, it is not yet possible to prepare a complete key for the determination of these organisms. Such a key, however, would be advantageous in routine identification. The phage test is simple and can easily be included among the routine methods, as propagation of phages can be standardized, the phage suspensions may be storable for long periods of time without loss in titre, and the typing results are easily read and interpreted.

Materials and methods

Brucella strains and identification tests. Partly Hungarian, partly foreign cultures including 117 *B. abortus*, 44 *B. suis* and 26 *B. melitensis* strains were examined. The conventional methods used for species determination were as follows. Growth in the presence of 1 : 50,000 thionine and 1 : 100,000 basic fuchsin on bromthymol blue (1 : 15,000) agar; H_2S production in Huddleson agar; catalase activity with NYIREDY's rapid test [11]; and urease activity with HUDDLESON's test [5]. Antigenic structure of the cultures was examined with monospecific abortus and melitensis sera. As only S forms are suitable for phage typing, all strains were tested with BURNET's thermoagglutination method. Cultures showing traces of agglutination were excluded.

Propagation of phages. The 10 phage strains were propagated on strain B19 using DROZEWKINA's method [3]. Phage suspensions at 1.2—1.5 ml and 5 hour Martin broth cultures at 0.6—0.8 ml aliquots were pipetted into 50 ml pH 7.2 Martin broth. The inoculated media

were kept at 4 °C for 4—6 hours, then incubated at 37 °C for 14—16 hours. During incubation the cultures were shaken several times. Bacterial cells were then removed by filtration through G5 glass filters. Propagation was repeated until a titre of 10^9 had been reached.

Phage titration was performed by ADAMS' double agar layer technique [1]. Strain B19 was cultured in 5 ml Martin broth for 6—12 hours, then 0.9 ml of the culture and 0.1 ml of each of 1 : 10—1 : 10,000,000 Martin broth dilutions of the phage suspension were mixed with 3 ml aliquots of 0.7 per cent agar cooled to 46 °C. The mixtures were layered over agar plates poured and dried previously. When the seed-layer hardened, the plates were incubated at 37 °C for 24—48 hours. The phage titre was determined by multiplying the number of plaques by the corresponding dilution.

Lytic activity was determined as recommended by the Brucella Subcommittee, with two phage concentrations. For the routine test dilution (RTD) the concentrated phage suspensions were diluted 10^{-4} with Martin broth; the undiluted phage suspension itself was designated 10,000 × RTD. The examined strain was suspended in Martin broth to contain approximately 500 million cells per ml, then a 1 ml aliquot of the suspension was distributed evenly on the surface of a well-dried agar plate. After removing the excess suspension, the plate was dried at 37 °C for 1—2 hours. Then, to separate the phages, the sterilized end of a cork borer 10 mm in diameter was sunk into the agar at 8—10 different sites. The circular fields obtained in this manner were seeded by means of a calibrated platinum loop with 0.02 ml amounts of RTD and 10,000 × RTD phage suspensions. Sterile Martin broth placed on one circular field of each plate served as the control. Plates inoculated with *B. abortus* were incubated in the presence of 10 per cent CO_2; *B. suis* and *B. melitensis* were incubated in normal atmosphere. All strains were examined on 3 parallel plates. Readings were made after 24 and after 48 hours' incubation at 37 °C.

The 10 phages used in these experiments were compared with phages isolated by other authors, 10/I, 212/XV, 371/XXIX (PARNAS), "Tb" (U.S.S.R.) and F25U (VAN DRIMMELEN).

Results

All phage strains were active against 111 (94.8 per cent) out of 117 *B. abortus* strains at both RTD and 10,000 × RTD concentrations. Three strains (2.6 per cent) were lysed by 10,000 × RTD only. No phage suspension reacted with 3 (2.6 per cent) strains.

Both phage suspension concentrations lysed 5 (11.4 per cent) suis-like strains. As regards phage sensitivity, these cultures were accordingly classified as abortus strains. However, in view of their high catalase activity and urease production characteristic of suis strains, these cultures could not be included in the abortus group. The majority of suis strains (27 out of 44, 61.4 per cent) were lysed by 10,000 × RTD phage suspensions. Twelve strains (27.2 per cent) were resistant to undiluted phages.

With both diluted and undiluted phage suspensions 5 (19.3 per cent) out of 26 melitensis strains showed lysis. Four of these cultures originated from Italy, 1 of them, which had been isolated from sheep, was a Polish strain. According to the standpoint of the Brucella Subcommittee as to the biological characters of abortus strains, the 4 Italian strains belonged to type 5, the Polish strain to type 7, of *B. abortus*. The remaining 21 melitensis strains (80.7 per cent) were resistant even to undiluted phages.

The 5 foreign phage strains were identical in lytic activity with our phages.

Discussion

It has been shown that, similarly to phages isolated in other countries, Hungarian phage strains are usually active against *B. abortus* cultures. The number of *B. suis* and especially *B. melitensis* strains lysed by these phages is considerably smaller. This finding is indicative of the identity of all Hungarian and foreign phages. This has been confirmed by previous studies showing that there was no difference among our phages as to adsorption value, adsorption rate constant, latent and active multiplication period, phage yields per infected cell, neutralization rate constant, and sensitivity to physical effects [10].

From the finding that the 10 Hungarian as well as the internationally recognized 5 foreign phages lysed mainly *B. abortus* strains, it has been concluded that, at least for the time being, we have only one brucella phage which is specific for the abortus group. This has been supported by the observations of foreign authors.

From previous results it would appear that there is no specific suis phage, or, at least, it has so far not been encountered, because our phages isolated from the gastric contents of a swine foetus and from the liquid manure of pigs as well as phage 212/XV obtained by PARNAS from a *B. suis* culture were not more active against suis strains than phages originating from cattle [10]. That the phage strain isolated from the gastric contents and from suis culture is not specific for *B. suis*, is also indicated by the fact that these phages propagate much better on abortus than on suis strains. Moreover, antiphage sera prepared with phages originating from cattle neutralized "suis" phages in a similar degree as bovine phages.

On the basis of the present findings a key has been constructed for the differentiation of Brucella species. Provided that the source of the examined strain is known (cattle, sheep, goats, pigs, hares, etc.) this key allows a reliable classification:

(1) Strains of bovine origin that are lysed by brucella-phages at RTD or 10,000 × RTD correspond to *B. abortus*.

(2) Strains which were isolated from pigs and hares and are lysed only by 10,000 × RTD suspensions or are resistant even to the concentrated phage, are regarded as *B. suis*. Phage resistant cultures of unknown origin may be *B. suis* or *B. melitensis*.

(3) Strains resistant to 10,000 × RTD, if isolated from sheep or goats, are classified as *B. melitensis*.

All three groups of brucella contain some intermediary strains. Thus phage typing does not make the classical methods entirely unnecessary. These are generally superfluous when the animal species from which the strain has been recovered is known. Otherwise the taxonomic position of the culture cannot be properly determined without performing conventional biological and serological tests in addition to phage typing.

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LYSOGENIC PROPERTIES OF VARIOUS STAPHYLOCOCCUS AUREUS PHAGE-TYPES

By

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(Received February 22, 1966)

Summary. Close association has been established between *Staphylococcus aureus* phage group I strains responsible for a hospital outbreak and phage type 52B strains occurring in the same department. Lysogenization with suitable phages converted the strains into the other phage type. When different phage pattern staphylococci are isolated from a hospital outbreak, It should be considered that they may originate from the same source.

In the course of staphylococcal outbreaks it has often been observed that screening examinations performed after the isolation of the epidemic organism frequently reveal variants differing from the agent responsible for the infections. On the basis of epidemiological findings it has been concluded that these variants may develop from the epidemic strain as a result of lysogenization.

First BURNET and LUSH [1], then SMITH [2] and ROUNTREE [3] have called attention to the lysogenicity of staphylococci and to the fact that the phenomenon may influence the phage type of the strain. LOWBURY [4] and WAHL and FOUACE [5] have observed that the spontaneous loss of the prophage causes an alteration in phage sensitivity. The mentioned authors have also studied artificial lysogenization, which is easy to carry out with staphylococci. It is connected with the change of phage type. In some cases this manifests itself in a resistance against the lysogenizing phage, in others the change is associated with a loss of sensitivity to a variety of phages.

Changes in the phage type of phage group I strains have been described by ASHESHOV and RIPPON [6], COMTOIS [7], ROSENBLUM and JACKSON [8] and SAKURAI *et al.* [9]. After lysogenization some type 80/81 strains became untypable. In outbreaks due to 80/81 strains phage types 52/52A/80/81 and 52/52A/80 were also observed. It was demonstrated that by lysogenization with suitable serological groups of phages these three types could be converted into each other. Lysogenization with serogroup A phages not only induced resistance against A phages but also shifted the strain sensitive to B phages. These phages were termed by ROUNTREE converting phages [10]. Later she showed that conversion was the result of the substitution of the prophage, in other words the defective prophage of strain 80/81 was able to recombine with converting phage A, but was unable to produce phages.

The purpose of the present paper is to give an account of the origin of staphylococcal strains different in phage type but similar in biochemical behaviour.

Materials and methods

Strains. The majority of *Staph. aureus* strains used in this study were isolated from throat or ear swabs taken from patients treated in the Children's Department of the 2nd Municipal Hospital, Szeged. Smaller part of the samples were received from the Children's Hospital, New-Szeged, and the Department of Paediatrics, University Medical School, Szeged.

Cultural examination. The materials were seeded into broth containing 4 per cent potassium rhodanate. Subcultures were plated on blood agar.

Phage-typing was performed as described by BLAIR and WILLIAMS [12]. In addition to the basic set of phages (29, 52, 52A, 79, 80, 3A, 3B, 3C, 55, 71, 6, 7, 42E, 47, 53, 54, 75, 77, 83A, 42D, 81, 187), and additional phages (42B, 47C, 52B, 69, 73, 78,D, B5, 77A), phages 304, 377, 756 and 950 isolated in the Phage Laboratory, State Institute of Hygiene, Budapest [13], and phages 13, 2042 and 1380 introduced in the Regional Public Health and Infectious Disease Station Phage Laboratory, Budapest [14], were also employed.

Biochemical reactions. The coagulase test was routinely performed with the slide method using citrated rabbit plasma. When the slide method gave negative results, the reaction was carried out in tubes with citrated rabbit blood. The tube test was read after 4 hours incubation. Fermentation of mannitol was examined in SKORKOVSKY's medium [15], phosphatase production was tested on phenolphthalein-phosphate-containing agar plates; both tests were read after 16 hour's incubation. Haemolysin production was read after 16 hours on agar plates containing 15 per cent ox blood.

Antibiotic sensitivity was tested by the use of penicillin, streptomycin, chloramphenicol, chlortetracycline, tetracycline, neomycin, polymyxin B and erythromycin discs (Institute for Serobacteriological Production and Research "Human", Budapest).

Detection of lysogenic strains. Phages were obtained by using the supernatant of 4-hour broth cultures. In parallel experiments the bacterial strain was inoculated into broth preheated to 56 °C, then after keeping at 56 °C for 2 minutes the suspension was cooled and placed in an incubator at 37 °C for 4 hours. The supernatants of the unheated and heated cultures were tested with an empirically selected series of indicator strains consisting mainly of cultures used for propagation (6, 7, 47, 53, 54, 77, 83A, 73, 52B, D, B5), and of a strain (162) isolated in our laboratory, which was sensitive to all of our phages. The degree of lysis was expressed as recommended by the Staphylococcus Reference Laboratory, London.

Results

The majority of strains had been isolated from secondary staphylococcal infections occurring among patients admitted in the course of a spring-time influenza epidemic to the Children's Department of the Municipal Hospital, Szeged. By routine phage-typing the strains were classified as belonging to phage group I. At RTD they were partly lysed by phage 29; at 1000 RTD different degrees of lysis were observed with phages 29/80/52/52A. Phage 52B lysed all strains at RTD.

The second group of staphylococcal strains included multiple antibiotic resistant phage type 52B cultures. These organisms were commonly found in various departments of the hospital.

Some representatives of these cultures were examined more thoroughly for biochemical and lysogenic properties (Table I).

The cultures were fairly similar in antibiotic sensitivity spectrum. They were generally resistant to antibiotics except neomycin and polymyxin B;

Table I

Properties of the examined strains

	Antibiotic sensitivity									Phage type		Biochemical reactions				
										Basic phages						
Strain	Penicillin	Streptomycin	Chloramphenicol	Chlortetracycline	Tetracycline	Terracyn	Neomycin	Polymyxin B	Erythromycin	RTD	1000 RTD	Additional phages RTD	Haemolysis	Coagulase*	Mannitol	Phosphatase
628	—	—	+	—	—	—	+	+	—	29	29/80(52)	52B	+	±	+	+
629	—	—	+	—	—	—	+	+	—		29/80	52B	+	±	+	±
645	—	—	+	—	—	—	+	+	—	(29)	29/80(52/52A)	52B	+	±	+	+
646	—	—	±	—	—	—	+	+	—	(29)	29/80(52/52A)	52B	+	+	+	+
647	—	—	—	—	—	—	+	+	—		80	52B	+	±	+	±
657	—	—	±	—	—	—	+	+	—	(29/80)	29/80(52)	52B	+	±	+	+
658	—	—	±	—	—	—	+	+	—	(29)	29/80(52/52A)	52B	+	±	+	+
660	—	—	+	—	—	—	+	+	—	(29)	29/80(52)	52B	+	±	+	±
945	—	—	+	—	—	—	+	+	—		29	52B	+	+	+	+
578										—	—	52B	+	+	+	+
591	—	—	—	—	—	—	+	+	—	—	—	52B	+	+	+	+
603										—	—	52B	+	+	+	+
614	—	—	—	—	—	—	+	+	—	—	—	52B	+	+	+	+
616	—	—	—	—	—	—	+	+	—	—	—	52B	+	+	+	+
617	—	—	—	—	—	—	+	+	—	—	—	52B	+	+	+	+
633	—	—	—	—	—	—	+	+	—	—	—	52B	+	+	+	±
642	—	—	—	—	—	—	+	+	—	—	—	52B	+	+	+	±
644	—	—	—	—	—	—	+	+	—	—	—	52B	+	+	+	±

+ = antibiotic sensitive; intensive enzyme production
± = moderately antibiotic sensitive; moderate enzyme production
— = antibiotic resistant
*± = coagulase reaction unstable; for explanation see text

part of the strains belonging to group I were moderately sensitive to chloramphenicol. All strains produced haemolysin and fermented mannitol. Coagulase production by group I strains was not constant: by the slide test in citrated rabbit plasma they reacted negatively, but by the tube test in citrated rabbit blood they showed a positive reaction in 2—4 hours. Phage type 52B strains gave positive reactions in both tests. The former group of strains exerted a weak phosphatase activity. Some weakly phosphatase-positive cultures occurred also among phage type 52B strains.

Table II

Host range of induced phages

Induced phages		Indicator strains											
Strain	Group	6	7	47	53	54	83A	42C	73	52B	D	B5	162
628'	I	++	++	++	—	—	—	—	—	—	++	++	—
629'	,,	++	++	++	—	—	—	—	—	—	++	++	—
645'	,,	++	++	++	—	—	—	—	—	—	++	++	—
646'	,,	++	++	++	—	—	—	—	—	—	++	++	—
647'	,,	++	++	++	—	—	—	—	—	—	++	++	—
657'	,,	++	++	++	—	—	—	—	—	—	++	++	—
658'	,,	++	++	++	—	—	—	—	—	—	++	++	—
660'	,,	++	++	++	—	—	—	—	—	—	++	++	—
945'	,,	++	++	++	—	++	++	—	++	++	++	++	—
578'	52B	++	++	++	—	—	—	—	—	—	++	+	—
591'	,,	++	++	++	—	—	—	—	—	—	++	++	—
603'	,,	++	++	++	—	—	—	—	—	—	++	++	—
614'	,,	++	++	++	—	—	—	—	—	—	++	++	—
616'	,,	++	++	++	—	—	—	—	—	—	++	++	—
617'	,,	++	++	++	—	—	—	—	—	—	++	++	—
633'	,,	++	++	++	—	++	++	—	++	++	++	++	—
642'	,,	++	++	++	—	—	—	—	—	—	++	++	—
644'	,,	++	++	++	—	—	—	—	—	—	++	++	—
915'	NT.	++	++	++	—	++	++	—	++	++	++	++	—
918'	,,	++	++	++	—	++	++	—	++	++	++	++	—
949'	,,	++	++	++	—	++	++	—	++	—	++	—	++

++ = more than 50 plaques; + = 20—50 plaques; ± = less than 20 plaques; — = no lysis.

Phage liberation experiments revealed that all strains were lysogenic. As to host range, their prophages were mostly identical. Only two strains carried prophages different from those of the other cultures. Thus it was supposed that the strains carried related if not identical phages and thus no cross-sensitivity or lysis could be expected. When the liberated phages were deposited onto plates seeded with the examined cultures, lysis was observed only with the two phages that differed in host range from the others (Table II).

In view of the similar biochemical properties of strains belonging to the two groups, it may be assumed that the difference in their phage type has developed as a result of lysogenization.

Table III

Phage pattern of parent and lysogenized cultures

Strains	Dilution of standard phages															Lysogenizing phages				
	29 (B)			52 (B)			52A (B)			80 (B)			52B (B)			633' (B)	915' (B)	918' (B)	949' (B)	945' (F)
	RTD	1000	cc	RTD	1000	cc	RTD	1000	cc	RTD	1000	cc	RTD	1000	cc	cc	cc	cc	cc	cc
645 "I"	+	++	++	—	+	++	—	+	++	—	++	++	++	++	++	++	++	++	++	++
645(633')	—	—	+	—	—	+	—	—	+	—	—	—	++	++	++	—	—	—	++	++
645(915')	—	—	+	—	—	+	—	—	+	—	—	—	++	++	++	—	—	—	++	++
645(918')	—	—	±	—	—	—	—	—	—	—	—	—	+	++	++	—	—	—	++	++
645(949')	—	—	±	—	—	—	—	—	—	—	—	—	+	++	++	++	++	++	—	++
645(945')	+	++	++	—	+	++	—	+	++	—	+	++	++	++	++	++	++	++	++	—
642"52B"	—	—	0	—	—	0	—	—	0	—	—	0	++	++	++	++	++	++	++	++
642(633')	—	—	+	—	—	—	—	—	—	—	—	—	+	++	++	—	—	—	++	++
642(915')	—	—	+	—	—	—	—	—	—	—	—	—	±	+	++	—	—	—	++	++
642(918')	—	—	+	—	—	+	—	—	—	—	—	—	±	+	++	—	—	—	++	++
642(949')	—	—	+	—	—	+	—	—	+	—	—	±	±	+	++	++	++	++	—	++
642(945')	—	++	++	—	—	++	—	—	+	—	+	++	++	++	++	++	++	++	++	—

++ = more than 50 plaques; + = 20—50 plaques; ± = less than 20 plaques;
0 = inhibition; (B), (F) = serological group of phages.

For inducing lysogenicity in the two groups, we had to choose phages exhibiting a broad-spectrum activity on the indicator strains and also lysing strain 52B used for propagation (the examined strains were also lysed by phage 52B). Suitable phages were obtained from strains 633 (type 52B), 945 (group I, type 29/52B) and from three untypable cultures. Heat-induced phages propagated on suitable standard strains were serologically grouped by the antiphage serum neutralization test and were finally used for the lysogenization of some representatives of group I strains. The lysogenized strains became naturally resistant to the lysogenizing phage and their liberated phages were identical in lytic spectrum with the lysogenizing phage.

Table III shows representatives of group I and 52B strains and their lysogenized variants. The lysogenized variants of phage group I strains differed from the parent cultures in phage pattern: when lysogenization had been performed with phages belonging to serogroup B, they were sensitive only to phage 52B; when lysogenization had been induced with phage 945' (serogroup F) there was no change in phage pattern. It should be noted that strain 945 yielding phage 945' belonged to phage group I.

Of the lysogenized variants of type 52B, only the culture lysogenized with phage 945' acquired a more definite sensitivity to group I phages; this, however, was less marked than that shown by phage group I strains. After lysogenization with serogroup B phages, only concentrated group I phages caused a slight lysis; the sensitivity of these cultures to phage 52B also decreased.

Discussion

The routine, less extensive phage-typing method which involves the use of additional phages only when the strains do not react with the basic set of phages, may not reveal epidemiological associations existing among different phage types. The fact that by lysogenization strains belonging to seemingly different phage groups can be converted into the other phage types indicates that the change may take place *in vivo*.

All phages used for lysogenization in the present study had originated from staphylococci isolated in the Children's Department of the 2nd Municipal Hospital or in the Department of Paediatrics, University Medical School, Szeged. As the two kinds of staphylococcal types were prevalent in the same communities, it may be assumed that alterations from one type to another occurred *in vivo*. Thus, type 52B, frequently observed in the Children's Department may have appeared during the outbreak in a modified form: 29/80/52B.

The epidemiologist should therefore be aware of the fact that strains with different phage pattern but with similar biochemical and biological properties may have a common origin.

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ACRIDINE ORANGE FLUORESCENCE OF TISSUE CULTURES INFECTED WITH AUJESZKY'S DISEASE VIRUS*

By

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of the Hungarian Academy of Sciences, Budapest

(Received February 28, 1966)

Summary. The behaviour of two strains (wild and vaccine strain) of Aujeszky's disease (Ay) virus has been examined in two kinds of cell cultures (calf testicle and porcine embryo kidney epithelium cells). In 24—28 hours after infection, both strains caused "chromatin-granulation" of nuclei, typical of DNA viruses, in both cell cultures. The wild Ay-virus strain caused the formation of giant syncytia sometimes containing 50—100 nuclei in both cell cultures, whereas the vaccine strain caused only the rounding off of the cells or if it caused syncytium formation, this was of a minor degree, consisting of 2—5 nuclei detectable on careful examination.

The cytopathic effect of Aujeszky's disease virus (Ay-virus) in tissue cultures has been described by numerous investigators [1—14]. In the infected tissue cultures Cowdry type A acidophilic nuclear inclusions were observed [1—3, 5, 14]. As Ay-virus behaved differently in the different tissue cultures, we examined its cytopathic effect in cultured cells by the acridine orange (AO) fluorescence method [5—19].

Materials and methods

Virus strains. (1) Wild Ay-virus strain maintained in calf testicle tissue culture for an indefinite time. In our laboratory it was carried through 3 passages in the same tissue culture. In the present experiments, material from the 3rd passage was used. Titre: 10^7 $TCID_{50}$/ml. (2) Ay-virus vaccine strain [10], maintained in calf testicle tissue culture since 1961. Titre: 10^7 $TCID_{50}$/ml.

Tissue culture. Primary monolayer cultures prepared from calf testicle and pig embryo kidney epithelial cells by trypsinization were used. The cell suspensions were distributed in sterile test tubes in each of which was placed a sterile glass slide measuring 7 cm by 0.7 cm. After inoculation of the virus, the slides were stained with AO, whereas the monolayers grown on the tubes' walls were stained with haematoxylin-eosin for control purposes.

AO-staining was performed according to Mayor [18].

Apparatus. For fluorescence assay, a Zeiss HBO 50 high pressure mercury vapour lamp was used as a light source. Light was transmitted through a BG 3/4 filter and a GG 9/OG 1 ocular filter.

Photography. Photos were taken on Orwo (Agfa) colour UT 15 or 16 diafilm strips, with an exposition time of 15 minutes, for a magnification of 400.

* Paper read at the Session of the Division for Bacteriology and Virology of the Hungarian Association of Microbiologists, Budapest, January 29, 1965.

Results

In non-infected calf testicle cell cultures, cellular cytoplasms stained brown, the nuclei green and the nucleoli orange, with AO (Fig. 1). In cultured calf testicle cells inoculated with wild Ay-virus, syncytia containing numerous, sometimes 50—100 nuclei, were formed in 24—28 hours after infection (Fig. 2). The nuclear chromatin became granulated and exhibited an intensive green fluorescence owing to the accumulation of deoxyribonucleic acid (DNA). The nuclei exhibited a slight swelling (Fig. 3). The cytoplasm's ribonucleic acid (RNA) content was apparently normal as it showed a brown shade entiiely identical with that seen in the non-infected cells. Seventy-two hours after the infection the whole cell layer was disrupted, its former continuity being indicated by long cytoplasmic threads connecting the degenerated cell groups. The cells were rounded off, their cytoplasms disappeared almost entirely and they arranged either single or in minor groups (Fig. 4).

In cultured pig embryo kidney cells, wild Ay-virus exhibited an essentially similar behaviour as in calf testicle cells.

In calf testicle tissue culture, the vaccine strain of Ay-virus caused as a rule only a disruption of the confluent cell sheat and a rounding off of the cells.

In pig embryo kidney cell culture, the vaccine strain of Ay-virus behaved similarly as in calf testicle cells, causing typical granulation of the cellular nuclei in 24—28 hours, while the cytoplasm remained apparently intact (Fig. 5). Owing to the accumulation of DNA, the nuclei appeared in a vivid green or even yellowish-green hue. Seventy-two hours after the infection the cell layer disrupted, the cells became rounded off and their cytoplasm appeared as a narrow border. In the rounded cells, however, 2—5 shrunken nuclei were observed on careful examination (Fig. 6).

Discussion

The examined two strains of Ay-virus caused nuclear changes typical of DNA viruses both in calf testicle and porcine embryo kidney cell cultures. In 24—28 hours after infection the nuclear chromatin became granulated and appeared in a green or yellowish-green colour owing to the accumulation of DNA. In calf testicle cell cultures, the wild strain of Ay-virus produced syncytia containing numerous, sometimes 50—100 nuclei. In pig embryo kidney cells, the syncytia contained much less nuclei. The vaccine strain of Ay-virus, however, produced no syncytia in either cell culture. Its cytopathic effect manifested itself in the appearance of polynuclear cells composed of 2—5 shrunken nuclei and of rounded off single cells in which the cytoplasm appeared only as a small border around the shrunken round nucleus showing an intensive yellow fluorescence.

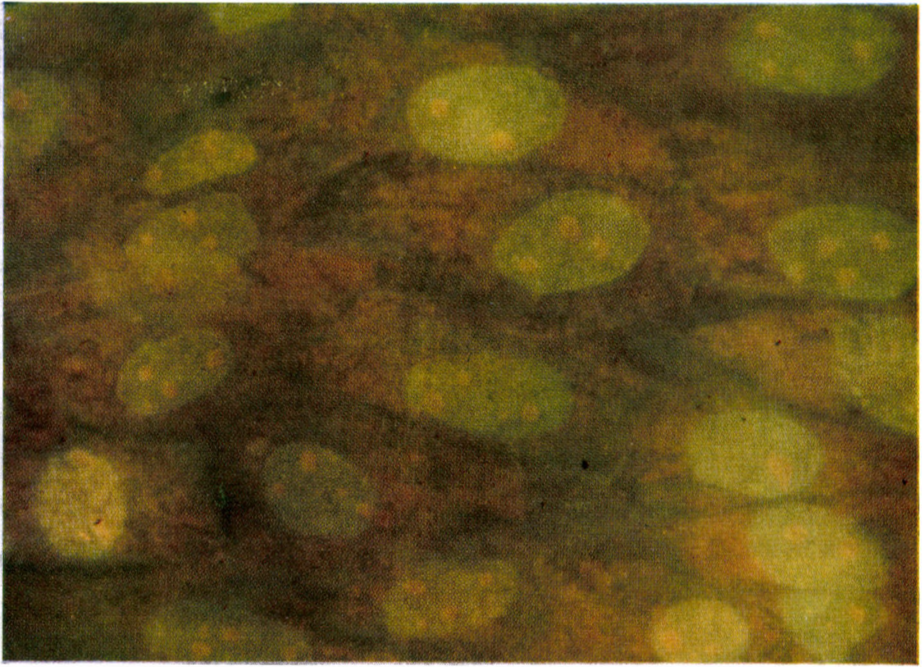


Fig. 1. Non-infected calf testicle cell culture. AO staining; magnification, ×400

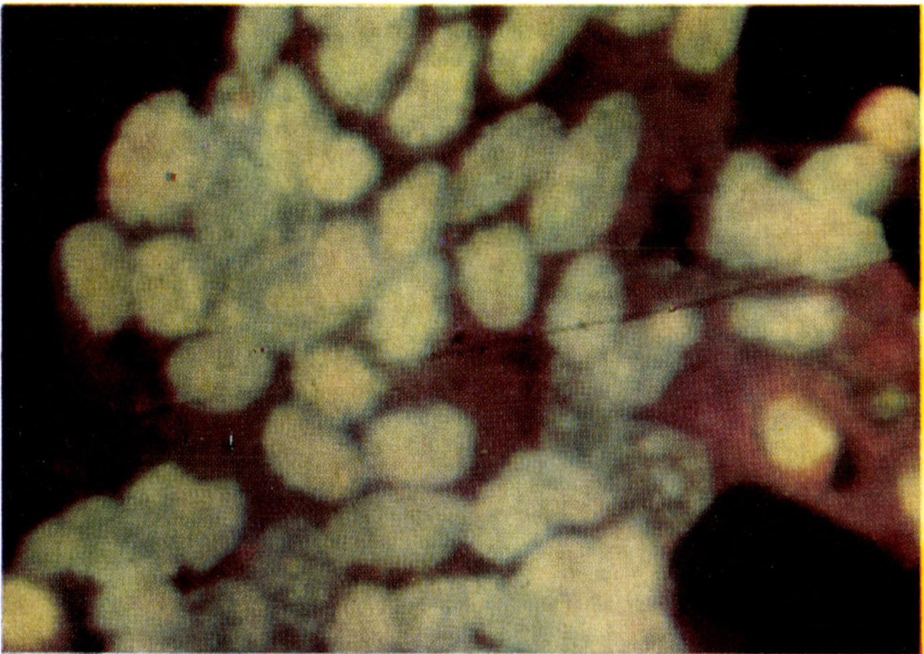


Fig. 2. Calf testicle cell culture 24 hours after infection with wild Ay-virus. Large syncytium with numerous nuclei. AO staining; magnification, ×400

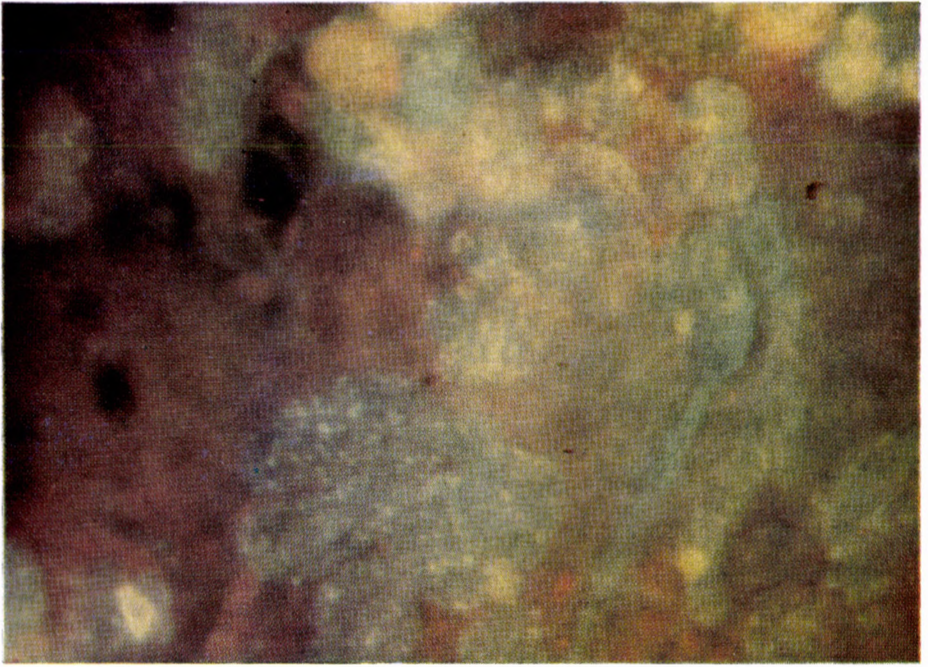


Fig. 3. Calf testicle cell culture 24 hours after infection with wild Ay-virus. Note "chromatin granulation", accumulation of DNA and swelling of nuclei. AO staining; magnification, ×400

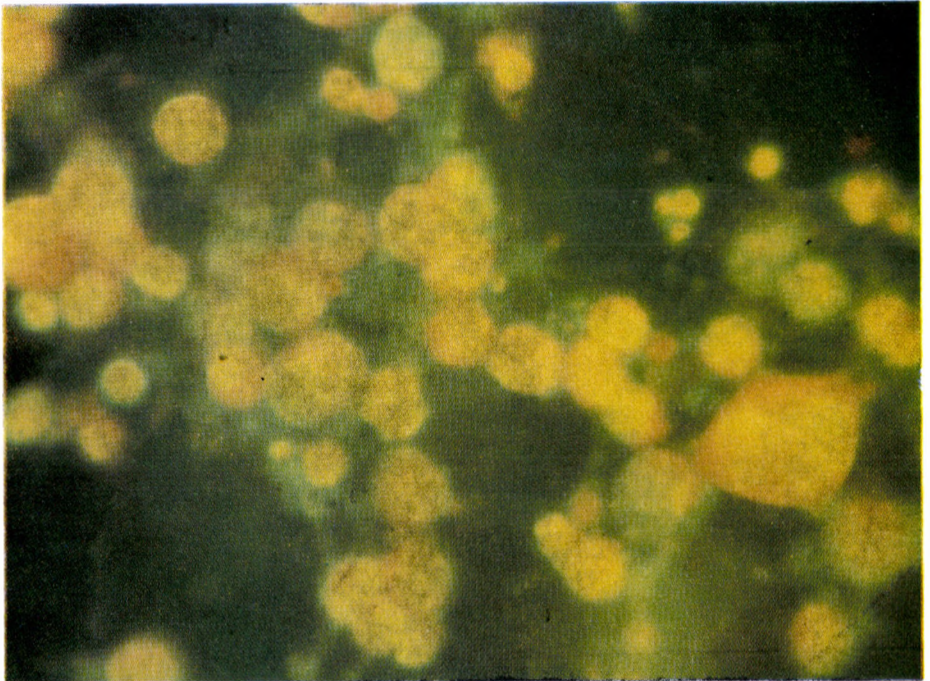
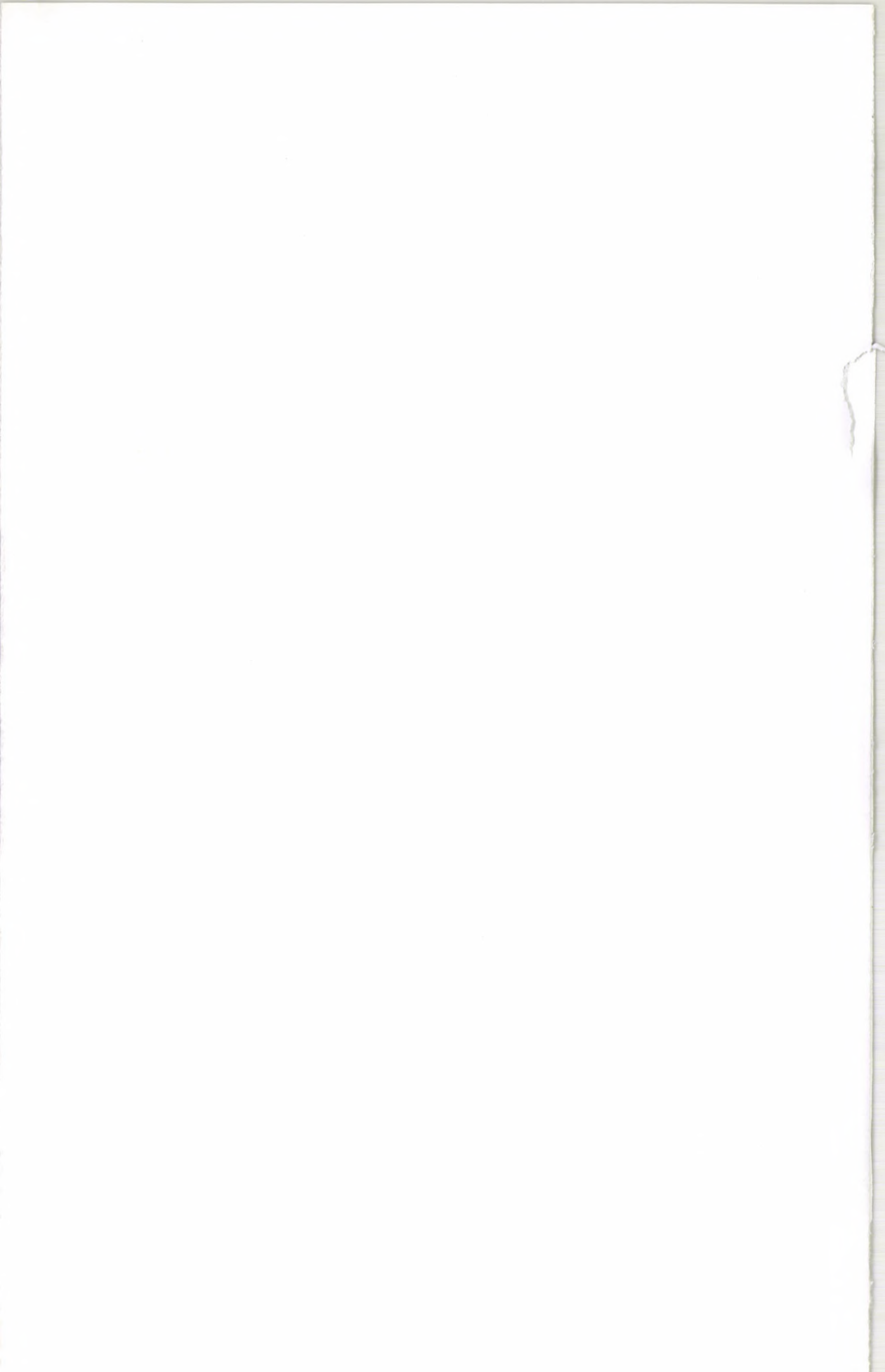


Fig. 4. Calf testicle cell culture 72 hours after infection with wild Ay-virus. Disruption of cell layer, rounding off of cells, disappearance of cytoplasm. AO staining; magnification, ×400



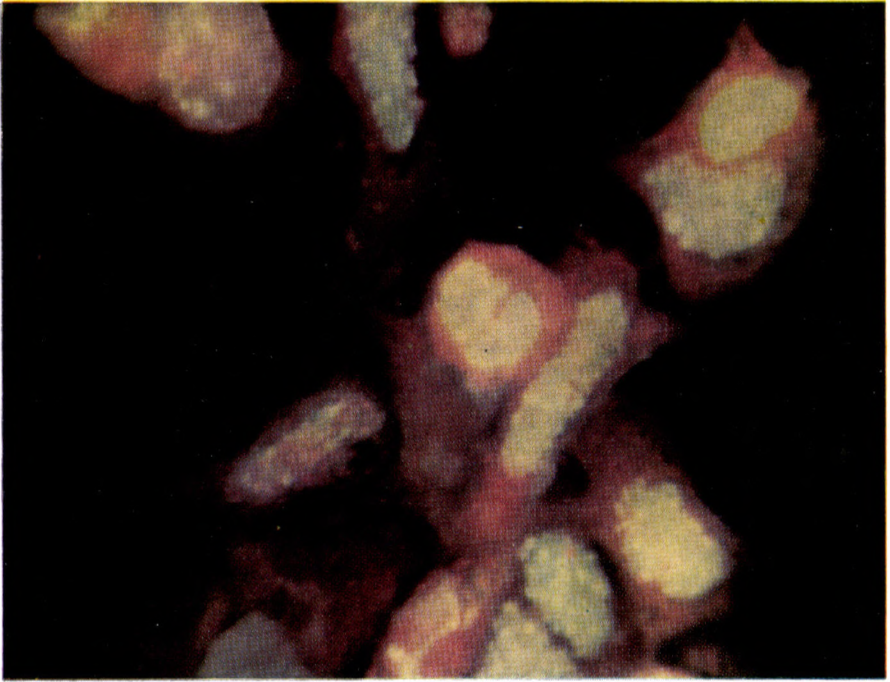


Fig. 5. Pig embryo kidney cell culture 24 hours after infection with the vaccine strain of Ay-virus. Note the typical "chromatin granulation" in nuclei with intact cytoplasm. AO staining; magnification, ×400

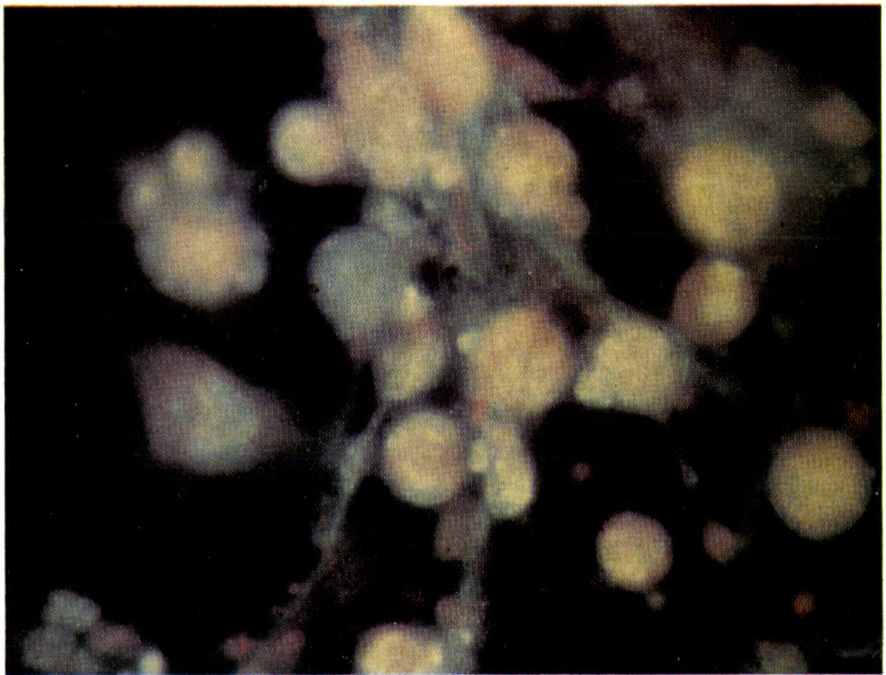
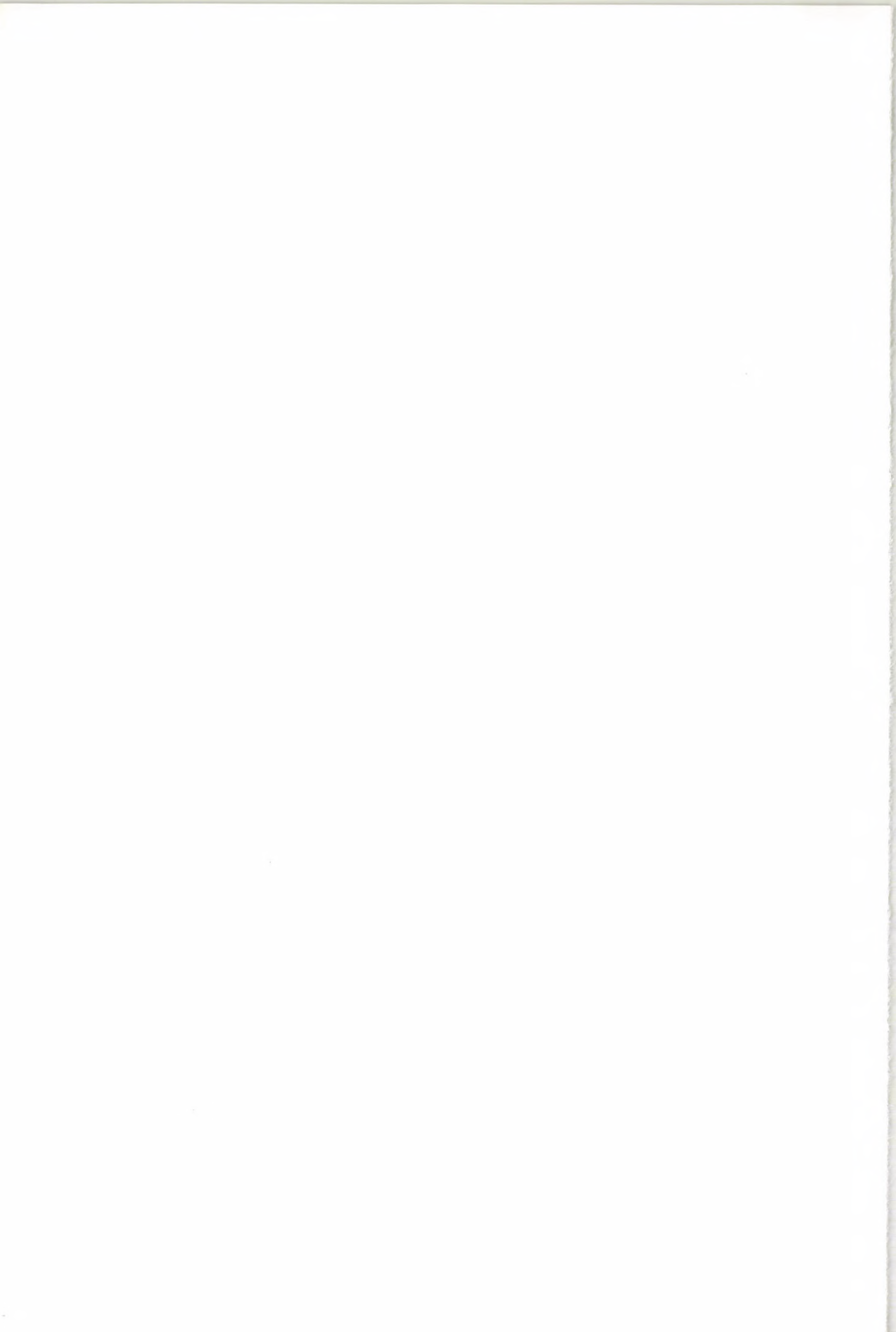


Fig. 6. Pig embryo kidney cell culture 72 hours after infection with the vaccine strain of Ay-virus. Note the disrupted cell layer, rounding off of cells containing each 2—5 shrunken nuclei. AO staining; magnification, ×400



Neither the wild nor the vaccine strain of Ay-virus produced nuclear inclusions under the present experimental conditions. This is incongruent with the findings of several other investigators [1—3, 5, 14]. Further experiments are in progress to clarify the cause of the inconsistency of our results.

Acknowledgement. We are indebted to Dr. J. KOJNOK (Phylaxia State Vaccine Institute, Budapest) for the vaccine strain of Ay-virus.

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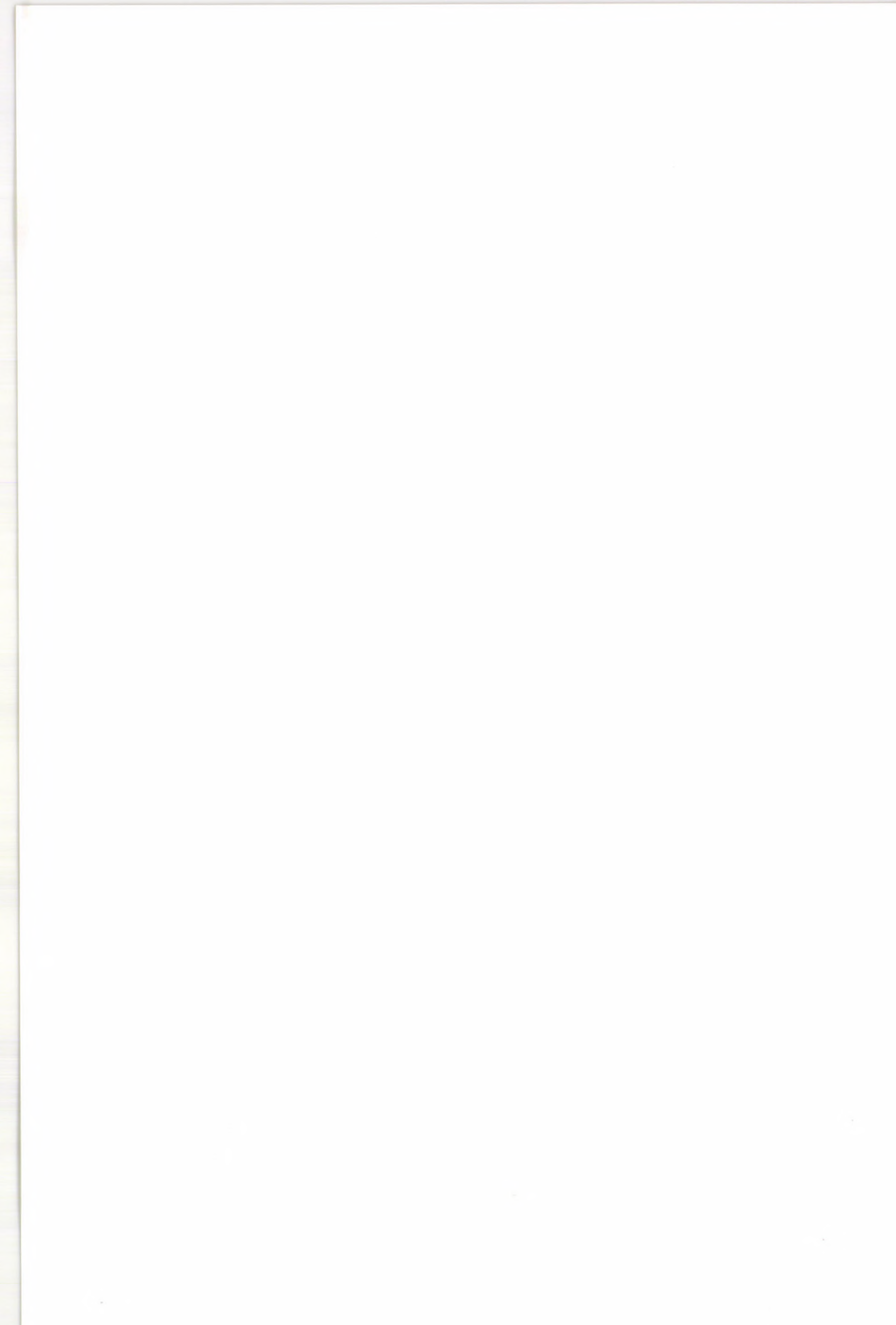

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Acta microbiol. Acad. Sci. hung. 13, 189—192 1966

FURTHER STUDIES ON HAEMAGGLUTINATING AGENTS OBTAINED FROM ACUTE HEPATITIS SERA

By

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(Received September 30, 1965)

Summary. Earlier findings have been confirmed in that in the allantoic fluid of chick embryos inoculated with the acute-phase sera of patients suffering from viral hepatitis a haemagglutinating agent develops similar to that contained in the serum. The agent can be maintained through numerous serial passages without loss of titre. Hepatitis convalescent sera mostly inhibit the haemagglutinating agent obtained either from the acute-phase sera or in the allantoic fluids. The sera of two rabbits immunized with an IH and SH agent, respectively, showed only homologous inhibition. Preliminary neutralization tests *in ovo* seem to be consistent with the results of the haemagglutination inhibition tests. The agents have proved to be highly resistant to heat; heating to 100° C for 20 minutes failed to inactivate their haemagglutinating capacity and their maintainability in serial egg passages.

It has been shown [1, 2] that acute-phase serum samples of patients with viral hepatitis agglutinate in high titres (1 : 32—1 : 6144) tannin-treated human Rh-positive group 0 erythrocytes. Sera of healthy subjects or of those suffering from other diseases including hepatic disease such as toxic hepatitis, give the reaction at most up to 1 : 8 dilution and the factor responsible for this agglutination is absorbable with papain-treated or untreated erythrocytes.

Examining the specificity of the reaction, we inoculated 11-day embryonated hen's eggs intra-allantoically with acute-phase hepatitis sera. The allantoic fluids harvested three days later were found to agglutinate the tannin-treated human erythrocytes. The haemagglutinating factor seems to be identical with the serum factor in all characteristics tested. It was successfully passaged in embryonated eggs, in two cases over 16, in two others over 20, consecutive passages. In the course of passages the haemagglutination (HA) titre tended to increase.

In the present study attempts have been made to characterize the HA agent.

Materials and methods

Sera. Acute-phase blood samples were taken under sterile circumstances from 99 hepaitis patients in the first five-day period following the first symptoms of the disease. The separated sera were tested for bacteriological sterility and kept in the refrigerator (0 — +4° C) for a period not longer than 48 hours. Before experiment the sera were heated to 60° C for 30 minutes.

Convalescent sera were taken from 13 patients with the clinical diagnosis of infectious hepatitis (IH) and from 5 with serum hepatitis (SH). These and serum samples from 5 healthy donors (controlled by the National Blood Transfusion Service, Budapest) were tested for HA-inhibiting (HI) antibodies against the haemagglutinating agent.

Immune sera were produced in rabbits. The IH antigen was a haemagglutinating allantoic fluid from the 9th egg passage of an agent derived from the serum of an IH patient; the SH antigen was an allantoic fluid from the 4th egg passage of an agent originally obtained from the serum of an SH patient. The immunization scheme has been described elsewhere [3]. The immune sera were absorbed with a normal allantoic fluid before used.

HA test with tannin-treated erythrocytes [1, 2]. Human Rh-positive group 0 blood was defibrinated by shaking with glass beads. The erythrocytes were separated by centrifugation and washed at least four times with saline. Subsequently, 8.5 ml of 0.5 per cent analytical quality tannic acid (Merck) dissolved in saline was added to 1.5 ml erythrocyte sediment. After vigorous shaking the erythrocyte suspension was placed in the incubator for 30 minutes. The sediment was washed once with saline and made up to 100 ml with the diluting fluid (see below) of 18—20° C. After shaking the conglomerated erythrocytes (2—10 per cent of the total) sedimented and the supernatant containing about 1 per cent homogeneously distributed erythrocytes was sucked off. The suspension was stored in sealed sterile ampoules at 0—4° C for at most six days. The diluent contained 0.4 per cent NaCl and 5 per cent glucose in Sörensen's phosphate buffer of pH 7.4. The erythrocytes were not treated with formalin either before or after tanninization.

HA titration was usually carried out in TAKÁTSY's Microtitrator [4]. The titres are expressed in the highest dilution showing positive reaction after 70 minutes at 10—15° C or 30 minutes at 10—15° C and 0 — +4° C overnight. The reaction was accepted as positive if the bottom pattern characteristic of agglutinated erythrocytes was seen.

Inoculation of eggs. Eleven-day-old embryonated eggs were inoculated intraallantoically and incubated at 37° C for further three days. The harvested allantoic fluids were tested for sterility and heated to 100° C for 15 minutes before tested for HA.

HI test. Three parallel twofold dilution series, from 1 : 2 to 1 : 16, were prepared in the wells of the Microtitrator plate from each of the sera to be tested for anti-haemagglutinin. To each dilution an equal volume of diluted haemagglutinating serum or allantoic fluid was dropped. The haemagglutinin content of the allantoic fluid amounted to 2 to 32 HA units in the tests carried out on different days. The mixtures were kept at 37° C for two hours, whereafter tanninized erythrocytes were added. In the following the procedure was the same as described for the HA test. Because of the fluctuation in the quantity of haemagglutinin, exact HI titres cannot be presented. The HI test was considered positive when HA did not develop in any of the wells of the three parallel series not even in the wells with the 1 : 16 diluted serum. Control tests with known negative and positive sera were performed in every case and the haemagglutinin (serum or allantoic fluid) used in the test was examined without serum as well.

Results

Table I summarizes the results of the egg passages initiated with acute-phase sera of patients suffering from viral hepatitis.

Eggs were inoculated with 99 acute-phase sera. The harvested allantoic fluid agglutinated tanninized red cells in 96 cases. In 3 cases no agglutination was observed. Out of the 96 agents 27 were subjected to further passages, in 24 cases with success. Two agents were subjected to two, one agent to three, two agents to 16 and two agents to 20 allantoic passages.

For control purposes serum samples obtained from 28 blood donors kept under the control of the National Blood Transfusion Service were inoculated into eggs. No HA was observed though 19 sera were subjected to two, 4 to six and 5 to eight serial passages. In addition, serum samples obtained from 43 patients suffering from diseases other than hepatitis were inoculated and subjected to 1—8 serial passages. In the first passage haemagglutinating allantoic

Table I

The history of haemagglutinating agents in embryonated eggs

Inoculum	No. of samples inoculated	Successful inoculation	Unsuccessful inoculation
Acute phase serum	99	96	3
1st passage allantoic fluid (AF)	27	24	3
2nd passage AF	7	7	—
3rd passage AF	5	5	—
4th—16th passage AF	4*	4	—
17th—20th passage AF	2**	2	—

*, ** Number of agents carried over 16 and 20 passages, respectively

Table II

Haemagglutination inhibition (HI) tests

Haemagglutinating fluids	Sera tested for HI: Rabbit immune sera		Convalescent sera		Control sera	
	anti-IH (1)*	anti-SH (1)	IH (13)	SH (5)	rabbit (1)	human (5)
Acute IH sera (6)						
No. of tests	6	6	78	30	6	30
Inhibition	4	—	46	1	—	—
No inhibition	2	6	32	29	6	30
Acute SH sera (4)						
No. of tests	4	4	52	20	4	20
Inhibition	—	3	15	8	—	—
No inhibition	4	1	37	12	4	20
IH agent in AF** (14)						
No. of tests	14	14	182	70	12	68
Inhibition	7	—	63	—	—	—
No inhibition	7	14	119	70	12	68
SH agent in AF** (9)						
No. of tests	9	9	117	45	9	45
Inhibition	—	8	30	20	—	1
No inhibition	9	1	87	25	9	44

* In parentheses, the number of sera and allantoic fluids tested
** AF = allantoic fluid

fluids were harvested in 7 cases, but the haemagglutinating agent could not be passaged and did not re-appear in the course of further passages. Finally, allantoic fluid samples from 74 noninoculated eggs were tested for HA. From six allantoic fluids eight serial blind passages were made, with negative results. Table II shows the results of HI tests.

Anti-IH and anti-SH rabbit serum prevented HA in the majority of the acute-phase IH and SH sera respectively, while cross reactions never occurred. Each of the convalescent sera inhibited the HA of some of the acute-phase sera. In a few cases, however, cross reactions were observed, irrespective of whether acute-phase serum or allantoic fluid was used as the haemagglutinating agent. Among the sera of healthy subjects a single one inhibited the SH agent (6th allantoic passage). The seropositive person did not remember any hepatitis infection and laboratory tests and clinical examinations provided no evidence of disease.

Preliminary studies have supplied data indicating that there may be some correlation between the HI reaction under study and the neutralization test *in ovo*.

Discussion

Both our earlier and the present studies have shown that the allantoic fluids of a great part of the chick embryos inoculated with serum samples obtained from patients with acute viral hepatitis display haemagglutinating activity similar to that elicited by the serum itself. The haemagglutinating agents could be maintained through numerous consecutive passages in embryonated eggs.

The nature of the agents has not been determined exactly. Their resistance to heat is remarkable. After being heated at 100° C for 20 minutes, the agents (50 allantoic fluids out of 52) still agglutinated tanninized human erythrocytes. The same treatment never prevented the agents from being maintainable in egg passages.

The fact that hepatitis convalescent-phase sera often inhibited the haemagglutinating agents and this inhibition seems to distinguish IH from SH points to a possible specific reaction of the organism against the haemagglutinating agents.

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SIMULTANEOUS INHIBITORY ACTION ON VIRUS MULTIPLICATION OF INTERFERON AND SOME NATURAL MUCOPOLYSACCHARIDES (HEPARIN, HYALURONIC ACID)

By

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(Received November 22, 1965)

Summary. Reproduction of influenza A (PR8) and parainfluenza type 1 (Sendai) viruses on chorioallantoic membrane pieces was not influenced by heparin and hyaluronic acid at concentrations of 10, 100, 1000 μg/ml and of 1, 10, 100 μg/ml, respectively. These substances had no influence on the interferon's inhibitory action on the growth of Sendai virus. The reproduction of Herpes simplex virus in chick embryo fibroblast cultures was inhibited by heparin, but not by hyaluronic acid. When heparin was added simultaneously with interferon, the inhibitory effect exhibited summation while hyaluronic acid had no influence on the interferon action.

It has been shown by several authors that the reproduction of Herpes simplex virus was inhibited by heparin in a variety of tissue cultures [1, 2, 3, 4]. The relevant results have been summarized by VAHERI [5].

Heparin is a polyanionic polysaccharide composed of glucosamine and glucuronic acid [6, 7]. Its virus inhibitory action has been ascribed to the sulphate groups responsible for the anionic character of the compound; they are supposed to attach to oppositely charged groups on the virion's surface, thus resulting in the formation of a heparin-virus complex incapable to penetrate into the susceptible cell [5]. According to recent concepts, the heparin present under natural conditions in the different tissues of the living organism, may be regarded as an unspecific, natural, *in vivo* protective measure inhibiting the spread of the invading virus into the susceptible organism through the above outlined mechanism [3, 5, 8, 9].

It has also been shown that interferon inhibits the reproduction of several viruses [10]. The action of interferon (as well as that of the recently described interferon-like substances) is intracellular. Several authors ascribe an important role to interferon in the recovery of the organism from virus infections, as its appearance is known to precede that of the specific antibodies [11, 12, 13, 14]. Several authors have characterized interferon as a basic protein [15, 16, 17].

It seemed to be of interest to study the possibility of an interaction between heparin or hyaluronic acid and interferon.

Materials and methods

Viruses. Strain PR8 of influenza A virus and strain Sendai of type 1 Myxovirus parainfluenzae were used. Both strains were maintained in chick embryo allantoic cavity by serial transfers and stored at —20° C until used.

Herpes simplex virus was isolated in this laboratory and has been maintained in HeLa cell cultures by serial transfer. The virus material was stored at +4° C until used.

Tissue cultures. For the experiments in roller drum, the method of HORVÁTH was used [18]; the virus was grown on aliquots of chopped chorioallantoic membrane.

Tube cultures of chick embryo fibroblasts were prepared by trypsinization. PARKER's medium 199 with 10 per cent calf serum was applied during outgrowth. As maintenance or diluent fluid a mixture of equal volumes of PARKER's medium and HANKS' solution with 10 per cent calf serum was employed.

Preparation of interferon. Eleven days old chick embryos were infected with PR8 virus by inoculation into the allantoic cavity. After 72 hours' incubation at 35° C, the allantoic fluids were harvested. The virus was destroyed and the interferon partially purified and concentrated by the method of ZEMLA and VILČEK [19], using chilled acetone for precipitation.

Heparin. A preparation of the G. Richter Pharmaceutical Works, Budapest, was used. Each 5 ml ampoule contained 50 000 μg/ml active substance. Dilutions were freshly prepared in the appropriate medium. Hyaluronic acid was a preparation of the Institute for Pharmaceutical Research, Budapest.

Titration of infectivity. The infective titres of PR8 and Sendai viruses were determined by the roller drum method of HORVÁTH [18]. Titres are expressed in terms of ID_{50}.

Quantitation of interferon. The method has been described earlier [20]. Twofold dilution series were prepared from the concentrates of interferon and added each to an ampoule in the roller drum. After 24 hours' incubation, 100 ID_{50} Sendai virus was added per ampoule. After 60 hours of additional incubation, the virus content was determined by the haemagglutination test, using TAKÁTSY's micro-method [21].

Interferon activity was expressed as the highest dilution still resulting in the inhibition of Sendai virus replication in half of the ampoules tested.

Heparin-interferon, and hyaluronic acid-interferon combinations. The action of the above combinations on Sendai virus was examined in the roller drum. Experiments with Herpes simplex virus were carried out in chick embryo fibroblasts.

Results

In preliminary experiments in roller drums the action of heparin and hyaluronic acid on the growth of PR8 and Sendai viruses was examined. No effect whatever was observed with heparin and hyaluronic acid within the concentration ranges 10—1000 μg/ml and 10—100 μg/ml, respectively. Neither was any effect observed on the activity of interferon in the same system, with either of the above substances in the concentration ranges given. The titre of the interferon preparation used in this experiment was between 1 : 16 and 1 : 32. These experiments were performed exclusively with Sendai virus.

The lack of action on the growth of Sendai virus of heparin suggested the necessity to examine the effect of a combination of heparin and interferon on a virus sensitive to both substances. The virus of Herpes simplex was chosen for this purpose and the effects of hyaluronic acid and interferon were also examined in the same system. The final concentrations of heparin and hyaluronic acid in these experiments were 2.5 μg/ml and 100 μg/ml, respectively. Interferon and heparin alone each reduced the virus titre by about 1 log unit.

The simultaneous addition of the two substances resulted in a titre decrease of about 2 log. Hyaluronic acid alone had no effect on the virus, its simultaneous application with interferon did not affect the latter's activity.

The effect of heparin and interferon on the reproduction of Herpes simplex virus was examined also in different pH ranges. The final concentration of heparin in these experiments was 2.5 μg/ml.

As shown in Table I, virus growth was more inhibited at pH 6.5 than at pH 7.2.

Table I

Multiplication of Herpes simplex virus in the presence of interferon and heparin, at pH 7.2 and 6.5

pH	Difference in infectivity titres as related to the control ($\log_{10}$) in the presence of		
	interferon	heparin	interferon + heparin
7.2	1.0	1.0	2.0
6.5	1.5	1.5	2.5

Discussion

The aspecific protective factors against viral infections of the organism are the increase of temperature, a slight lowering of pH, further interferon and certain tissular mucopolysaccharides [9, 22, 23]. Of the mucopolysaccharides heparin might be considered the most important. The substance is present in blood as well as in the tissues. Its storage and release during inflammatory processes have extensively been studied [24, 25]. Virus infection causing local tissular reactions is supposed to induce a release of heparin.

The polyanionic heparin may attach to a wide variety of basic substances, among them to proteins [26, 27, 28, 29, 30]. The pH and the ionic composition of the medium have a remarkable influence on these reactions [31, 32, 33]. According to present concepts [3, 5], the antiviral activity of heparin has the same mechanism of action. The heparin-virion complex is supposed to be unable to penetrate into the sensitive cells.

Viral infections are known to result in certain cases in the production of interferon. This substance is a basic protein, thus it may be supposed to react with heparin. This would probably impair the activity of interferon. The present experiments have shown the absence of such a reaction. In the case of Herpes simplex virus, the inhibitory action of heparin and interferon was more intensive than that of the two substances applied separately. Hyaluronic acid failed to inhibit the activity of interferon.

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EFFECT OF HEPARIN ON HERPES SIMPLEX VIRUS INFECTION IN THE RABBIT

By

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(Received March 5, 1966)

Summary. The influence of exogenous heparin has been examined on the growth of Herpes simplex virus (HSV) in the skin of rabbits. Heparin administered 1 or 18 hours prior to infection was found to inhibit in dependence on the concentration the growth of several HSV strains and the development of cutaneous lesions. Under the experimental applied conditions a definitely neurovirulent strain of HSV was not inhibited by heparin. The possible mechanism of the protective action of heparin against HSV infection and the correlation between neurovirulence and heparin sensitivity are discussed.

Simultaneous injection of Herpes simplex virus (HSV) and hyaluronidase has been found to result in the development of well-defined characteristic local lesions [1]. The lesions disappeared spontaneously after a period of 8 to 10 days, as a result of the development of specific immunity [2] and other non-specific factors inhibiting viral growth and pathogenicity [3]. Of the latter factors, heparin may have a decisive importance. The present paper is a report on studies concerned with the effect of exogenous heparin on the replication of HSV strains and their pathogenicity in animals.

Materials and methods

Viruses. Strains PM, TM, DE, GE and CsB were isolated from clinically typical lip mucosa lesions. Strain OB was isolated from the contents of vesicles having developed repeatedly on the thigh. All strains were isolated in human embryonic fibroblast cell cultures, and all but OB caused in both embryonic fibroblast and HeLa cell culture the development of diffusely localized small rounded refractile cells. Human embryonic fibroblast and HeLa cell cultures infected with strain OB underwent cytopathic (CP) changes characterized by focal appearance of small rounded refractile cells and polynuclear giant cells. All strains were stored at +4° C until used.

Preparation of inocula for animal experiments. The strains stored at +4° C were inoculated into human embryonic fibroblast cell cultures. After the generalized CP effect had developed, the cells were scraped from the flasks' walls and suspended in the original medium. Cells were disintegrated by sonication using a MSE apparatus (1.5 A, 3/4", Titan sonicator, in 10 ml volume). Cell debris were removed by centrifugation. Uninfected cultures treated in an identical way served as controls. The inoculum was always prepared in a way to ensure the presence of 20,000 $TCID_{50}$ virus in 0.5 ml. For growth as well as for dilution of viruses PARKER's No. 199 medium was used throughout.

Inoculation of rabbits. Male rabbits weighing 2.5 kg and bred in this Institute were used. The back of the animals was depilated. The depilated area was divided into four circles, 6 cm in diameter each. Into the centre of each circle 30 I.U. (0.2 ml) hyaluronidase solution and immediately afterwards 0.5 ml of the inoculum specified above was injected. Heparin was

injected 1 or 18 hours prior to virus infection into two individual circles on the same animal. Three rabbits were used in each experiment with the individual virus strains and three with the same strains and heparin. The development of lesions was examined daily. In the absence of heparin, the lesions attained their maximum on the fourth post-infection day. The lesions showed oedematous swelling and hyperaemia with nodules and vesicles.

Determination of virus content of the skin. At appropriate points of time, the circles with the lesions were cut out of the skin together with the fascia. The skin-fascia samples were washed free of blood, minced with scissors and homogenized with seasand of analytic purity in PARKER's 199 medium. Ten per cent suspensions were prepared, and centrifuged for 5 minutes at 1000 rpm. The pH of the supernatant was adjusted to pH 7.2 by bubbling CO_2 through the fluid. A series of tenfold dilutions was then prepared and 0.1 ml each were inoculated into human embryonic fibroblast cell culture tubes. Adsorption was allowed to take place at 37° C for 1 hour. 0.9 ml of PARKER's 199 medium was then added to each tube. The infected cultures were incubated at 37° C and examined microscopically after 3 days. $TCID_{50}$ was calculated according to REED and MUENCH [4].

Determination of virus content in the central nervous system. Brain and spinal cord of animals exhibiting neurological symptoms were removed and processed in a manner similar to that described for the skin. The method of virus assay was also identical.

Chemicals. Hyaluronidase solution (Organon, Oss, Holland) contained 150 I.U. per ml. Heparin (Gedeon Richter Pharmaceutical Works Ltd., Budapest) contained 25,000 I.U. per 5 ml. Both substances were diluted appropriately in PARKER's 199 medium.

Histological examination. Excised skin specimens were fixed in 4 per cent formalin for 48 hours. To prevent dissolution of mucopolysaccharides, one specimen was always fixed in a solution containing 6 per cent cetylpyrimidium chloride in 4 per cent formalin for 48 hours. Fixed specimens were embedded in paraffin and cut into 7 μ sections. The preparations were stained with haematoxylin-eosin and azan stains. For the detection of acid mucopolysaccharides, an 0.1 per cent solution of toluidine blue (pH 2.0) was used. Part of the preparations were digested with hyaluronidase for 18 hours at room temperature.

Results

Table I shows the effect of different doses of heparin on the growth of strain PM of HSV in rabbit skin and on the development of cutaneous lesions. This strain had identical characters both *in vivo* and *in vitro* with all the other HSV strains examined (except for strain OB).

Table I

Effects of different amounts of heparin on the replication of an HSV strain (PM) in rabbit skin

Dose of heparin in μg injected one hour before infection	Diameter in mm of skin lesion on 4th day*	Virus content of the lesion* (log $TCID_{50}$/ml)
0	35	6.00
40	35	6.00
400	25	5.75
800	25	5.25
1200	0	4.50

* Average of three experiments

As shown in Table I under the used experimental conditions inhibition was demonstrable only with 1200 μg heparin inoculated into the rabbit's skin. The extension of the lesions was related to the demonstrable amount of virus.

Fig. 1 shows the growth curve of the same virus as measured by determining the virus content of skin specimens treated or not treated with heparin prior to infection.

As shown in Fig. 1, most of the inoculated amount of virus was recovered from the heparin-treated skin specimens, while much less from the

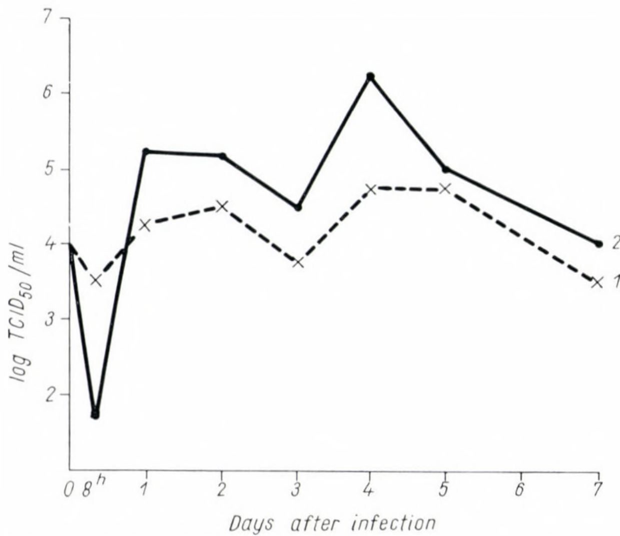

Fig. 1. Replication of a HSV strain (PM) in rabbit skin pretreated (1) and not pretreated (2) with 1200 μg of heparin, as indicated by virus assay in human embryonic fibroblast cultures. The individual plots are averages from three experiments

untreated ones, as determined eight hours after infection. Later no significant virus replication took place in the heparin-treated animals. After the 5th day, the amount of virus in heparin-treated and untreated skin specimens was very similar, both exhibiting a decreasing tendency.

In Table II are shown the effects of heparin concentration (1200 μg) on the growth of some other strains of HSV. The presence or absence of neurological symptoms as well as the demonstrability of the virus in the central nervous system have also been included in Table II.

According to the data in Table II, all the examined strains were found to have grown appropriately in the rabbit skin and produce typical lesions on the fourth day. Except for strain OB, none of the strains were able to grow appropriately in the presence of heparin; consequently the lesions were also absent. During a 1-month period of observation none of the strains were found

to have produced demonstrable neurological changes. Virus was not demonstrable in the central nervous system after 7 to 14 days. Strain OB, however, was not inhibited by 1200 μg heparin under the present experimental conditions. Attempts to recover virus from the central nervous system were successful; the animals developed serious encephalitis and myelitis after 7 to

Table II

Replication of HSV strains in rabbit skin infiltrated with 1200 μg of heparin

Designation of strain	Diameter in mm of lesion on 4th day		Virus content of lesion log $TCID_{50}$/ml on 4th day		Neurological signs*	Virus isolation from central nervous system
	after pretreatment with					
	Heparin	Parker's 199	Heparin	Parker's 199		
PM	—	35	4.5	5.8	Absent	Negative
TM	—	30	4.5	5.5	Absent	Negative
DE	—	30	4.0	5.2	Absent	Negative
GE	—	30	4.6	5.8	Absent	Negative
CsB	—	25	4.5	5.5	Absent	Negative
OB	25	25	5.6	5.7	Present**	Positive***

* One-month observation period
** General weakness, depression, fever, spasms on the 7—14th day
*** 10,000,000 $TCID_{50}$/5 g

Table III

Replication of an HSV strain (PM) in rabbit skin infiltrated 18 hours prior to infection with heparin, resp.* PARKER'S *No. 199 medium*

Pretreatment	Lesion on 4th day	Virus titre on 4th day (log $TCID_{50}$/ml)
2000 μg of heparin	0	4.50
PARKER'S No. 199	+	5.75
No pretreatment	+	5.80

* Average of three experiments

14 days. Thus, the cutaneously inoculated virus attained high titres also in the central nervous system.

In Table III is shown the inhibition of viral growth and the development of lesions by 2000 μg heparin administered 18 hours prior to infection. The PM strain was used and heparin was administered directly into the skin region designated for being infected.

Under the present experimental conditions, heparin inhibited both the growth of virus and the development of skin lesions.

Fig. 2 demonstrates a histological preparation stained with toluidine blue. The specimen had been obtained 18 hours after the injection of heparin and the preparation was digested with hyaluronidase.

As shown by Fig. 2, metachromatically staining acid polysaccharides were present in large amounts in the proliferating connective tissue cells. The substance is supposed to represent heparin incorporated by the cells. The connective tissue matrix failed to exhibit this type of staining.

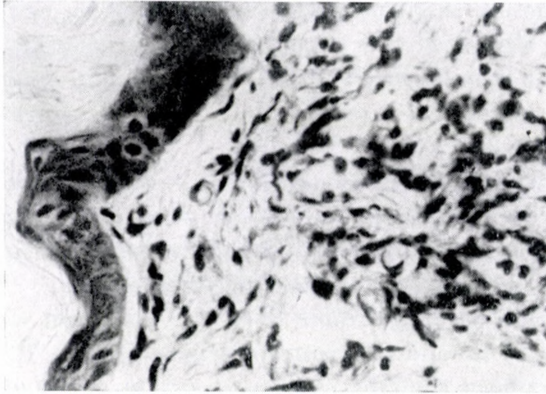

Fig. 2. Incorporation of exogenous heparin by connective tissue cells of rabbit skin 18 hours after the intracutaneous injection of heparin. Stained with toluidine blue at pH 2.0, after digestion with hyaluronidase. Magnification, × 800

Discussion

Several authors have studied the effect of heparin on the growth of HSV *in vitro* [5, 6, 7, 8, 9, 10]. To our best knowledge, no studies *in vivo* have been performed with this virus, though numerous other viruses had been examined in animal experiments in respect of their inhibition by heparin or other polyanions [11, 12, 13, 14].

The present experiments have shown that in the heparin-pretreated rabbit skin the growth of HSV is inhibited in dependence on the applied concentration of heparin. A correlation has also been observed between the development of lesions and the grade of virus multiplication. This fact is in agreement with the observations of Force *et al.* [1], who have found that only replicating HSV produced lesions in the rabbit skin. Heparin concentrations inhibitory for virus growth inhibited also the development of lesions.

In accordance with Higginbotham's [15] similar observations we found connective tissue cells to take up heparin. We therefore supposed that the heparin-virus complex may enter the connective tissue cells and may even develop in them. The further course of infection may depend on further events

within the cell (inactivation of the virus, possible latent infection or replication). The supposed role of heparin in viral infections has been discussed previously [9, 21].

One may ask how far these animal experiments may be considered a model of natural infection. Large amounts of heparin were found to inhibit virus replication, but under natural conditions no comparable amounts of virus may gain access to the organism. As shown by others and also by us [9, 10] in experiments *in vitro* growth of HSV was inhibited by 0.1 to 10 μg/ml of heparin. Considering that the plasma is supposed to contain 1 to 2 μg/ml heparin [16] and, also, that this concentration may considerably increase at the site of infection, it may be supposed that in certain cases of natural viral infection heparin might have some influence on the further course of the process.

The capability of the individual HSV strains to invade the nervous system might be quite different GOODPASTURE *et al.* [17, 18] have reported about a strain producing encephalitis in rabbits when inoculated by corneal scarification or by inoculation into the masseter muscle.

The HSV strain used by FORCE *et al.* [1] multiplied well in the skin of rabbits, but failed to invade the central nervous system. The neurovirulence of HSV strains has been thought by several authors to be related with some particularities of the cytopathic effect. KOHLHAGE *et al.* [19] described that HSV strains causing the appearance of small giant cells in tissue culture exhibited an encephalogenic character. A similar observation has been made by WHEELER [20]. The OB strain isolated in this laboratory and applied in the present study grew well in the skin of rabbits, produced encephalitis and myelitis within a few days following the infection and was not inhibited by heparin at the concentrations applied in the present experiment. As far as one may judge from the behaviour of a single such strain, there might be some relation between the neurovirulence or neurotropy and the sensitivity to heparin of a given strain of HSV.

Acknowledgements. We are indebted to Dr. B. MÁNDY for preparation and evaluation of histological sections. The skilful technical assistance of Mrs. Zs. HERPAY, Mrs. L. JENKEY and Miss É. MIKÓ is duly acknowledged.

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OLIGOSACCHARIDE UTILIZATION BY SAPROPHYTIC CLAVICEPS STRAINS

By

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Summary. The oligosaccharide utilization of alkaloid positive and negative Claviceps strains isolated from rye and grass has been examined. Three strains were shown to possess β-fructo-furanosidase and one another endosaccharase. The former enzyme was shown to have transfer activity even in Claviceps strains. Strains freshly isolated from the sclerotium exhibited high trehalase activity. The utilization of maltose was demonstrable in some strains depending on uptake, respectively induction. None of the strains utilized lactose or melibiose.

Since the 'twenties of this century, the saprophytic cultivation of Claviceps strains has been studied in order to develop facilities for the production of ergot alkaloids by fermentation. The first important result of these studies was the demonstration of the dependence of saprophytic alkaloid production on the actually used strain. Among the strains producing alkaloid in the sclerotial phase of their parasitic life cycle, very few were found to be able to produce alkaloid when maintained under saprophytic conditions [1]. Alkaloid production *in vitro* was more frequent with Claviceps species parasitic in wild grasses than with species of the secale type. In case of clones obtained from one and the same sclerotium, the incidence of alkaloid positivity *in vitro* was increased by treatment with mutagens [2].

With the strains capable of producing alkaloid *in vitro,* it has become possible to produce in superficial or shaken cultures or in fermentors the following substances: agroclavine and elimoclavine [1, 3], lysergic acid amide and lysergic acid-methylcarbinolamide [4], ergometrine [5] and other alkaloids. For cultivation, mainly Abe's medium [1] or its modifications with mono- or oligosaccharides either alone or in combinations, as main component, were used. Within certain limits, the attainable alkaloid level is related with the amount of the added saccharide. The importance of the saccharide actually used for the production of alkaloid has prompted us to study the sugar decomposing and utilizing activity of alkaloid positive and negative Claviceps strains of different origin. The present paper is a report on our studies with oligosaccharides mainly saccharose.

Materials and methods

Strains. (a) *Claviceps sp. Ix 1326* mutant. The strain was isolated and selected from a mutagen-treated culture of a strain typically producing ergotoxin on rye. The mutant produced ergotoxin and ergometrin *in vitro.* (b) *Claviceps sp. T. 20.* The strain produced typically ergotamine on rye, being alkaloid-negative *in vitro.* (c) *Claviceps sp. Se 1. Sesleria coerulea Ard.* sclerotium (from the collection of T. Pócs) yielded in this laboratory a strain alkaloid negative *in vitro.* (d) *Claviceps sp. B 35.* The strain originated from *Pennisetum typhoideum* Rich. and produced agroclavine and elimoclavine *in vitro.* It was kindly supplied by Prof. A. Tonolo.

Maintenance of the strains and preparation of cultures. The strains were maintained on saccharose glycine agar by transfer at one or bi-monthly intervals. Maintenance cultures were transferred to a liquid enrichment medium and the mycelia obtained were homogenized. An amount of mycelium suspension representing a 5 per cent final concentration was transferred to the following medium: saccharose, 10 per cent; succinic acid, 1 per cent; KH_2PO_4, 0.25 per cent; $MgSO_4$, 0.25 per cent; $FeSO_4$, 0.9 mg per 100 g; $ZnSO_4$ 0.3 mg per 100 g. The pH was adjusted to 5.5 with ammonium hydroxide. The cultures were incubated for 96 hours at 24° C in a rotary shaker.

Activity of live cells or acetone-dried cells or cell-free extracts was examined under semi-anaerobic conditions [6], to facilitate demonstration of cleavage products. The bulk of mycelia obtained after 96-hour incubation was sedimented by centrifugation, washed three times in saline and used for the preparation of the following specimens: (a) 13 g (wet weight) of mycelia were suspended in a final volume of 40 ml; (b) acetone powder corresponding to 13 g wet weight of mycelia was suspended in 40 ml buffer; (c) 13 g wet weight mycelium was ground with quartz-sand in the presence of a buffer aliquot. After centrifugation, the mycelium-free supernatant was made up to 40 ml with buffer. Each of the above specimens was distributed to tubes in 4 ml aliquots.

As aerobic controls, (d) washed mycelia were suspended in buffer at the ratio given above and incubated in a rotary shaker.

The sugars examined were saccharose, raffinose, maltose, lactose, and trehalose at an initial concentration of 2 per cent. With aerobic incubation the utilization of glucose, fructose, and galactose, corresponding to cleavage products, was examined, too.

The medium was adjusted to pH 5.5 with 1/15 *M* phosphate buffer. The substrate was added at 0 time and samples were taken at 0, 1, 2, 4, 6 and 8 hours. The enzyme was inactivated by boiling for 2 minutes. Cleavage, or saccharide utilization was followed up by chromatography of 5 μl of a centrifuged supernatant on Macherey—Nagel 214 filter paper. Chromatograms were run in n-buthanol : acetic acid : water (4 : 1 : 1) solvent by the ascending technique and developed with benzidine.

Results

Claviceps sp. Ix 1326 mutant. All the three types of mycelium preparations (a, b and c, see above) decomposed saccharose, raffinose and trehalose under semi-anaerobic conditions. The activity of live mycelia and of cell-free extracts was relatively low. Maltose was decomposed only by the acetone powder while neither lactose nor melibiose were decomposed by any of the preparations.

Cleavage of saccharose yielded beside glucose and fructose also a benzidine positive product with an R_f value lower than that of saccharose. The latter were tentatively designated as oligosaccharides. None of the added or produced saccharides were metabolized by any of the samples (Fig. 1).

Cleavage of saccharose and raffinose was more intensive in shaken than in semi-anaerobic cultures (Fig. 2). The oligosaccharides formed from saccha-

rose as well as the produced and added glucose were utilized, whereas melibiose, fructose and galactose were not. The live cell suspension was inactive against maltose even under aerobic conditions.

Claviceps sp. T. 20. When incubated under semi-anaerobic conditions, the acetone-treated cells showed a slight saccharose and raffinose decomposing

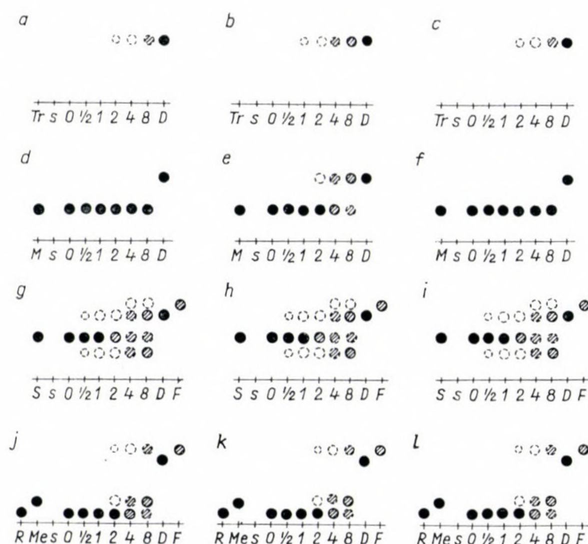

Fig. 1. Chromatographic analysis of oligosaccharide splitting by Claviceps sp. Mutant Ix 1326. Semi-anaerobic incubation. (a) live cells + trehalose; (b) acetone treated cells + trehalose; (c) cell free extract + trehalose; (d) live cells + maltose; (e) acetone-treated cells + maltose; (f) cell-free extract + maltose; (g) live cells + saccharose; (h) acetone-treated cells + saccharose; (i) cell-free extract + saccharose; (j) live cells + raffinose; (k) acetone-treated cells + raffinose; (l) cell-free extract + raffinose. The first one or two and the last one or two samples on the chromatograms are standards and the sample preceding 0 time is the control of the culture

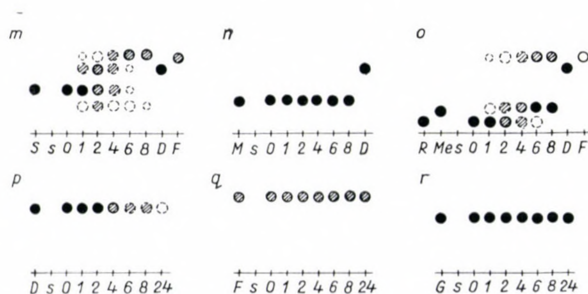

Fig. 2. Chromatographic analysis of oligosaccharide splitting by Claviceps sp. Mutant Ix 1326. Aerobic incubation. (m) live cells + saccharose; (n) live cells + maltose; (o) live cells + raffinose; (p) live cells + glucose; (q) live cells + fructose; (r) live cells + galactose. For other explanations, see Fig. 1

activity. Trehalose was decomposed by all the three preparations. No saccharide was consumed under semi-anaerobic conditions (Fig. 3).

In shaken cultures, decomposition of saccharose and raffinose was more intensive and glucose was utilized. Neither galactose nor fructose was consumed by this strain (Fig. 4).

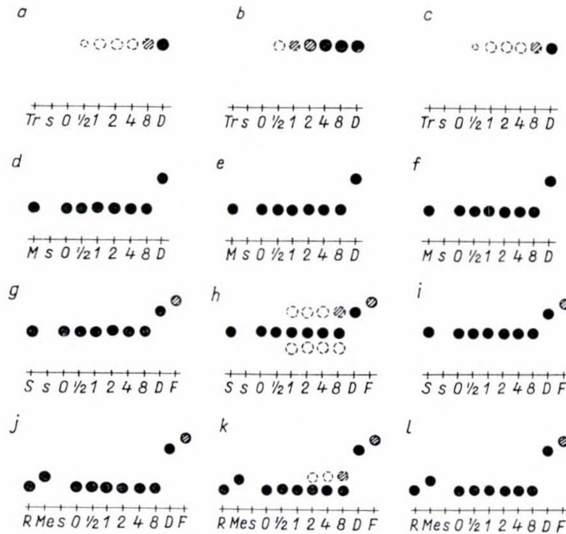

Fig. 3. Chromatographic analysis of oligosaccharide splitting by Claviceps sp. T. 20. Semi-anaerobic incubation. (a) live cells + trehalose; (b) acetone-treated cells + trehalose; (c) cell-free extract + trehalose; (d) live cells + maltose; (e) acetone-treated cells + maltose; (f) cell-free extract + maltose; (g) live cells + saccharose; (h) acetone-treated cells + saccharose; (i) cell-free extract + saccharose; (j) live cells + raffinose; (k) acetone-treated cells + raffinose; (l) cell-free extract + raffinose. For other explanations, see Fig. 1

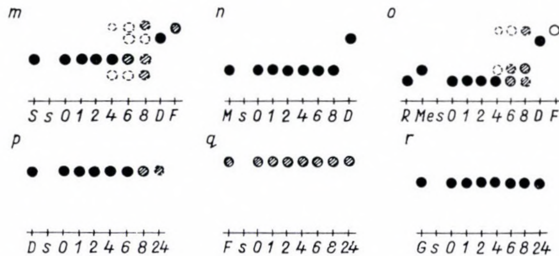

Fig. 4. Chromatographic analysis of oligosaccharide splitting by Claviceps sp. T. 20. Aerobic incubation. (m) live cells + saccharose; (n) live cells + maltose; (o) live cells + raffinose; (p) live cells + glucose; (q) live cells + fructose; (r) live cells + galactose. For other explanations, see Fig. 1

Claviceps sp. Se 1. Under semi-anaerobic conditions, all the three preparations exhibited moderate cleavage of saccharose and raffinose, and intensive decomposition of trehalose. None of them was active against maltose, lactose or melibiose.

Parallel to the cleavage of saccharose, oligosaccharides were produced. No sugar consumption was demonstrable (Fig. 5).

In shaken cultures, cleavage of saccharose and raffinose was remarkably more intensive and the oligosaccharides produced as well as glucose and moderately also fructose were utilized.

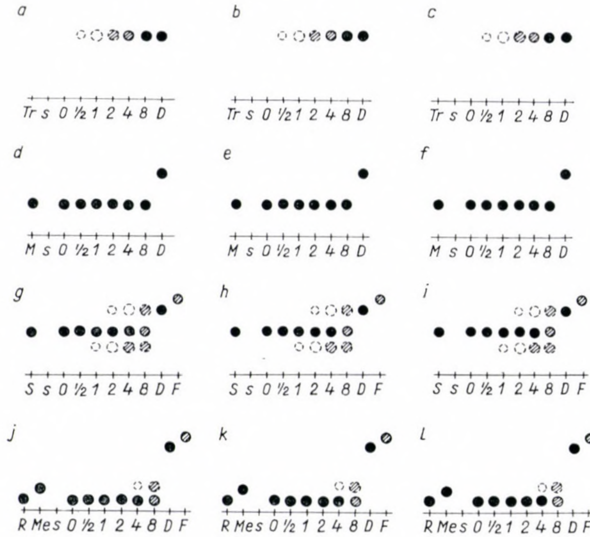

Fig. 5. Chromatographic analysis of oligosaccharide splitting by Claviceps sp. Se. 1. Semi-anaerobic incubation. (a) live cells + trehalose; (b) acetone-treated cells + trehalose; (c) cell-free extract + trehalose; (d) live cells + maltose; (e) acetone-treated cells + maltose; (f) cell-free extract + maltose; (g) live cells + saccharose; (h) acetone-treated cells + saccharose; (i) cell-free extract + saccharose; (j) live cells + raffinose; (k) acetone-treated cells + raffinose; (l) cell-free extract + raffinose. For other explanations, see Fig. 1

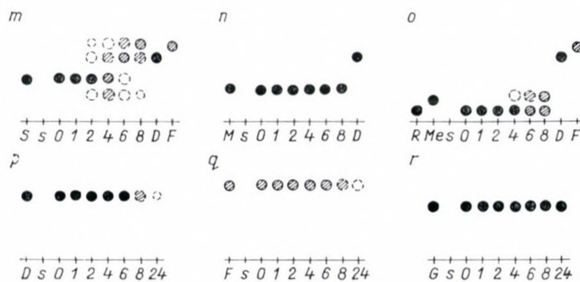

Fig. 6. Chromatographic analysis of oligosaccharide splitting by Claviceps sp. Se. 1. Aerobic incubation. (m) live cells + saccharose; (n) live cells + maltose; (o) live cells + raffinose; (p) live cells + glucose; (q) live cells + fructose; (r) live cells + galactose. For other explanations, see Fig. 1

The strains exhibited no activity against galactose (Fig. 6).

Claviceps sp. B 35. Under semi-anaerobic conditions, saccharose was definitely decomposed by the cell-free extract, the acetone powder was remark-

ably less active, while live mycelia were perfectly inactive. No cleavage of raffinose, trehalose, maltose or lactose was observed. No sugar utilization occurred under semi-anaerobic conditions (Fig. 7).

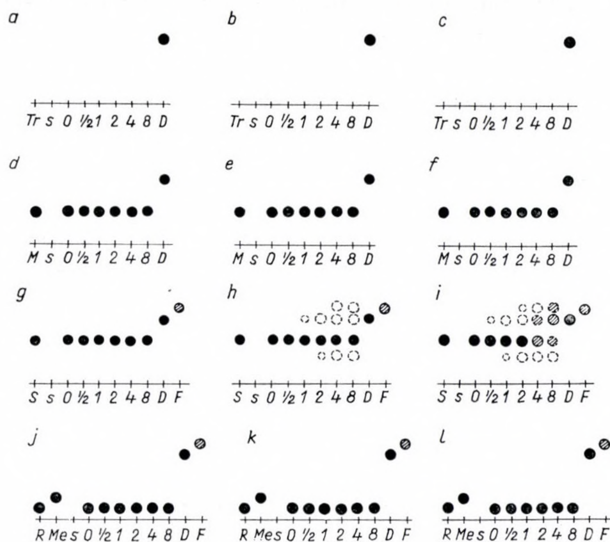

Fig. 7. Chromatographic analysis of oligosaccharide splitting by Claviceps sp. B. 35. Semi-anaerobic incubation. (a) live cells + trehalose; (b) acetone-treated cells + trehalose; (c) cell-free extract + trehalose; (d) live cells + maltose; (e) acetone-treated cells + maltose; (f) cell-free extract + maltose; (g) live cells + saccharose; (h) acetone-treated cells + saccharose; (i) cell-free extract + saccharose; (j) live cells + raffinose; (k) acetone-treated cells + raffinose; (l) cell-free extract + raffinose. For other explanations, see Fig. 1

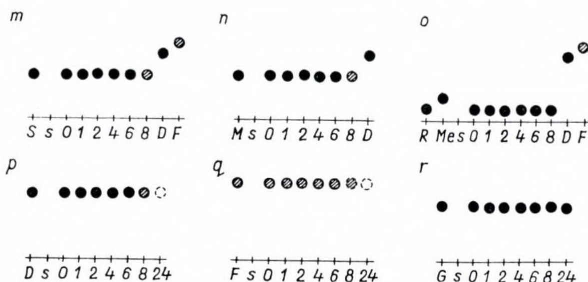

Fig. 8. Chromatographic analysis of oligosaccharide splitting by Claviceps sp. B. 35. Aerobic incubation. (m) live cells + saccharose; (n) live cells + maltose; (o) live cells + raffinose; (p) live cells + glucose; (q) live cells + fructose; (r) live cells + galactose. For other explanations, see Fig. 1

In shaken cultures, consumption of saccharose as well as of maltose was demonstrable. No cleavage products of saccharose were obtained. Out of the added monosaccharides, glucose and, to a moderate degree, also fructose, were utilized (Fig. 8).

Discussion

Claviceps Ix 1326 mutant. On the basis of the saccharose cleavage observed with all preparations of this strain, the presence of invertase was demonstrable. The cleavage of raffinose, the products obtained, and the oligosaccharide production parallel to the decomposition of saccharose suggested the presence of β-fructofuranosidase, a lipid solvent-resistant [6, 7, 8] enzyme localized outside the permeable barrier of the cell [9]. The produced oligosaccharides may be regarded as products of the transfer reaction first described in connection with yeast invertases [10] on the basis of their close relationship with saccharose and its cleavage products, and their R_f values. Beside the transfer activity of yeast invertase, the transfer ability of Euascomycetes-invertases has also been shown [11, 12]. With this observation a new family has been included in the subclass. The transfer products thus formed are chromatographically separable into four components, to be described in detail elsewhere.

Invertase activity showed an increase on aerobic incubation. This may have been a direct action or else the indirect result of the utilization of glucose produced under such conditions. Another consequence of glucose uptake was the gradual disappearance of the formed oligosaccharides. Under identical conditions a further increase of the rate of saccharose cleavage and transfer product utilization may be achieved by increasing the rate of glucose utilization, for example by adding ammonium salts of organic acids. Under such conditions a certain fructose uptake was also demonstrable, thus our statement concerning the inactivity against fructose should be completed by admitting the influence of experimental conditions. According to EDELMAN [12], the presence of glucose may have an influence on invertase activity by permitting a simultaneous partial saccharose re-synthesis parallel to saccharose decomposition with glucose as an acceptor in the trasfer reaction.

According to GOTTSCHALK [13], PHILLIPS [14], NOVÁK and ZSOLT [15], the cleavage of trehalose is indicative of the presence of trehalase, no other α-glucosidase being able to decompose this sugar. The enzyme has been known to be firmly bound [16, 17] as shown also in our studies by the high activity of acetone powder preparation. A similarly bound acetone-resistant trehalase has already been described with *Procandida (Candida) albicans* [15].

Different enzymes have been known to cleave maltose [18, 19, 20, 21], thus our studies do not allow for any other conclusion but to the presence of an acetone resistant endomaltase. The lack of maltose consumption by live cells may be explained by the lack of uptake of this particular sugar.

Claviceps sp. T 20. The results obtained with saccharose and raffinose under semi-anaerobic conditions pointed to the presence of an invertase. Activity was nevertheless poor and only with the acetone powder. Invertase productivity of this strain was clearly evident in shaken cultures.

A fundamental difference was demonstrable in the activity of the invertases of the two strains obtained from rye. With alkaloid negative strains, intact cells cleaved saccharose only under aerobic conditions, while with alkaloid positive strains, activity decreased slightly under semi-anaerobic conditions. No such difference was demonstrable with acetone powder preparations. This would suggest that the bounding of the substrate by the enzyme (active substrate uptake or enzyme liberation) requires an active aerobic process in the live cell. This process seemed less important with the alkaloid positive strain, an observation in accordance with the morphological differences of the hypha cells of the two strains, suggesting certain differences in their cell walls. Hypha cells of the alkaloid negative strain formed thin, highly refractile hair-tress-like bundles, whereas those of the alkaloid positive strain were short, stout, often swollen spherical cells. In microscopic preparations, rupture of the cell wall and cytoplasmic leakage has frequently been observed. This morphology has been regarded characteristic of the alkaloid positive strains also by TONOLO [4].

The two strains were different in the intensity of trehalose decomposition and in maltose cleavage.

Claviceps sp. Se 1. The strain possesses an invertase and, in intact cells, aerobic conditions are not required for the enzyme's full activity. This phenomenon appears to be quite particular, the strain being alkaloid negative. It should, however, be noted, that the strain freshly isolated from a sclerotium showed characteristics similar to those of strain T 20 and moderate semi-anaerobic saccharose and raffinose decomposition only appeared after repeated transfer in the laboratory. In the Figures only the latter state has been included, as the different strains isolated at different occasions could be compared only after a certain number of passages in the laboratory.

The number of transfers following the strain's isolation from a sclerotium appeared to have a certain influence on the function of other enzymes. Trehalose decomposition was the most active in freshly isolated Se 1 strain, whereas in T 20 and Ix 1326 it decreased in the said order. Strain B 35 isolated in 1954 [3], is entirely lacking this activity. The saccharide characteristic of the sclerotium is trehalose; strains recovered therefrom will comprehensively exhibit reduced specific enzyme production on maintenance on other carbon sources. Finally, the enzyme activity is lost.

Claviceps sp. B 35. The enzyme responsible for saccharose decomposition by this strain differs from those described for the other three strains. It does not decompose raffinose, its activity in aceton powder is relatively slow even under aerobic conditions as compared to cell-free extracts.

The enzyme is acetone sensitive. This type of endosaccharase has first been described in yeast by LINDEGREN *et al.* [22, 23], later also by us in further types of yeasts [8, 25, 26, 27].

The strain can be differentiated from the other examined strains besides its failure to attack trehalose also by the utilization of maltose by aerobic incubation of live cells. Considering the method of inoculum preparation, induction by maltose cannot be excluded.

All the strains examined failed to attack lactose, melibiose and galactose.

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ISOLATION OF ANTIBODIES BY GEL-FILTRATION

By

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Summary. Separation of rabbit antibodies against ovalbumin with a yield of 66—80 per cent has been achieved by dissociating ovalbumin—antiovalbumin precipitates in N acetic acid, followed by gel-filtration on Sephadex G-200. The isolated antibodies sedimented at 6.4 *S* in the ultracentrifuge and were identified as γ G-globulins by immunoelectrophoresis. Separation of antibodies resulted in practically no loss of precipitin properties against ovalbumin, while a fourfold activity over whole immune serum was observed in anaphylaxis experiments.

Separation of antibodies from immune sera may be accomplished by specific precipitation employing the homologous antigen, followed by dissociation of the dissolved precipitate and by separation of the dissociated components at the pH of dissociation [1], this last step being the most critical one.

Employing the gel-filtration technique of PORATH [2, 3, 4], substances different in molecular weight may be separated over wide pH ranges, as exemplified by the separation of lysosyme—antilysosyme or ribonuclease—anti ribonuclease, on Sephadex G-75 [5]. On the other hand, attempts to separate ovalbumin and antiovalbumin on the same gel type were unsuccessful.

FLODIN and KILLANDER [6] and FIREMAN *et al.* [7] succeeded in isolating serum proteins on molecular weight basis employing Sephadex G-200. Most recently, BOYNS and HARDWICKE [8] have reported on the separation by Sephadex G-200 of bovine serum albumin—rabbit antibody complexes dissolved in antigen excess. The data presented below deal with the separation on Sephadex G-200 of ovalbumin-antiovalbumin complexes dissociated at acid pH, resulting in the isolation of antiovalbumin antibodies.

Materials and methods

Proteins. (a) Ovalbumin prepared at our Institute was crystallized three times and found homogeneous by paper electrophoresis (OA). Immunodiffusion analysis revealed the presence of three contaminating components [9]. The molecular weight of the main component of ovalbumin preparation was 41,000, as determined in the ultracentrifuge. (b) Ovalbumin separated by gel-filtration was labelled with ^{131}I [10], ^{131}I (OA). (c) Normal rabbit γ G-globulin was prepared at our Institute [11].

Antiserums. (a) Rabbit immune serum against three times crystallized ovalbumin (OA—IS). (b) Goat immune serum against whole rabbit serum.

Antigen-antibody complex. One part of OA—IS was mixed with one part physiological saline and with the amount of gel-filtrated OA required for maximum precipitate formation, as determined in previous quantitative precipitation studies. The mixture was kept at 37° C for 2 hours and centrifuged at 4° C. The sediment was washed twice with cold physiological saline and dissolved in 1 to 2 ml N acetic acid. Any insoluble precipitate was centrifuged off and discarded.

Gel-filtration on Sephadex G-200. 1.0—1.5 g Sephadex G-200 (Lot No. To-6471) was allowed to swell in 0.1 *M* NaCl, washed and filled in a glass tube of 50 cm by 1.0 cm. The column was washed with pH 2.4 N acetic acid for 2 days. Protein solutions or ovalbumin—antiovalbumin complexes were applied in 0.2—2.0 ml samples and elution was carried out with N acetic acid at room temperature at a flow rate of 20 ml/hour. Fractions of 2.0 ml were collected and tested for protein concentration by absorbance measurements at 280 mμ in an Unicam SP-500 spectrophotometer, employing calibration curves taken with rabbit γ G-globulin and OA of known N-content. Radioactivity of eluates was determined in a *Frieseke—Hoepfner* apparatus, employing an FH 421/Z6 type NaJ(Tl) well-type scintillation detector. Activities thus obtained were converted into N-values on the basis of activity measurements of ^{131}I—OA solutions of known protein content. Eluate fractions for further analysis were dialysed against pH 7.0 physiological saline. The precipitate was removed by centrifugation and the supernatant was tested for protein content.

Immunoelectrophoresis was performed according to the SCHEIDEGGER microtechnique [12], at a voltage gradient of 3.05 V/cm in a pH 8.2 diethylbarbiturate buffer for 5 hours.

Ultracentrifugation was carried out in a Phywe U type apparatus.

Quantitative precipitation studies were done according to HEIDELBERGER, as described previously [13].

Passive sensitization. Antiovalbumin isolated by gel-filtration was injected intracardially to guinea pigs of 170 to 380 g body weight, in doses of 0.48, 0.24, 0.12 or 0.06 mg/kg body weight. The animals were shocked by 4.0 mg OA given intrajugularly after 48 hours interval [14].

Results

The filtration diagram of OA is illustrated by curve 1 in Fig. 1. Beside the ovalbumin peak with the maximum at the 34th ml two minor peaks at the 14th and 20th ml, respectively, can be seen resulting from contaminating components of higher molecular weight. In order to avoid the disturbing effect of these components on antibody isolation, antiovalbumin precipitation was carried out with ovalbumin samples purified by gel-filtration. Curve 2 visualizes the elution of normal rabbit γ G-globulin with the maximum protein content in the 22nd ml. The mixture of ^{131}I—OA and normal rabbit γ G-globulin yielded the filtration diagram illustrated by curve 3. The first peak at the 22nd ml coincides with curve 2 and corresponds to rabbit γ G-globulin. The dotted line (curve 4) exhibits the N-values of eluted ^{131}I—OA. The peak obtained at the 34th ml permits identification of the second flat peak of curve 3, resulting also from ovalbumin. The ascending slope of γ G-globulin contains no ovalbumin. The first 4 ml eluates after the globulin peak contain 4 to 5 per cent of ovalbumin, while eluates corresponding to the descending slope of curve 3 comprise increasing amounts of ovalbumin. Rabbit γ G-globulin and OA were mixed in proportions corresponding to the expected antigen—antibody ratios.

Based on the possibilities of separation as illustrated above, isolation of antiovalbumin was attempted from the dissociated antigen—antibody complex.

Antibodies were precipitated quantitatively from immune serum, the resulting ovalbumin—antiovalbumin complexes were dissociated at pH 2.4 and applied to the Sephadex G-200 column.

Fig. 2 illustrates the filtration diagram of the complex (curve 5) and the ^{131}I—OA content of eluates, based on activity measurements (curve 6, dotted line). The first peak of curve 5 at the 15th—16th ml corresponds very

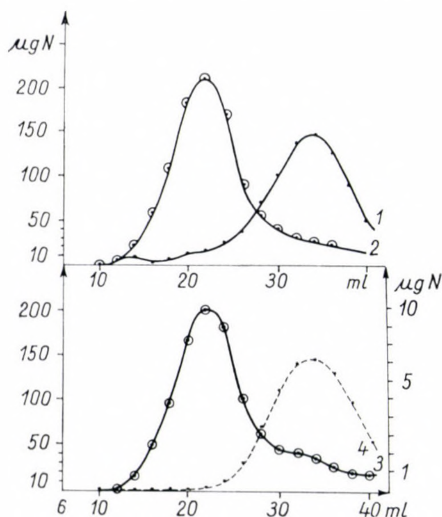

Fig. 1. Fractionation of proteins on Sephadex G-200 gel
Curve 1: Filtration diagram of OA
Curve 2: Filtration diagram of normal rabbit γ G-globulin
Curve 3: Filtration diagram of a mixture of normal rabbit γ G-globulin and ^{131}I—OA
Curve 4: Protein values of eluted ^{131}I—OA, expressed in μg N. Right scale refers only to Curve 4

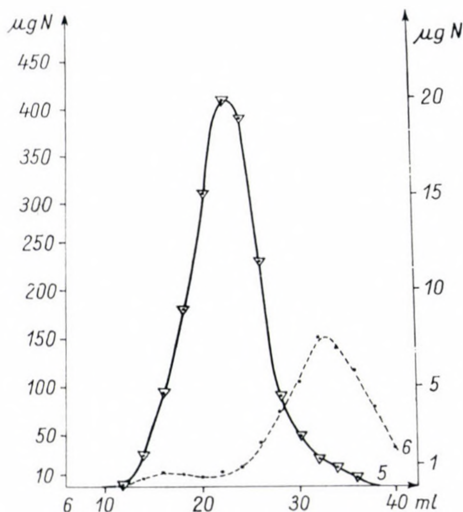

Fig. 2. Fractionation of proteins on Sephadex G-200 gel
Curve 5: Filtration diagram of dissociated ovalbumin-rabbit antiovalbumin complex
Curve 6: Protein values of eluted ^{131}I—OA expressed in μg N. Right scale refers only to Curve 6

likely to dissolved but not fully dissociated antigen—antibody complexes, since it coincides with a minor peak seen also on the ^{131}I—OA curve. The highest peak with the maximum at the 22nd ml corresponds to rabbit γ G-globulin. Dissociated ^{131}I—OA results in a third peak at the 33rd ml. Fractions between the 18th and 26th ml were collected and the resulting 10 ml solution was considered to be purified antiovalbumin, containing only about 5 to 12 per cent of ^{131}I—OA, as determined in 8 separate experiments.

The purified antiovalbumin sedimented with s_{20}, v = 6.4 S in the ultracentrifuge. It was subjected to immunoelectrophoretic analysis, employing the gel-filter γ G-fraction partly as antibody, partly as antigen. Results of these studies are illustrated in Fig. 3.

In the first picture the upper antigen well contained three times crystallized OA which was not subjected to gel-filtration. After electrophoresis, the ovalbumin yielded a broad precipitation band with the homologous antiserum filled into the central well. Besides, 3 arcs indicating the presence of contaminating proteins were observed. In the second arrangement the same OA preparation was subjected to electrophoresis, while the antibody well contained

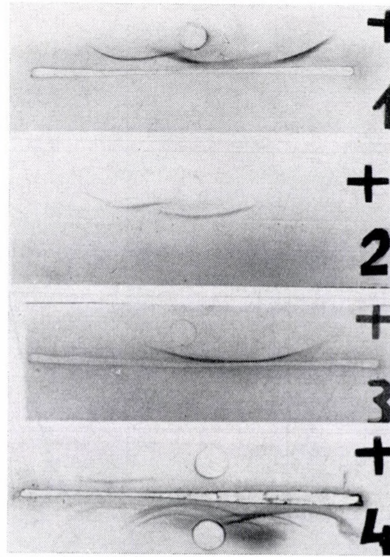

Fig. 3. Immunoelectrophoresis of rabbit antiovalbumin isolated by gel-filtration on Sephadex G-200. For detailed explanation see in text

the supernatant resulting after the absorption of antiserum against ovalbumin with ovalbumin purified by gel-filtration. The arc of ovalbumin is absent, while the contaminating protein bands are well distinguished. The third picture illustrates the reaction of the same OA preparation against antiovalbumin isolated by gel-filtration. One single arc corresponding to ovalbumin can be seen with no indication of contaminating antigen—antibody systems. Thus the antibody fraction isolated by gel-filtration contained purified, homogeneous antiovalbumin. In the fourth picture the upper antigen well was filled with gel-filtrated antiovalbumin, the lower antigen well contained 1 : 8 diluted normal rabbit serum as a control. After electrophoresis, the central well was filled with goat immune serum against whole rabbit serum. As seen, the isolated antiovalbumin corresponds to γ G-globulin, with no detectable traces of other immunoglobulins.

Next, the gel-filtered antiovalbumin was tested in quantitative precipitation experiments, employing purified OA added in pH 7.0 buffered physiological saline.

Table I

Quantitative precipitation studies with antiovalbumin preparations isolated on Sephadex G-200 from individual rabbit immune sera

No. of preparation	Protein content of gel-filtered antibody fraction, μg N/0.5 ml	Protein content of precipitate derived from gel-filtered antibody fraction, μg N/0.5 ml	Antigen—antibody molecular ratio of gel-filtered antibody fraction	Antigen—antibody molecular ratio of whole immune serum
1.	50	45	2.46	3.1
2.	100	105	3.06	3.1
3.	78	85	2.46	2.6
4.	65	52	2.02	2.26
5.	75	80	2.80	2.26

Table II

Anaphylaxis experiments on guinea pigs passively sensitized with antiovalbumin isolated by gel-filtration on Sephadex G-200

Body weight of guinea pigs g	Sensitizing dose of antibody μg N/kg	Eliciting dose of antigen, mg	Reaction after challenge
260	480	4	Fatal shock within 2 min.
310	480	4	Fatal shock within 1 min.
360	240	4	Fatal shock within 2 min.
277	240	4	Fatal shock within 2 min.
380	120	4	Fatal shock within 2 min.
370	120	4	Fatal shock within 2 min.
338	120	4	Fatal shock within 4 min.
355	120	4	Severe shock, survived
372	120	4	Fatal shock within 2 min.
306	120	4	Fatal shock within 2 min.
270	120	4	Fatal shock within 2 min.
226	120	4	Fatal shock within 2 min.
213	120	4	Fatal shock within 2 min.
228	120	4	Fatal shock within 2 min.
256	60	4	Mild shock symptoms
246	60	4	Severe shock, survived
337	60	4	Severe shock, survived
173	60	4	Fatal shock within 2 min.
178	60	4	Severe shock, survived

According to the data compiled in Table I, the purified antiovalbumin retained its precipitin activity to nearly 100 per cent. Antigen—antibody molecular ratio measured at the point of equivalence was found somewhat lower than in the case of whole immune serum.

Finally, guinea pigs were passively sensitized with different amounts of isolated antiovalbumin and challenged with 4.0 mg OA 48 hours later. Results of passive anaphylaxis experiments are shown in Table II.

Lethal anaphylactic shock could be elicited not only after the usual sensitizing dose of 480 μg antibody-N/kg body weight, but also in animals sensitized with only 240 or even 120 μg antibody-N/kg body weight. Since in previous experiments the minimum sensitizing dose of immune serum for lethal anaphylaxis proved to be 480 μg antibody-N/kg body weight for the used guinea pig strain [14], our present studies indicated a fourfold increase in biological activity of antiovalbumin antibodies purified by gel-filtration.

The final question to be answered refers to the yield of antibodies after gel-filtration. Losses during preparation may be attributed to the following circumstances. (1) After quantitative precipitation the precipitates obtained cannot be dissolved completely in N acetic acid. (2) Only a 10 ml combined portion of eluates is considered to be purified antiovalbumin, while some antibody is obviously contained also in other fractions. (3) During neutralization of the 10 ml combined eluate precipitates are formed, consisting mainly of ovalbumin having remained in the eluate but comprising also antibody molecules. The summed effect of these factors resulted during preparation in an antibody loss of about 21—34 per cent. Considering that antiovalbumin isolated by gel-filtration retained nearly 100 per cent of its precipitin activity, the described method allowed to isolate and recover about 66 to 80 per cent of the antibodies of whole antiovalbumin immune serum.

Discussion

In our introductory experiments Sephadex G-200 gel was found suitable for the separation of ovalbumin from normal rabbit γ G-globulin. The thus isolated rabbit γ G-globulin retained only 4 to 5 per cent of the ovalbumin content of the mixture. It seemed therefore likely that Sephadex G-200 would separate also the components of dissociated ovalbumin—rabbit antiovalbumin complex on a molecular weight basis. The prerequisite for separation is the complete dissociation of the antigen—antibody complex, as demonstrated by BENNETT and HABER [15].

In the present experiments antigen—antibody complexes were dissociated by *N* acetic acid. Attempts were, however, also made to promote dissociation by changing the pH of the solutes. Thus, the precipitates were dissociated

according to LEE *et al.* [16] in alkaline medium. The resulting marked reduction in flow rate, however, has made us to abandon this modification. Complete dissolution of precipitates could be achieved at pH 1.0 in 0.1 *N* hydrochloric acid. The quantity of ovalbumin remaining in the γ G-globulin fraction during gel-filtration was not inferior to that observed in the case of dissociation in *N* acetic acid. On the other hand, 0.1 *N* hydrochloric acid is close to the degree of acidity that would exert a deleterious effect on Sephadex gel. Considering these two circumstances, dissociation in hydrochloric acid was disregarded.

As demonstrated in Table II, a one-fourth dose of antiovalbumin separated by gel-filtration and dissolved in physiological saline was only required to sensitize guinea pigs for lethal anaphylactic shock, as compared to sensitization with whole immune serum [14]. This discrepancy may have its explanation in a competetive effect between normal gamma globulin and immunoglobulin molecules of native rabbit immune serum, similar to that observed by BIOZZI, HALPERN and BINAGHI [17] in passive cutaneous anaphylaxis experiments on guinea pigs.

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ALLERGIC TYPE SKIN REACTIONS IN ANIMALS WITH ALLOXAN DIABETES

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Summary. Attempts have been made to elicit by the intracutaneous administration of antigen-antibody complexes a passive *Arthus* phenomenon and passive cutaneous anaphylaxis in rabbits and mice with alloxan diabetes. These skin reactions of allergic type were prevented or suppressed by the induction of alloxan diabetes. Hyperglycaemia after glucose administration had a similar inhibitory effect. The decrease of the skin reactions might have been due to the suppression of histamine release and impairment of granulation tissue formation by hyperglycaemia.

Several authors have observed the influence of carbohydrate metabolism on the intensity of reactions provoked by dextran or ovomucoid [1—4]. Hyperglycaemia had a pronounced anti-inflammatory effect, while hypoglycaemia promoted inflammation. In our previous studies on animals with alloxan diabetes, Jancsó's test [5] and Shwartzman phenomenon were less intense than in normoglycaemic controls [6].

The experiments to be reported had the aim of clarifying the influence of alloxan diabetes and glucose induced hyperglycaemia on allergic skin reactions, such as the local passive *Arthus* phenomenon, inflammation evoked by antigen-antibody complexes, and passive cutaneous anaphylaxis.

Materials and methods

Skin reactions were performed on rabbits and mice. Alloxan (Reanal, Budapest) in doses of 100 mg per kg body weight was injected intravenously to rabbits twice in a 24 hour interval, while the mice received a single intraperitoneal dose of 250 mg per kg body weight. Transient hyperglycaemia was induced in rabbits by the intravenous or subcutaneous injection of 20 per cent glucose solution. Blood sugar levels were determined according to Hagedorn and Jensen.

Three times crystallized ovalbumin, and antiovalbumin rabbit immune sera were used throughout. The local passive *Arthus* phenomenon was elicited by the intracutaneous injection of antiserum, 60 minutes prior to the administration of antigen into the same skin area. Ovalbumin was applied in a triple dose as compared to the antigen amount at the point of equivalence in quantitative precipitation studies.

Antigen-antibody complexes were prepared by mixing the ingredients in ratios corresponding to the point of equivalence, followed by incubation for 2 hours at 37° C and 48 hours at 4° C. The resulting precipitate was exhaustively washed with physiological saline in the cold, dissolved in a 3—4-fold excess of antigen at room temperature and injected into the back skin of rabbits. The resulting reaction was read after 24 hours.

The local passive *Arthus* phenomenon and the skin reaction evoked by antigen-antibody complex were evaluated according to the scheme of COCHRANE and WEIGLE [7] as follows. +: mild oedema and erythema, up to 15 mm in diameter; ++: mild to moderate oedema and erythema, 20 to 30 mm in diameter; +++: moderate oedema and erythema of 30 to 40 mm with haemorrhage measuring 5 to 15 mm; ++++: marked oedema and erythema of more than 40 mm, with haemorrhage measuring more than 15 mm, or necrosis.

Passive cutaneous anaphylaxis experiments were performed on mice. Injection of 1.5—2.0 μg N of antigen into the abdominal skin was followed 2 hours later by the intravenous administration of 1.5 mg antibody-N in a 0.5 per cent EVANS blue solution. The resulting blue coloration read after 45 minutes on the internal skin surface was evaluated according to diameter as follows. + = 5—10 mm; ++ = 10—15 mm; +++ = 15—20 mm; ++++ = above 20 mm.

All experiments included appropriate controls; in these tests normal rabbit serum was administered instead of the anti-ovalbumin immune serum, with uniformly negative results.

Results

Local passive *Arthus* phenomenon was elicited in rabbits, with immune sera of varying titres. As demonstrated in Table I, normoglycaemic control animals responded with marked skin reactions. On the other hand, rabbits with alloxan diabetes and a blood sugar level of above 250 mg per 100 ml

Table I

Local passive ARTHUS *phenomenon in control rabbits*

No. of animal	Antibody-N mg	Antigen-N mg	Blood sugar, mg per 100 ml	Reaction intensity
1	0.17	0.078	85	++++
2	0.17	0.078	113	+++
3	0.21	0.060	85	++++
4	0.21	0.060	113	+++
5	0.24	0.14	85	++++
6	0.24	0.14	113	+++
7	0.24	0.14	75	+++
8	0.24	0.14	105	++
9	0.48	0.20	75	++++
10	0.48	0.20	105	++++
11	0.65	0.18	85	++++
12	0.65	0.18	113	+++

(Table II) exhibited mild or no reaction. Hyperglycaemia due to glucose administration exerted a similar suppressing effect on the local passive *Arthus* phenomenon.

Antigen-antibody complexes were prepared from immune sera of various antibody content. After the intradermal injection of these complexes normo-

Table II

Local passive ARTHUS *phenomenon in rabbits with alloxan diabetes or glucose induced hyperglycaemia*

No. of animal	Antibody-N mg	Antigen-N mg	Blood sugar, mg per 100 ml	Reaction intensity
13*	0.17	0.078	183	+
14	0.17	0.078	143	+
15	0.17	0.078	385	—
16	0.21	0.060	385	—
17	0.21	0.060	385	—
18	0.24	0.14	183	+
19	0.24	0.14	266	—
20	0.24	0.14	280	—
21	0.24	0.14	385	—
22	0.48	0.20	280	—
23	0.48	0.20	266	—
24	0.65	0.18	183	+
25**	0.40	0.30	183	+++
26	0.40	0.30	385	—
27	0.48	0.20	280	+
28	0.48	0.20	183	++
29	0.65	0.18	266	+
30	0.65	0.18	266	+

* Rabbits with alloxan diabetes
** Rabbits with glucose induced hyperglycaemia

Table III

Skin reaction evoked by antigen-antibody complex in control rabbits

No. of animal	Antibody-N mg	Blood sugar, mg per 100 ml	Reaction intensity
31	1.22	105	++++
32	1.22	97	++++
33	1.22	110	+++
34	1.32	84	++++
35	1.32	90	++++
36	1.32	84	++++
37	1.64	102	++++
38	1.64	84	++++
39	1.68	97	++++
40	1.68	112	++++
41	2.52	112	++++
42	2.52	104	++++

Table IV

Skin reaction evoked by antigen-antibody complex in rabbits with alloxan diabetes or hyperglycaemia induced by glucose

No. of animal	Antibody-N mg	Blood sugar, mg per 100 ml	Reaction intensity
43*	1.22	279	++
44	1.22	340	—
45	1.22	295	+
46	1.37	349	—
47	1.37	322	—
48	1.37	315	—
49	1.64	385	—
50	1.64	355	—
51	1.68	385	—
52	1.68	340	—
53	2.52	315	—
54	2.52	263	+
55**	1.64	385	—
56	1.64	220	++
57	1.68	183	++++
58	1.68	185	+++
59	2.52	385	—
60	2.52	185	+++

* Rabbits with alloxan diabetes
** Rabbits with glucose induced hyperglycaemia

glycaemic rabbits almost without exception displayed a maximum response (Table III), whereas hyperglycaemic rabbits failed to exhibit the characteristic skin reaction (Table IV).

In order to clarify whether or not the observed suppressing effect was specific to the rabbit, a series of experiments was performed in mice, this species being very suitable for the induction of both alloxan diabetes and passive cutaneous anaphylaxis [8]. As demonstrated in Table V, marked diabetes fully prevented the development of passive cutaneous anaphylaxis in mice.

Discussion

In our previous studies, diabetes or glucose induced hyperglycaemia was found to suppress or inhibit JANCSÓ's test, but to exert no effect on anaphylactic shock in the dog or the guinea pig [6]. The role of histamine in the pathogenesis

Table V

Passive cutaneous anaphylaxis in control and diabetic mice

No. of animal	Blood sugar, mg per 100 ml	Reaction intensity
61*	104	+++
62	54	++++
63	52	++++
64	64	+++
65	80	++++
66	70	++++
67**	518	—
68	418	—
69	408	+
70	478	—
71	522	—
72	452	—
73	542	—
74	442	+
75	460	—
76	466	—
77	520	—
78	474	—

* Control mice
** Mice with alloxan diabetes

of anaphylaxis in these species is undoubted. We suggested that hyperglycaemia, while exerting a definite inhibitory effect on protracted histamine release, could not be operative when histamine liberation occurred promptly, *e.g.* in the case of acute anaphylaxis.

In the present experiments allergic skin reactions were suppressed or prevented by alloxan diabetes or glucose-induced hyperglycaemia. Since histamine plays an important role in the development of allergic skin lesions [8], diabetes and hyperglycaemia are likely to exert their inhibitory action by interfering with histamine liberation, as suggested also in the literature. According to Goth *et al.* [9], diabetes markedly inhibits histamine release induced by dextran. Fowler, Szegő and Sloan [10] found a significant drop in intrinsic histamine release in the uterus of rats with alloxan diabetes. As reported by Sheldon and Bauer [11], and recently by Sacra and Adamkiewicz [12], hyperglycaemia prevented disruption of mast cells and suppressed the toxic action of the histamine liberator compound 48/80.

The impairment of granulation tissue formation by diabetes and hyperglycaemia [11, 13, 14] may be a related phenomenon. Besides the inhibition of histamine release, that effect may contribute to the marked depression of allergic skin lesions during hyperglycaemia.

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ENHANCING EFFECT OF HUMAN ERYTHROCYTE EXTRACTS ON THE SUSCEPTIBILITY OF MONKEY KIDNEY CELLS TO CERTAIN ENTEROVIRUSES

By

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Summary. Previous studies showed that the multiplication of certain mutants of haemagglutinating echoviruses was enhanced in primary monkey kidney (MK) cultures treated with human erythrocyte extracts (HEE). The practical applicability of this phenomenon was studied in comparative virus isolation experiments made in HEE-treated and untreated MK cultures. From faecal specimens of 175 healthy infants altogether 53 virus strains were isolated; of these 32 were detectable in both kinds of cultures; one in untreated, and 20 in HEE-treated ones. Of the latter strains 13 belonged to haemagglutinating enterovirus types. Treatment of MK cultures with HEE is recommended for routine diagnostic tests, as the virus isolation rate was significantly increased by this simple procedure.

Investigations of the action of cell extracts on haemagglutinating echoviruses led to the discovery that extracts of human cells — including erythrocytes — contain substances which enhance the multiplication of certain haemagglutinating viruses in primary monkey kidney cells. This effect was first demonstrated in the case of haemagglutinating (H^+) virus lines derived by the terminal dilution technique from prototype strains of echovirus type 3, 6, 7 and 12, and of some echovirus strains present in faecal samples [1]. Further studies on mutants of echovirus type 19 have revealed that just those H^+ virions (mutant No. 6) are sensitive to this action of human cell extracts which appear to represent the natural kind of this serotype [2]. Thus, it seemed of interest to study the practical usefulness of this phenomenon in virus isolation experiments. Faecal samples of 175 healthy children under 2 years of age were tested for enteroviruses by inoculating them into normal monkey kidney cell cultures and those treated with human erythrocyte extract.

Materials and methods

Collection and preparation of faecal specimens. Faecal samples were taken from 175 healthy infants between 3 months and 2 years of age, in December, 1964, *i.e.* before the start of a nation-wide vaccination campaign with live poliovirus vaccine. These samples constituted part of the material taken for virological control of vaccinations. The infants lived in 12 counties of Hungary. Only one or two of them belonged to the same child community. The specimens were sent on by ordinary mail, and were prepared for virus isolation experiments as described previously [3].

Virus isolation experiments were performed on primary monkey (rhesus) kidney (MK) cell cultures prepared by a minor modification of YOUNGNER's method [4]. 7 to 9 day-old tube cultures were used. All faecal specimens were inoculated into 6 tube cultures 3 of which were pretreated immediately before inoculation with human erythrocyte extract (HEE) as follows. After the growth medium had been poured off, 0.1 ml of HEE was added to the cultures and these were allowed to stand in slanted position for 30 minutes, at room temperature. HEE was prepared as described previously [1] and diluted to contain 1 HAI units in 0.025 ml titrated against 8 HA units of echovirus type 7 H^+ line [1]. Both HEE-treated and untreated cultures were inoculated with 0.1 ml of faecal specimens each and after 30 minutes of incubation at room temperature 0.9 ml PARKER's medium No. 199 was introduced.

The cultures were incubated at 37°C for 14 days and checked for the appearance of cytopathic alterations every other day. On the 7th day of incubation the medium of all negative cultures was replaced by a fresh one. Blind passages were carried out with media of cultures showing toxic or uncharacteristic alterations. Harvests from HEE-treated and untreated cultures were passaged in respective cultures. The cultures were investigated for 14 days in blind passages, too.

Virus isolation was attempted also in primary human embryonic kidney cell cultures from specimens which previously yielded virus in HEE-treated MK cell cultures only. The method of inoculation, maintenance, and observation of these cultures was identical with that of MK cultures.

Identification of the isolates was carried out by the CPE neutralization test in the cell system in which isolation had been made. In the case of haemagglutinating strains the identification was first attempted with the haemagglutination inhibition test (HAI). Rabbit immune sera prepared against poliovirus type 1—3, coxsackievirus type A-7, A-9, B 1—6, echovirus type 1—9, 11—27, 29, 30, 32, adenovirus type 1—7 and reovirus type 1, were used. The schedule of immunization was described previously [3]. HA and HAI tests were carried out in TAKÁTSY's Microtitrator apparatus [5].

Results

Virus isolations from faecal specimens of infants in untreated and HEE-treated MK cultures are summarized in Table I. Of 175 specimens 53 gave positive results. All but one of specimens yielding virus in untreated MK cultures were positive in HEE-treated ones, too, but in the latter 20 additional

Table I

Results of virus isolation experiments in untreated and HEE-treated monkey kidney cell cultures

		Isolation experiment in untreated cultures		Total	
		positive	negative	No	%
Isolation experiment in HEE-treated cultures	positive	32	20	52	30
	negative	1	122	123	70
Total	No	33	142	175	
	%	19	81		100

samples proved to be positive. Thus, the number of isolates in HEE-treated cultures was more than one-and-a-half times more than that obtained in untreated ones.

Types and frequency of the viruses isolated in untreated and HEE-treated cultures are shown in Table II. Of the 20 strains isolated in HEE-treated cultures, 13 belonged to the echovirus group, 1 to the coxsackievirus group, 2 to the reovirus group; 4 remained unidentified. The latter viruses were ether resistant. Their isolation was repeatedly successful in HEE-treated cultures, thus they had very probably originated from faecal specimens and not from the cell cultures. The majority (15) of the additionally isolated strains

Table II

Type-distribution of strains isolated in untreated and HEE-treated monkey kidney cell cultures

Virus types		Number of strains isolated in	
		untreated	HEE-treated
		cultures	
Echo	3	0	1
	5	0	1
	6	3	4
	7	3	3
	8	2	2
	11	2*	4
	12	2	5
	13	1	1
	14	4	4
	19	0	4
Coxsackie	B-2	6	6
	B-3	4	5
Reovirus		1	3
Coxsackie B-2 + Echo 12		1	1
Unidentified		4	8
Total		33	52

* One of these strains failed to be isolated in HEE-treated cultures

belonged to virus types capable of agglutinating human erythrocytes (echovirus type 3, 6, 11, 12, 19; coxsackievirus B-3, reovirus).

Isolation experiments in human embryonic kidney cultures made with specimens from which virus had only been isolated in HEE-treated cultures gave positive results in as few as 12 cases. One echo 11, two echo 12, one coxsackie B-3, one reovirus and 3 unidentified viruses failed to be isolated in human embryonic kidney cells.

HEE-treated cultures were more favourable than untreated ones not only with respect to the number of isolates but also to the average interval between inoculation and the first sign of CPE, which interval amounted to 4 days in contrast to the 9 days in untreated cultures. Moreover, toxic alterations occurring after inoculation of faecal suspensions were significantly less frequent in HEE-treated cultures.

Discussion

It has been shown earlier [1] that MK cells treated with HEE or extracts of permanent human cell lines yielded higher titres of the haemagglutinating mutants selected from prototype strains of certain echovirus types. A later analysis of the virion population of echovirus type 19 revealed that out of the nine mutants obtained (six H^+ and three H^-) only one H^+ mutant was enhanced by HEE. This mutant seemed, however, the most important from the practical point of view because in contrast to the prototype strain freshly isolated strains consisted predominantly or exclusively of such virions which thus might be regarded to represent the natural kind of virion predominating the virus population multiplying in the human intestinal tract [2]. We supposed that virions of all of those serotypes the growth of which was enhanced by HEE in MK cultures might have been similar in nature, *i.e.* such virions may occur also in other echovirus types. As this kind of virion was observed to grow poorly in MK cells except when HEE had been added to the cultures, we assumed that the isolation rate will be increased when HEE-treated MK cultures are used in virus isolation experiments. The present investigations have justified that expectation since among the 175 faecal specimens there were 20 which contained virions able to multiply only in HEE-treated cultures. Of the 20 strains isolated in this way, 15 gave HA with human erythrocytes and 13 of the latter belonged to the enterovirus group. Thus, it is suggested that this particular kind of virions occur mostly in haemagglutinating enteroviruses.

It is not known which component of the cell extracts acts as a multiplication-enhancing factor, but it is contained only in cells of human origin. The same extracts also inhibit the HA of echoviruses, and treatments destroy-

ing this action deprive the extracts from the multiplication-enhancing effect. There is also a positive correlation between the two actions [6], but the available data are not sufficient for stating that the two effects are due to the same compound. As to the mode of action, our further studies [6] revealed that the component of human cell extracts promotes the penetration of virions which in the absence of human cell extracts are able to adsorb to, but unable to penetrate into, the MK cells.

LEE *et al.* [7] observed that the human kidney cell culture is the optimal system for isolation of viruses present in the human intestinal tract. Human kidney cells surpassed the MK cells "not only in number of positive isolations but also in the range of viruses isolated". As our experiments have shown, HEE-treated MK cells are more susceptible to certain viruses than human kidney cells. There are, however, no data, whether the range of isolable viruses is widened by HEE-treatment of MK cells. Nevertheless, it is recommended to use HEE-treated MK cultures instead of untreated ones for routine diagnostic purposes, because this simple procedure ensures significant increase in isolation rate.

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STUDIES ON THE INITIAL PHASES OF POLIOVIRUS REPRODUCTION CYCLE

II. COMPARATIVE STUDIES ON HeLa AND PERMANENT MONKEY KIDNEY CELLS

By

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Summary. Cells of HeLa and of a permanent monkey kidney line (PMK III/1) were treated with versene or trypsin for different periods of time prior to their infection with type 1 (Mahoney) poliovirus. Virus reproduction cycles were compared in cells suspended in HANKS' balanced salt solution (HBS) with, or without, bovine albumin (BA).

In identically infected permanent monkey kidney (PMK III/1) cells the final virus yield was regularly 10 times higher in HBS + BA than in plain HBS. Pretreatment of cells with versene or trypsin did not affect the difference, only the absolute yields decreased moderately parallel to the duration of trypsinization. The highest absolute titres were obtained in versenized cells.

In versenized or protractedly (30, 60 minutes) trypsinized HeLa cells, yields were identical in HBS and HBS + BA. The absolute titres were low. Trypsinization for 5 to 20 minutes resulted in a tenfold increase of the final yield in cells suspended in HBS + BA.

Wherever the tenfold difference of yield occurred also the lag period was shortened in HBS + BA.

HeLa cells seem to have three, whereas PMK III/1 cells two, different kinds of receptor.

According to a recent survey by HOLLAND [1], most studies on the conditions of adsorption and eclipse of enteroviruses in general and of polioviruses in particular have been performed on HeLa cells. Recent studies in this laboratory [2] on the permanent monkey kidney cell line PMK III/1 have revealed an infection-enhancing effect of bovine albumin (BA), in a system comprising type 1 poliovirus infected cells suspended in HANKS' balanced salt solution (HBS). Permanent monkey kidney cells being less frequently used by other authors, it seemed to be of interest to check our observations also on the poliovirus-HeLa cell system under identical experimental conditions.

Materials and methods

HeLa cells were obtained from the M. R. C. Laboratory Hampstead, in 1964, and maintained since that time in this laboratory by serial transfers in a medium consisting of PARKER's medium No. 199 (P 199) and 10 per cent calf serum.

One day prior to each experiment, the growth medium was replaced by serum-free P 199.

Trypsin treatment of cells. An 0.25 per cent solution of Difco trypsin in Ca and Mg free PBS was used. Monolayers of HeLa or PMK III/1 cells in culture bottles were washed three times with Ca and Mg free PBS. Trypsin solution heated to 37° C was then added and the bottles were incubated at that temperature. After one minute, the cells were suspended by shaking and incubation was continued as required in the actual experiment. Trypsin treatment

was stopped by adding to the suspension 9 volumes of chilled HBS. The cells were washed 3 times with HBS in the cold prior to addition of virus.

Versene treatment of cells. Monolayers washed 3 times with Ca and Mg free PBS were overlaid with an 0.1 per cent solution of versene in PBS. Incubation took place at 37°C for 15 minutes with occasional shaking. Suspended cells were washed 3 times with HBS at room temperature.

Other details concerning materials and methods have been described previously [2].

In all the present experiments, cells were infected with an input multiplicity of 1 of strain Mahoney, type 1 poliovirus.

Experimental

In the first experiment, the viral cycle was examined in HeLa cells suspended by versenization or trypsinization for 20 minutes at 37°C. Time of onset, course of replication and final yield of the viral cycle were compared

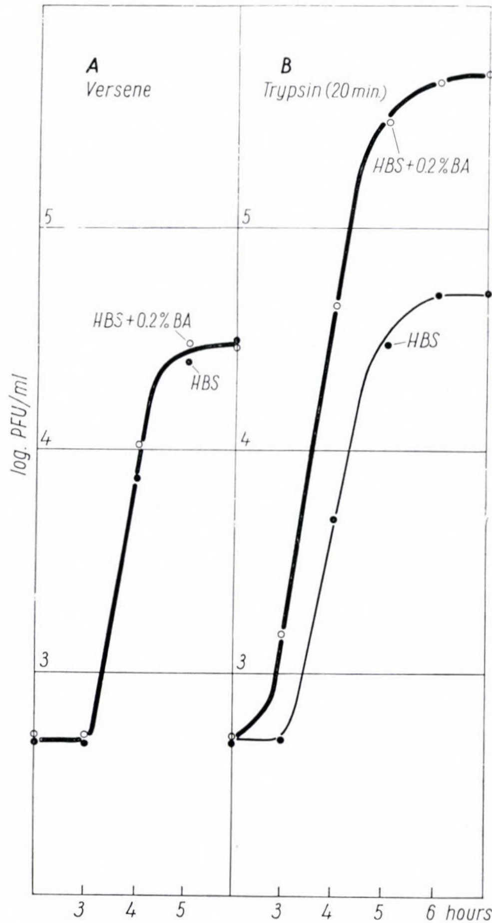

Fig. 1. Growth of poliovirus in versenized or trypsinized HeLa cells in Hanks' balanced salt solution with and without bovine albumin

in cells suspended after adsorption of virus in HBS with or without BA. Results are shown in Fig. 1.

Equally low yields and retarded onset were observed in both media when versenized HeLa cells were used. Trypsin treatment resulted in a tenfold increase of the yield and a shortening of the lag phase in cells suspended in

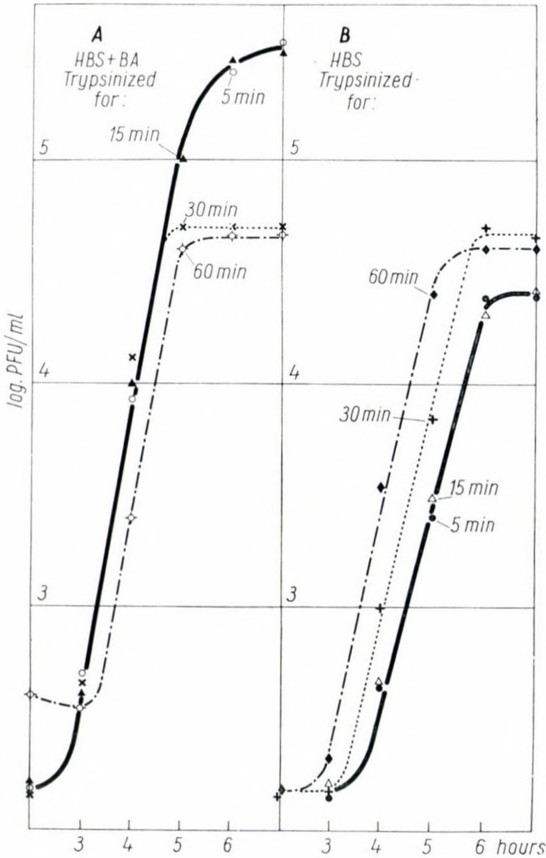

Fig. 2. Growth of poliovirus in HeLa cells trypsinized for different periods and suspended in Hanks' balanced salt solution with and without bovine albumin

HBS + BA following the infection. In plain HBS, both the yield and the time of onset resembled those observed with cells versenized prior to infection.

Trypsin treatment thus appeared either to remove some inhibitor(s) of infection or to activate (demask) certain efficient receptors. The time of onset and the final yield of the viral cycle were, therefore, examined in cells treated with trypsin for different periods of time prior to the adsorption of virus. Results are shown in Fig. 2.

HeLa cells trypsinized for 5 to 15 minutes yielded in a single cycle ten times more virus after a one hour shorter lag in HBS + BA than in plain HBS. Trypsinization for 30 and 60 minutes reduced the yield in HBS + BA to the level observed in plain HBS. Duration of trypsin treatment had, however, a remarkable effect on the lag phase of viral cycle in cells maintained in plain HBS, since the longer the period of trypsinization, the shorter was the lag phase (Fig. 2B). Trypsin treatment for 30 minutes abolished the infection enhancing effect of BA, but did not affect the lag phase. After 60 minutes,

Table I

Comparison of absolute and relative** final poliovirus yields in HeLa and PMK III/1 cells in HBS + BA and plain HBS*

Cells	Medium	Versene		Final yield in cells reated prior to infection with trypsin							
				for 5 min.		for 15 min.		for 30 min.		for 60 min.	
		absolute	re-la-tive	abs.	rel.	abs.	rel.	abs.	rel.	abs.	rel.
HeLa	HBS+BA	2.5×10^4	1	3.5×10^5	14	3.5×10^5	14	5×10^4	1.1	5×10^4	1.25
	HBS	2.5×10^4		2.5×10^4		2.5×10^4		4.5×10^4		4×10^4	
PMK III/1	HBS+BA	1.2×10^6	12	5×10^5	10	3×10^5	12	5×10^5	10	1×10^5	13
	HBS	1×10^5		5×10^4		2.5×10^4		5×10^4		7.5×10^3	

* PFU/ml

** $\frac{\text{Yield in HBS + BA}}{\text{Yield in HBS}}$

however, the effect of BA disappeared completely as regards reduction of lag and increasing final yield (Fig. 2A).

To clarify whether this effect of trypsin treatment was specific for HeLa cells, the same experiments were performed also with PMK III/1. Results are compared in Table I.

As compared to versenization, trypsinization for 5, 15 and 30 minutes reduced the final virus yield in PMK III/1 cells by 2 to 3 factors, without, however, affecting the tenfold difference between the yields in HBS + BA and plain HBS. Trypsinization for 60 minutes caused an about tenfold reduction of yield as compared to the versenized system, but still did not alter the roughly tenfold difference between yields in the two types of media.

With HeLa cells trypsinized for 5, 15 or 20 minutes, an about tenfold increase of yield was observed in the presence of BA. Yields were independent

of the presence of BA in versenized cells as well as in those trypsinized for 30 to 60 minutes. Among all the systems tested, versenized HeLa cells produced the lowest absolute amounts of virus.

Discussion

There is a fair amount of information available on the nature and many characteristics of the enterovirus receptors of different normal and malignant primate cells *in vitro* and *in vivo* [1]. The specific substance itself has been identified as a lipoprotein able to react with the appropriate enteroviruses also in the form of a solubilized "inhibitor" [3, 4]. The amount of receptors for enteroviruses is high in the membrane of HeLa cells, while relatively low in cultured monkey kidney cells [5]. Adsorption studies in appropriate systems using live untreated HeLa cells have shown that large numbers of virions may rapidly attach to most of the cells [6]. Nevertheless, a discrepancy was observed between the number of attached and virtually eclipsed virions and of those effectively initiating infection [7, 8]. Later it was discovered that the majority of virtually eclipsed virus was actually rejected in an "inactive" form into the fluid phase of the system. The "inactive" virions were demonstrated to have formed a stable complex with receptors and were rejected in this form [8]. Part of the firmly attached virions remained on the cell surface throughout the whole replication cycle initiated by a truly eclipsed virion [9].

Adsorption studies on monkey kidney cells have revealed very little, if any, rejection of "inactive" receptor-virion complexes [10]. Evidence is available concerning the possibility of prolonged persistence of firmly attached but not eclipsed fully active enterovirus virions on the surface of cultured monkey kidney cells [11].

Last but not least it should be remembered that the sensitivity to polioviruses of HeLa and monkey kidney cells is mostly quite similar, thus both may reasonably be supposed to eclipse comparable numbers of virions in systems suspended in a rich nutrient medium with 5 to 10 per cent serum content.

Using haemagglutinating and non-haemagglutinating mutants of certain enteroviruses, extracts of HeLa cells were found to enhance the efficiency of infection of monkey kidney cells by virions producing normally low titres in these cells [12].

In situ receptors of HeLa cells were shown to be sensitive to the action of trypsin as measured by the cells' capacity measurably to reduce the titre of a given virus suspension [13]. Even extensively trypsinized HeLa cells will fully regenerate their receptors within a short time [14]. HeLa cultures are known to produce considerable amounts of s lubilized receptors [12, 15] sitting on blebs extensively released parallel to the ageing of cells.

Considering the above, the present results have been interpreted as follows. The PMK III/1 cells carry a relatively small amount of receptors which is, however, easily available for the virus and exhibits a considerable resistance to trypsin. HeLa cells, on the other hand, possess large amounts of receptors. A major part of these is localized on the readily detachable blebs covering large areas of the surface, whereas the rest is to be found on the smooth areas of the cell surface. The former may act as "virtual inhibitors" of infection. The virions bound by them are rejected in an "inactive" form together with the whole bleb. Trypsinization for short periods (up to 20 minutes) appears to remove the detachable receptors from HeLa cells, thus facilitating sterically the attachment to receptors on the residual smooth areas. The latter type of adsorption offers a higher probability of a true eclipse, resulting in the infection of the cell. Versenization of cells fails to remove the detachable receptors which may, therefore, act as virtual inhibitors of infection.

This concept would explain the differences observed in the time of onset and final yield in versenized or trypsinized HeLa cells incubated after the infection in HBS + BA. In plain HBS, however, low but reproducible yields were obtained apparently independently of pretreatment as well as of the absence or presence of BA in the incubation medium. The latter phenomenon was observed also with PMK III/1 cells. Thus it appeared that part of the receptors of primate cells exhibits a special avidity. Attachment to an "avid" receptor results in a ready eclipse independently of the presence or absence of an infection-enhancing factor like BA. In HeLa cells trypsinized for 30 and 60 minutes only these "avid" receptors will remain intact, while the "rejectable" as well as the "common" receptors (*i.e.* those requiring an enhancing factor for true eclipse) are destroyed.

PMK III/1 cells fail to have "rejectable" receptors, while they possess both "common" and "avid" ones. In both cell lines, the number of "avid" receptors is ten times lower than that of the "common" ones. The relative resistance to trypsin of "common" receptors of PMK III/1 cells remains to be explained. No evidence is available to decide whether the differences in the behaviour of receptors are of a quantitative or a qualitative nature.

Further studies are in progress in this laboratory to test this tentatively proposed hypothesis.

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STUDIES ON THE INITIAL PHASES OF POLIOVIRUS REPRODUCTION CYCLE

III. ACTION OF FATTY ACIDS AND TWEEN 80

By

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Summary. Suspended cells of a permanent monkey kidney cell line (PMK III/1) infected with type 1 (Mahoney) poliovirus yielded low amounts of virus in plain Hanks' balanced salt solution (HBS). Addition of 0.2 per cent bovine albumin (BA) caused a tenfold increase of the yield. The possible similar action of fatty acids regularly present in BA and of Tween 80, present in Parker's No. 199 (P 199) medium has been studied.

Linoleic and oleic acids completely suppressed virus reproduction when applied at 10^{-4} *M* final concentration. At the same concentration, arachidic and stearic acids exhibited about 50 per cent of the activity of BA. Palmitic acid was somewhat less effective. At 10^{-5} *M* concentration, as referred to BA, the activity of arachidic acid was 90 per cent, that of linoleic and oleic acids was 40 per cent, while stearic and palmitic acids exhibited 32 and 22 per cent activities, respectively. Arachidic acid showed 50 per cent activity at 10^{-7} *M* concentration. Oleic acid had 30 per cent activity, whereas stearic and palmitic acids were practically inactive at this concentration.

Tween 80 was inhibitory at 150 μg/ml concentration, inactive at 1.5 μg/ml and as active as BA at 15 μg/ml concentration.

The activity of fatty acids within the concentration range of 10^{-4} to 10^{-7} *M* appeared to depend on the concentration and the chain length. The higher the concentration and the longer the chain, the higher was the activity. The inhibitory action of unsaturated fatty acids at high concentrations (10^{-4} *M*) suggested the possible involvement of some events sensitive to oxidation-reduction processes.

The presence of the tested fatty acids and of Tween 80 in the first hour of the cycle was necessary for ensuring the production of full final yield. Addition at later points of time failed to have any effect.

These substances are supposed to be factors facilitating the penetration of attached virions. A working hypothesis is presented.

As already reported [1], an infection enhancing action of bovine albumin (BA) was observed in a system comprising suspended permanent monkey kidney cells (PMK III/1) infected with type 1 (Mahoney) poliovirus by brief exposure at an input multiplicity 1. The basal maintenance fluid was Hanks' balanced salt solution (HBS). A preliminary assay with K-linoleate pointed to the probable importance of fatty acid contaminants of BA in the effect observed.

The present report deals with some further studies of some saturated and unsaturated fatty acids and the detergent Tween 80.

Materials and methods

Cells, media, methods of infection, of one-step growth experiment and of virus assay have been described previously [1].

Chemicals. Tween 80 was a product of Hilltop Labs., Cincinnati, Ohio, U.S.A. Appropriate dilutions were prepared in HBS.

Fatty acids. Stock solutions of 10^{-2} *M* of the fatty acids listed below were prepared in 96 per cent ethanol. Dilutions were also prepared in ethanol, so as to allow for a further 1 : 100 dilution in HBS to obtain the final concentration required in the actual experiment. Arachidic (eicosanoic) acid, Fluka A. G. Switzerland. Stearic (octadecanoic) and palmitic (hexadecanoic) acids, Institute of Nutrition, Budapest. K-linoleate (octadeca-9,12-dienoic acid), Serva, Germany. Oleic (octadeca-9-enoic) acid, Institute for Pharmaceutical Research, Budapest. All substances including ethanol were tested for acute toxicity at final concentrations twice the highest one used in the one-step experiment. In monolayer tube cultures of PMK III/1 cells incubated with the substances (five parallel tube cultures each) for 72 hours at 37° C, no microscopically detectable toxic effects were observed.

Experimental

Final yields of type 1 (Mahoney) poliovirus in PMK III/1 cells were identical in both PARKER's No. 199 (P 199) and HBS + BA media. On the basis of some preliminary assays this was considered an indirect evidence of the possible role of Tween 80 included at 15 μg/ml concentration into P 199 medium as a detergent facilitating the solution of cholesterol and fat soluble vitamins. An experiment with K-linoleate appeared to support the assumption that substances with detergent activity may be involved in the "infection enhancing" action of BA. The latter has been known to be regularly contaminated with fatty acids.

The experiment performed with Tween 80 and K-linoleate at different concentrations yielded results shown in Fig. 1.

The effect of linoleate was essentially identical with that already reported. Tween 80, at 150 μg/ml concentration, completely suppressed virus reproduction. At a concentration of 1.5 μg/ml it had no influence whatever on the final virus yield in HBS. At 15 μg/ml concentration, Tween 80 was found to have an effect essentially identical with that of BA. Thus the role of Tween 80 in P 199 appeared to be clarified.

At appropriate concentrations in HBS, linoleate and Tween had not only elevated the final yield, but also reduced the lag phase in a way similar to that observed with BA. The relatively limited concentration range in which linoleate and Tween 80 (containing oleic acid) were active, suggested the possibility that at higher concentrations the presence of unsaturated bonds might have been responsible for the inhibition.

Oleic acid, present in Tween 80 and carrying a single double-bond, as well as some saturated fatty acids of different chain lengths were, therefore, also examined. Results of these comparative studies are shown in Table I.

In general, the activity of saturated fatty acids appeared to be in direct relation to concentration and chain length. At concentrations of 10^{-5} to

10^{-7} *M*, arachidic and palmitic acids exhibited the highest and lowest activities, respectively. The activity of stearic acid was intermediate. Arachidic acid was moderately active at 10^{-4} *M*. Until further information will be available,

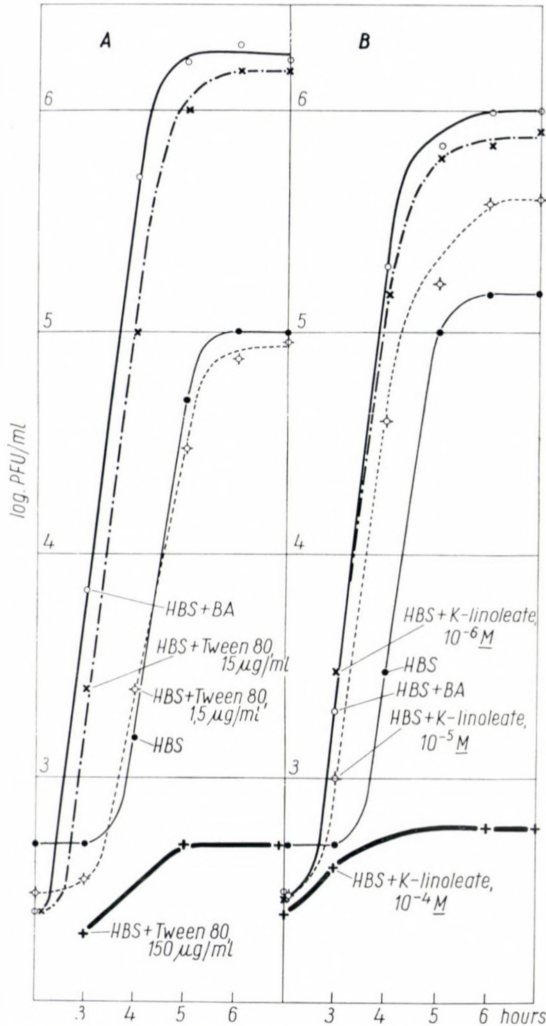

Fig. 1. Growth curve of poliovirus strain in the presence of Tween 80 (a), and K-linoleate (b), at various concentrations

we cannot tell whether this was the result of its poor solubility in HBS or of its true inhibitory action at that concentration.

The initially supposed inhibitory action of double bonds appeared to be confirmed also by the fact that at 10^{-4} *M*, both linoleate and oleic acid were strongly inhibitory, whereas stearic acid was distinctly active. At 10^{-5} *M*

Table I

Per cent final yield in Hanks' balanced salt solution supplemented with different fatty acids as referred to that in Hanks' plus bovine albumin

Fatty acid			Final yield (per cent) at fatty acid concentration (*M*)			
Name	Chain length in C atoms	Double bonds	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Arachidic acid	20	0	52	95	67	52
Stearic acid	18	0	52	32	22	11
Oleic acid	18	1	0.0006	45	41	30
Potassium linoleate	18	2	0.005	40	70	n. d.
Palmitic acid	16	0	40	22	11	11

n. d. = not done

Table II

Increase of final virus yield in the presence of different substances added at different points of time

Substance added	Time of addition (minutes)				
	0	30	60	120	180
Bovine albumin 0.2 per cent	10.0	5.0	2.0	1.0	1.0
Arachidic acid 10^{-5} *M*	10.0	8.0	5.0	1.5	1.0
Potassium linoleate 10^{-5} *M*	4.3	n. d.	n. d.	1.0	n. d.
Tween 80 20 μg/ml	9.7	4.4	3.0	1.5	1.0
Hanks' balanced salt solution only	1.0	—	—	—	—

n. d. = not done

concentration, all the three fatty acids containing 18 C atoms exhibited comparable activities, irrespective of the presence or absence of double bonds. Thus the inhibitory action of double bonds appeared to be "diluted out" and only the detergent activity manifested itself. The activity of fatty acids with double bonds appeared to exceed that of stearic acid at lower (10^{-6} and 10^{-7} *M*) concentrations.

Independently of their effects, all the tested fatty acids were uniformly reducing the lag phase in the same degree as 0.2 per cent BA did. To clarify whether fatty acids and BA were in fact required at the same phase of the viral cycle, experiments were performed in which 10^{-5} *M* arachidic acid or lino-

leate, or 20 μg/ml Tween 80 were added to the system at different phases of the viral cycle. Results as referred to a control system suspended in HBS + BA are shown in Table II.

The activity of BA as well as that of each of the tested substances was the lower the later the addition had taken place. From the second hour on, no activity whatever of either substance was demonstrable. This observation suggested the necessary presence of these substances during some early events of the viral cycle between the phases of adsorption and true eclipse. This assumption has been based on the fact that adsorption of virions was always allowed to take place prior to the transfer of cells into the experimental system (0 minute) and that photosensitivity has been shown by WILSON and COOPER [2] to be lost after 90 to 120 minutes.

Discussion

The present paragraph contains a general discussion of all the three papers of this series [1, 10] and a tentative working hypothesis.

The vague schedule of a possible new approach to the problem of penetration of polioviruses into susceptible cells seems to emerge from the experimental results obtained recently in this laboratory. We are perfectly aware of the incompleteness of information available, yet are unable to resist the temptation to describe some speculations in the form of a tentative working hypothesis.

As stated in a recent survey by PHILIPSON [3], studies on the early phase of attachment (adsorption) have long shown the general importance of ionizable groups of the virion and the receptor. Examination of the electrophoretic motility under different environmental conditions yielded important data on the average net charges of several different kinds of virions and cells as well as on the changes induced by their mutual interactions. In an earlier work from this laboratory [4, 5, 6], the number and nature of ionizing groups of influenza virus have been determined by potentiometry. Reaction of the basic groups with formaldehyde was shown to result not only in the disappearance of the corresponding parts of the titration curve, but also in a parallel loss of infectivity and haemagglutinating activity. Thus in this case, the electrostatic (Coulomb) nature of forces involved has been directly demonstrated in the attachment of the virus to the receptor.

The present studies have been performed in a poliovirus—host cell system. Adsorption of virus at an input multiplicity of 1 was allowed to take place in a highly concentrated system (10^8 virions and cells per 0.5 ml) for 10 minutes at room temperature and for an additional five minutes at 37° C. This method was supposed to limit the interaction to the formation of electro-

static bonds. This phase of enterovirus—host cell interaction has repeatedly been shown by others [7, 8, 9] to be reversible by appropriate methods.

The virion — cell complex thus formed was transferred into different media and the one-step growth curves were registered. In plain HBS the penetration was poor as shown by the low final yield and the delayed onset of the cycle. The presence of BA or of an appropriate fatty acid in the same system resulted in a shortening of the lag phase and an optimally tenfold increase of the final yield.

Comparative studies on HeLa cells [10] have shown that a similar effect of BA was only demonstrable with cells trypsinized for a short period (5 to 20 minutes). Versenized HeLa cells produced but poor yields after a prolonged lag phase, independently of the presence or absence of BA.

It was supposed that in untreated PMK III/1 and HeLa cells trypsinized for a short time there were two similar receptors present, *viz.* one allowing for penetration without BA ("avid receptor") and another, requiring the presence of BA for the successful penetration of a virion ("common receptor"). The third type, the "virtually inhibitory" receptors, observed in versenized HeLa cells, are thought to be those localized on blebs being released together with the attached virions, and thus deprived of their chance to initiate infection.

The studied fatty acids, as well as Tween 80, were found to have an effect similar to that of BA. The activity of the individual fatty acids at appropriate concentrations appeared to be related with the chain length and the number of double bonds present. This importance of chain length suggested the requirement of fairly strict steric conditions for full effectiveness. It has been thought therefore that fatty acids may be involved in the formation of certain hydrophobic *van der Waals* type bonds between the virion and the receptor.

As to possible mechanism of action of fatty acids, we propose the following facts to be considered.

In contrast to some other enteroviruses, the fine structure of the icosahedral capsid of poliovirus has scarcely been elucidated. Nevertheless it is quite certain that it is constructed, like any other icosahedral virion, of a certain number of identical equivalent symmetrically packed structural units and of exactly 12 vertices. In the vertices, the arrangement of structural units results in their "quasi equivalence", thus the icosahedron's 12 vertices are likely to carry some "extra" electrostatic charge. This would suggest the supposition that these points might play the leading role in the orientated attachment of the virion to certain appropriate oppositely charged groups, tentatively designated as "receptor sites" within a receptor region of the cell.

The receptor sites may be distributed within a receptor region in a patternized or random way. This would definitely influence the possible modes of attachment of a virion. It is easily conceivable that the orderly or random distribution of sites has little influence on the probability of a one site one

vertex collision between a receptor and a virion. This factor has, however, an increasing importance if simultaneous collisions of two or three sites with two or three vertices are required.

Supposing a random distribution of sites, the probability of accidental fitting of two or three sites with two or three vertices of a virion must be extremely small. The probability of a two-vertices two-sites hit will remarkably increase if sites are distributed in a nearly regular triangular plane net with their mutual distances varying within a narrow range close to the length of one edge of the actual virion. In this system the simultaneous fitting of three sites to three vertices has still a very low probability, the accidental occurrence of equilateral triangles in such a net being dependent merely on chance. Should the sites be localized at the intersection points of a perfectly regular equilateral triangular plane net, comprising triangles exactly corresponding to those forming the facets of the viral icosahedron, the fitting of two vertices with two sites would necessarily involve the fitting of the third vertex, too (Fig. 2). Naturally, the one-vertex one-site hit, supposed to be the main type of the first reaction between virion and receptor, would allow also for a wide variety of non-fitting landings in any of the above systems.

Vertex to site attachment is supposed to involve *Coulomb* forces. These forces are known to be quite weak though having relatively wide ranges; thus an appropriate orientation as well as a stable attachment of a virion to a receptor would very probably require the simultaneous and cooperative function of several charged groups at minimally two vertices and two receptor sites.

A virion, landed on one of its facets, and attached through its three corresponding vertices to three appropriately arranged receptor sites seems therefore to represent a lowest state of energy, *i.e.* an optimal attachment. Should the attachment be of a two-vertices two-sites type, the *Coulomb* forces on the third vertex of the icosahedron's involved triangle fail to participate in the stabilization of attachment. This state is, therefore, regarded as suboptimal. If only one vertex reacts with one site, the attachment is highly labile in respect of both the virion's statics and the strength of the involved forces. Thus, apparently, the statistical probability of a given type of attachment is inversely related to its stability.

The model proposed would explain the experimentally evidenced relative average stability of attachment as well as its reversibility by factors efficiently influencing the net and total charges of the surfaces involved [3]. Current concepts on the probable chemical composition and molecular structure of enterovirus receptors [11] do not seem to contradict this model.

On the basis of general information available on the interaction of proteins, it appears reasonable to suppose that the simultaneous reaction of three receptor sites with three vertices of the virion may result in important conformational changes in the involved area of the receptor region. This, by itself

may represent a satisfactory stimulus for the initiation of extensive molecular and fine structural changes in the cell membrane. Should the attachment occur between two vertices and two sites only, this stimulus may conceivably be below the threshold required for further changes. In the latter case, therefore, some additional factors may be necessary to induce appropriate changes in the cell membrane.

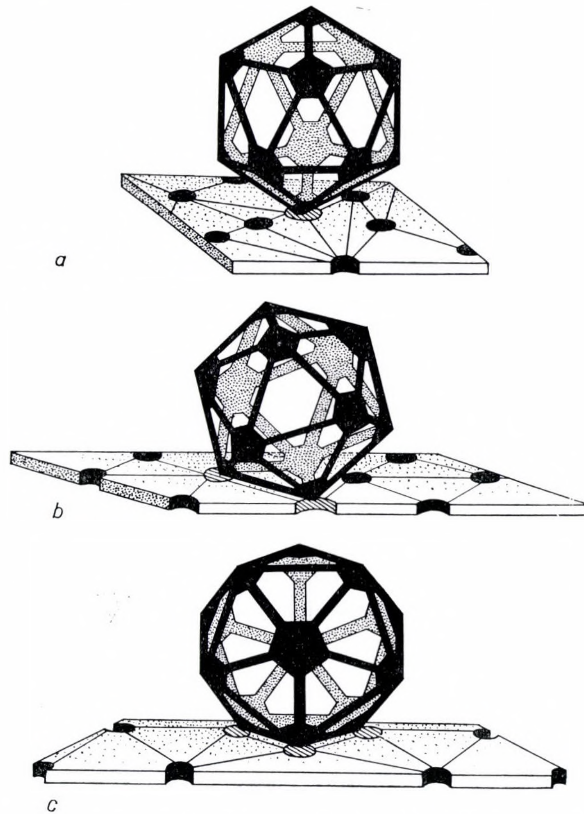

Fig. 2. Possible ways of the virion's attachment to the cell's receptor sites. (a) One vertex—one site hit. Random distribution of sites. (b) Two vertices — two sites attachment. Closely regular distribution of sites. (c) Three vertices — three sites attachment. Perfectly regular distribution of sites

Considering the results of our experiments with cell-virion complexes, suspended in the presence or absence of BA or fatty acids, the relatively low number of virions initiating infection in plain HBS may be those which are accidentally attached at three vertices simultaneously ("avid receptors"). A major part of the virions attached at two vertices only did not initiate infection in the absence of an appropriate factor, *viz.* BA or fatty acid ("common receptors"). In this case, fatty acids appear to be required to stabilize

attachment through *Debye, Keesom* or *London* forces. These forces are known to decrease by the 7th power of the distance. Thus a fairly close adjacency of molecules is required for the manifestation of these forces. The corresponding triangular facet of an icosahedral virion attached to the receptor through two

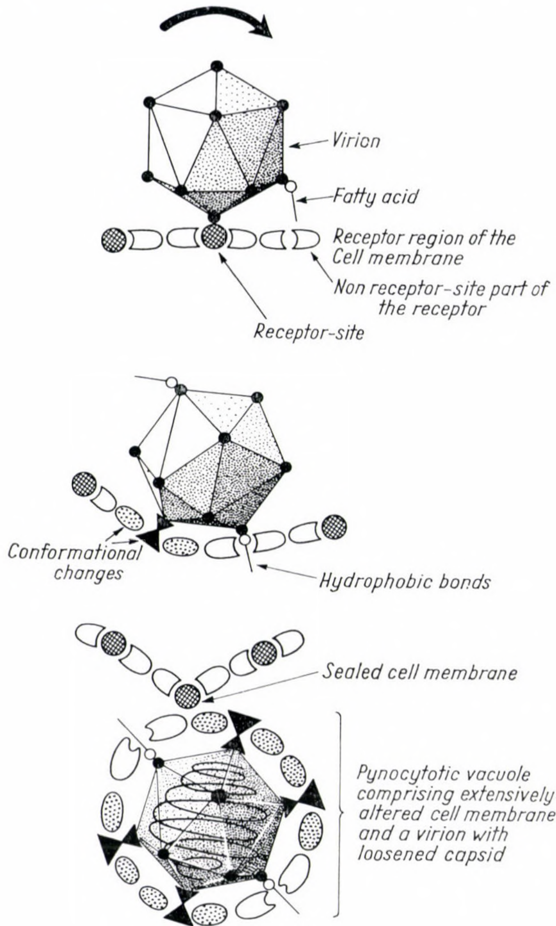

Fig. 3. Supposed mechanism of the virion's engulfment

vertices appears to lie close enough to the cell wall to allow for such interactions. Regions near the third unattached vertex of this facet appear liable to react with the fatty acid's polar end. Interactions between the non-receptor-site parts of the receptor region with the unattached vertex of the virion charged with fatty acids may result in orienting and polarizing effects sufficient to increase above the threshold the stimulus produced by a two-vertices two-sites attachment. The interactions involving fatty acids are considered non-specific in the strict sense of the term.

As shown in Fig. 3, it has been supposed that, as a result of conformational changes of the receptor sites and consequent ultrastructural changes in the neighbouring molecules, the cell membrane starts to develop an invagination at the area of virus attachment. Consequently, the presence of further receptor sites in the neighbouring area may conceivably result in the formation of further virion-vertex to receptor site attachments, facilitating further invagination of the cell membrane through appropriate changes.

In the absence, scarcity or irregular distribution of receptor sites in the receptor region, the above described effect is supposed to be induced by the fatty acid-mediated mechanism. This assumption appears the more probable as enterovirus receptors have been shown to be lipoproteins [11]. The process of invagination by any of the suggested mechanism continues step by step until a complete pinocytotic vacuole has been formed and the unimpaired parts of the cell wall are sealed together. Parallel to the development of a vacuole, the involved area of the cell membrane as well as the virion's capsid undergo increasing alteration, thus by the time of the vacuole's full development, both its wall and the enclosed virion's capsid develop sensitivity to the proteolytic enzymes released from lysosomes. This "non-specific" enzyme action would represent the end of penetration and the start of true eclipse.

Dependence of the infection enhancing action on the fatty acid's chain length appears to point to the strict sterical requirement of the formation of hydrophobic bonds involved in the receptor-virus interaction. At certain concentrations, linoleic and oleic acids exhibited activities comparable to that of saturated stearic acid. At higher concentrations, however, the two fatty acids with double bonds were strongly inhibitory. This phenomenon and the role of SH-groups observed by others [3] and by ourselves [1] strongly favoured the supposition that certain oxidation-reduction reactions or the necessity of protection of certain sensitive, active groups might be of considerable importance in the process of penetration.

The full effectiveness of the infection-enhancing activity of the studied factors is limited to a short period, *viz.* the first 30—60 minutes of the cycle. This seems to suggest the possibility that after a certain time the virions attached in a suboptimal way undergo some alteration rendering them inaccessible to the infection-enhancing factor. Such active, not penetrating virions have repeatedly been shown to be recoverable by different methods or to initiate infection of the same cell after appropriate treatment [12]. The latter experiments were, however, performed under conditions fundamentally differing from those used by us.

Studies performed in both PMK and HeLa cells [10] appear to support, or at least not to exclude, the validity of the proposed model. As to the role of "virtually inhibitory" receptors of HeLa cells, we have already offered an explanation. The release of blebs carrying attached virions and

resulting in a low efficiency of infection are in accordance with the observations of FENWICK and COOPER [13] and JOKLIK and DARNELL [14]. Blebs appeared to be removable by short trypsinization.

Comparative studies performed in HBS and P 199 media [1] suggested that the main constituent of P 199 in respect of virus reproduction may well be the detergent Tween 80. This observation correlates well with that of DARNELL and LEVINTOV [15], who found that poliovirus was synthesized from the intracellular metabolite pool and not from the extracellularly available substances.

Recent studies by STRANDSTRÖM [16] on the effects of lysolecithin and some other phospholipids on the interaction of host cells and attenuated poliovirus suggest that these substances may also act in a way essentially similar to that observed in this laboratory with fatty acids and Tween 80.

Based on the present working hypothesis, further studies are in progress to produce additional evidence to fill the gaps of our hypothesis with experimental data.

Acknowledgements. The excellent technical assistance of Mrs. K. PERÉNYI and Miss J. NYIREDY is appreciated.

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PATHOGENESIS OF THE WASTING SYNDROME FOLLOWING NEONATAL THYMECTOMY

By

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Summary. (1) The wasting syndrome following neonatal thymectomy appears earlier and is more severe in mice infected intracerebrally with lymphocytic choriomeningitis (LCM) virus at the time of weaning.

(2) All the mice surviving neonatal thymectomy and the subsequent intracerebral LCM virus infection proved to be virus carriers. Immunologically, however, they did not behave uniformly. About half of them gave an immune response while the others proved to be tolerant. Irrespective of their immunological status, most of the mice showing the wasting syndrome had died by the 80th postinfection day.

(3) Based on the corresponding literature and our own experience we assume that in the state of immunological depression the organism can only give a nonspecific response, *i.e.*, develop the wasting syndrome, to various antigenic stimuli.

(4) A possible analogy between the human neonatal and senile atrophies and the pathogenesis of the wasting syndrome are discussed.

The decisive role of the thymus in the development of immunological competence has been generally accepted. Mice thymectomized immediately after birth develop immunological depression and, within several weeks, the wasting syndrome [1, 2]. To study the immunological depression following neonatal thymectomy, other investigators [3—6] and ourselves [7—9] have found lymphocytic choriomeningitis (LCM) virus infection to be an appropriate model. We had supposed and later have shown experimentally that mice in the state of immunological depression due to neonatal thymectomy fail to develop fatal meningitis, the characteristic response of normal animals to LCM infection. In spite of this, only part of such mice survived for longer periods, *viz.*, 2—10 weeks, after infection; the rest died at the same time as the non-thymectomized infected animals, *i.e.*, 8—10 days after infection, without displaying meningeal symptoms. These informative experiments still failed to reveal the cause of the early deaths and supplied no experimental data on the immunological status of the virus carrier mice showing the wasting syndrome. We only assumed that LCM virus might aggravate the wasting syndrome.

The aim of the present experiments was to elucidate the cause of the early death and the immunological status of mice surviving for longer periods in spite of developing the wasting syndrome.

* Deceased in 1965.

Materials and methods

Experimental animals, thymectomy and virus: see our earlier report [9].

Re-isolation of virus. To re-isolate virus, samples of brain, blood, liver and ascitic fluid were inoculated into mice intracerebrally. The brain and liver samples were ground with quartz sand and suspended in saline. Then the tissue fragments were allowed to sediment and the supernatants were used for inoculation. Heparinized blood samples were taken from the orbit, under ether anaesthesia.

Excretion test. Three adult mice were kept for 3—6 weeks together with each of the mice to be tested for virus excretion. The test animals were subsequently challanged with 100 to 300 LD50 of virus each. From the orbit of the survivors blood was taken for complement-fixation test.

Complement-fixation (CF) test. LCM antigen was kindly supplied by *Italdiagnostic Ltd.* The test was performed according to the original prescription. Immune serum was prepared as recommended by Traub [10]. The CF titre of this serum was 1 : 256 while the negative sera showed no fixation at 1 : 4 dilution. The highest dilution tested was 1 : 16.

Immunization. Mice were immunized by a single intraperitoneal injection of 0.5 ml of a concentrated influenza A-2 antigen (haemagglutination titre: 1:20,000). To titrate haemagglutination-inhibiting antibodies, Takátsy's Microtitrator technique was used.

Histological technique. Sections from the brain, spleen, liver and thymus of experimental animals were stained with haematoxylin-eosin. In the spleen attention was paid to the lymphocyte count, the number of follicles, the degree of atrophy and the signs suggestive of reticulosis and/or fibrosis.

Control animals. Non-operated mice of the same age served as controls in every experiment. The organs of these animals were subjected to the same histological examination.

Experimental

A hundred and sixty mice thymectomized on the first day after birth were divided at weaning into two groups: in group "O" (operated) the consequences of neonatal thymectomy were observed, whereas group "O+LCM" served for the observation of the joint effect of neonatal thymectomy, and intracerebral LCM virus infection at the time of weaning (three weeks of age). Litter-mates of the mice included in the two experimental groups, a total of 160, were sham-thymectomized or used as non-operated controls. The period of observation after weaning was 120 days.

Data concerning the development of the wasting syndrome are summarized in Figs 1 and 2.

Fig. 1 shows the mortality with wasting disease and the incidence of spleen atrophy and of reticulosis. In group "O", 32 per cent of the mice died within 120 days after weaning. All these showed gross and microscopic signs characteristic of the wasting disease. The rest (68 per cent) were still alive and apparently healthy on the 120th day when they were killed and prepared for histological examination. Severe atrophy of spleen was found in 16 per cent, whereas pronounced reticulosis in the spleen was present in 28 per cent. In 24 per cent no histological abnormality was found.

The severe splenic atrophy was considered a histological sign of wasting disease, therefore the mice with this finding were classified with those having developed the wasting disease. Poor or lacking immune response to influenza A-2 antigen supported this classification.

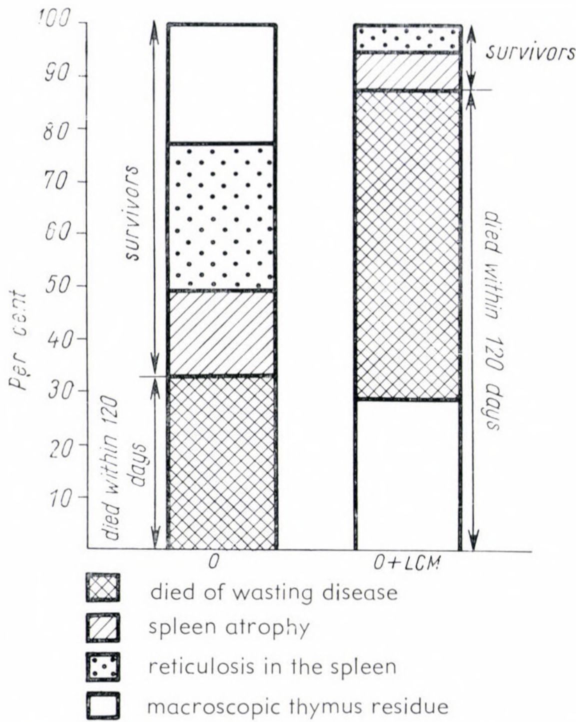

Fig. 1. Mortality, splenic atrophy and reticulosis in the spleen in thymectomized mice and in mice thymectomized and infected with LCM virus

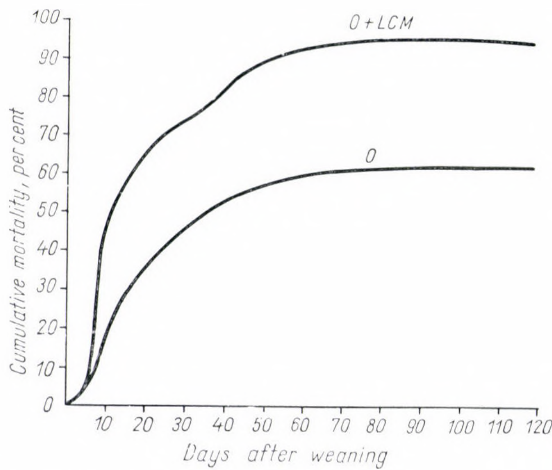

Fig. 2. Cumulative mortality of mice showing the wasting syndrome. "O" = thymectomy on the first day after birth; "O + LCM" = thymectomy on the first day after birth + intracerebral LCM infection at weaning

According to this interpretation, 48 per cent of our thymectomized not-inbred mice developed the wasting syndrome.

The 24 per cent of the "O" mice, those showing no histological abnormality, had residual thymus tissue satisfactory to prevent any sign of the wasting syndrome.

In group "O+LCM" 65 per cent of the mice had died with the wasting syndrome by the 120th postinfection day. Only 11 per cent survived. These had severely atrophied spleen (6 per cent) or showed reticulosis in the spleen (5 per cent). Thus, 71 per cent of the thymectomized and subsequently infected mice were considered to have developed wasting disease. In the remaining 24 per cent the thymectomy proved to be incomplete. These mice nevertheless died because their residual thymus tissue was sufficient to develop the immunological conflict after LCM virus infection. Thus, these mice died with the characteristic meningeal symptoms, simultaneously with the non-operated controls. At autopsy the thymus residue could be found in every case. The mice showed the cerebral lesions characteristic of LCM infection.

The cumulative mortality curves for groups "O" and "O+LCM" are shown in Fig. 2. In both groups the majority of deaths occurred 8 to 70 days after weaning. In group "O+LCM" a striking number of mice died between the 8th and 20th days. Only from the 20th day on show the two curves a parallel course.

The thymectomized mice which died 8 to 15 days after infection seemed to be normal. They showed neither convulsions nor any gross or microscopic sign of meningitis. However, each of them had an atrophic spleen. On the other hand, the mice which died after the 15th day, exhibited symptoms of wasting disease: such as diarrhoea, dermatitis, humped back, *etc.*

The thymectomized mice that had survived LCM infection by at least three weeks were tested for virus carriership. It was attempted to re-isolate the virus from the blood, in several cases from the ascitic fluid and, when an animal had died, from its brain and liver. Virus was re-isolated from 56 mice. The samples taken 20 to 70 days after infection were all positive. The five negative results were yielded by samples taken at a later time.

For virus excretion, 12 mice were examined during the period 30 to 45 days after infection and four mice from the 75th to the 120th postinfection day. In the earlier period seven mice, in the later period two of the four mice were found to excrete virus.

The thymectomized mice that had survived LCM infection were tested for CF antibodies to LCM virus on each of the 20th, 75th, 125th and 162nd days. On the 20th day 50 per cent had antibodies detectable up to 1 : 16 dilution. The others were negative even at 1 : 4 dilution. By the second bleeding (75th day) most of the mice had died with the wasting syndrome, irrespective of their antibody status on the 20th day.

Of the mice that had survived infection by 120 days only one (No. 16) failed to give antibody response, even after superinfection. This mouse was excreting virus and the virus could be re-isolated from its blood, too. No thymus residue, either gross or microscopic was found in it. Its spleen was severely atrophied.

Table I

Some data of six thymectomized mice surviving for long periods after LCM virus infection

Designation of mouse	Examinations performed on day												Histology	
	20		75				125				162		Thymus residue	Atrophic spleen
	CF	Vi	CF	VE	Vi	Ch	CF	VE	Vi	Ch	CF	VE		
16	<1 : 4			+	+	—	<1 : 4						0	+++
51			>1 : 16					+	+	—	>1 : 16		+	++
39	>1 : 16	+		+	—		>1 : 16						+	++
26	>1 : 16	—		—	—	—	>1 : 16						±	++
78			>1 : 16					—	—	—	>1 : 16		±	—
A							>1 : 16	—			>1 : 16	—	0	—

CF = serum CF titre
Vi = re-isolation of virus
VE = virus excretion
Ch = challenge

The other mice that were still alive on the 120th postinfection day had CF antibodies at least up to 1 : 16 dilution. Their carriership was fluctuating, they had a minimal thymus residue and severely atrophied spleen, or reticulosis in the spleen.

Discussion

In the present study 48 per cent of the thymectomized mice (group "O") developed the wasting syndrome; two-thirds of these (32 per cent) had died within 120 days after weaning, whereas the remainder (16 per cent) survived the observation period. Most of the deaths occurred between the 8th and 70th days after weaning, in a nearly even distribution.

Among the thymectomized and subsequently infected mice (group "O+LCM") 71 per cent developed the wasting syndrome. Most of these (65 per cent) had died within 120 days after weaning *(i.e.,* after infection), only 6 per cent survived that period. In this group, too, most of the deaths occurred between the 8th and the 70th days, but the distribution in time, unlike that in group "O", was uneven, showing a well-pronounced cumulation from 8 to 20 days after infection.

In brief, the wasting syndrome due to thymectomy on the first day of life developed more frequently and more rapidly in group "O+LCM" than in group "O".

It has, however, been reported that the LCM virus itself may cause some wasting in animals with insufficient immune apparatus. HOTCHIN [11] infected immunologically immature mice in the neonatal phase of physiological immune depression. During the subsequent 10—15 days he observed loss of weight, wasting and death. Presumably in the present experiments the same effect of the LCM virus had manifested itself during the persistent immunological depression due to the neonatal thymectomy. This effect led to the high mortality between the 8th and the 20th postinfection days.

The animals alive three weeks after infection all carried the virus, but did not behave uniformly from the immunological point of view. Fifty per cent of them produced antibodies, the others were tolerant immunologically. This apparently heterogenous result might be explained in the sense of the experiments of HOTCHIN and VOLKERT [11, 12]. According to these authors normal mice are in the status of immunological depression in the first 2—12 hours after birth. Antigen administered in this period induces tolerance. After the 6th or 7th day of life, on the other hand, the response to antigenic stimuli is specific, *i.e.*, in the case of the LCM virus it includes an autoimmune process. Between the 12th hour and the 6th day there is a period when probably the thymus plays the most important role. Antigen administered in this period induces either tolerance or an antibody response, depending on the time of administration within the period and the intensity of the antigenic stimulus. Our mice were thymectomized during the first 24 hours after birth, but, since the hour of birth had not been recorded, part of the animals were certainly operated upon as late as during the second 12 hours, when according to HOTCHIN and VOLKERT even total thymectomy may fail to cause tolerance. Thus, the mice operated upon in the second half-day might have become either tolerant or capable of producing antibodies. Furthermore, thymectomy in this transitory period might have prepared the mice for the wasting effect of the LCM virus.

We summarize our opinion as follows. Intracerebral LCM virus infection of non-operated control animals leads to an immunological conflict accompanied by lymphocytic choriomeningitis, which is an autoimmune process of fatal outcome [13, 14]. In successfully thymectomized mice the autoimmune reaction either fails to ensue or proceeds in an unusual way. The mice that died 8 to 15 days postinfection had not enough time to develop the typical wasting syndrome; atrophy of the lymphatic apparatus was the only sign characteristic of the syndrome. In these cases the immediate cause of death might have been a developing wasting syndrome and/or an LCM virus infection modified by the persisting immunological depression.

In the mice which died 15 to 20 or even more days after infection the wasting syndrome was predominant as the joint consequence of the persistent virus multiplication and the lack of thymus. The virus could be re-isolated from all the animals that died in this period. Fifty per cent of these were similar to the tolerant mice of TRAUB [10]: they carried the virus without producing antibodies. However, TRAUB's tolerant mice were symptom-free, whereas our experiments and those of other investigators have unequivocally evidenced that persistent carriership is not a physiological state in these animals. They sooner or later develop symptoms not specific for the virus, instead, characteristic of the wasting syndrome. According to present knowledge these symptoms are autoimmune in nature [16, 17]. Most of our carrier mice died of wasting disease; among those surviving 120 days two were found to carry virus and even these were ill and died soon. The non-carrier survivors showed only microscopical signs of the wasting disease.

The pathogenesis of the wasting syndrome is a debated question. Recent studies have called attention to the role of various antigens [17, 18], which may originate from any sort of pathogenic or nonpathogenic microorganisms, *e.g.*, from some members of the intestinal flora [19]. Germfree mice fail to develop the wasting syndrome after neonatal thymectomy [17, 20], though their being in immunological depression is clearly proven by the successful take of heterografts [17]. The same was observed when streptococcus or staphylococcus antigen had been given to mice in the physiological state of neonatal immunodepression [21]. In the present studies 48 per cent of the mice thymectomized on the first day after birth (group "O") developed wasting disease owing to natural antigenic stimuli. In group "O+LCM", besides antigens occurring at random, a massive dose of LCM virus antigen and the persistence of this virus played a role in inducing the wasting disease, which thus occurred more frequently and developed more rapidly. In consequence of this, most of the carrier mice died, whereas two-thirds of the mice that survived 120 days were not carriers. It should be noted that pathogenic bacterium could never be isolated from the mice which had died of wasting disease, in spite of the occasional occurrence of hepatic necrosis and ascites.

Based on the present experiments and literary data we may state that in the status of immunological depression of various origin (neonatal age, neonatal thymectomy, cortisone treatment, X-ray irradiation, *etc.)* the most diverse antigens [11, 17, 19, 20, 22] may induce the same nonspecific response, which results in wasting disease usually fatal in outcome. Unlike this, the organism possessing a thymus and an intact immune apparatus responds to antigenic stimuli in a specific way, usually protecting the organism against the agent. In the organism rendered immunologically depressive by thymectomy or any other intervention, foreign antigens induce processes which

are considered to be autoimmune in nature [23]. In our opinion these processes impair the whole organism and their consequences are independent of the specificity of the antigen.

This interpretation of the wasting syndrome's pathogenesis might furnish an explanation of some conditions of human importance, *viz.*, those resembling wasting disease and developing on the grounds of immunological depression. Such are for instance some conditions due to infectious diseases including certain forms of atrophy of premature and newborn infants. It would be reasonable to extend this conception to the old organism as well. It is well-known that while the immunological reactions of the organism are declining in old age, the organism is exposed to extrinsic antigens to the same extent as in young age. In the state of senile immunological depression various antigenic stimuli may initiate a process resembling wasting disease and eventually leading to the well-known senile atrophic alterations. Experiments concerning this question are in progress.

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HAS THE NERVOUS SYSTEM A ROLE IN THE ENDOTOXIN-INDUCED ALTERATION OF CELL METABOLISM?

By

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National Institute of Public Health (Director: T. BAKÁCS), *Budapest*

(Received July 12, 1966)

Summary. After parenteral administration of endotoxin, no difference was demonstrable in the metabolism of cells with and without nerve supply.

In a Balassa memorial lecture in 1948, G. MANSFELD stated that the action of bacterial endo- and exotoxins requires the presence of nerve supply. He believed that, like the centripetal migration of tetanus exotoxin, any endotoxin entering the central nervous system is proceeding centrifugally along a neuron to reach the target cell [1]. This concept, daring and bright as it was, has neither been proved nor disproved ever since. In fact, no metabolic process of the cell has been known to be particularly enhanced or inhibited by endotoxin action. Assay of the effect of endotoxin on cell metabolism has begun in 1960 [2—8]. In this context, one of the most important findings has been that endotoxins enhance aerobic glycolysis in various kinds of cells. In possession of this information we had a sufficient basis to start investigation of the influence in vivo of parenterally administered endotoxin on the aerobic glycolysis of cells with and without nerve supply.

Materials and methods

Albino rats from the inbred strain of our Institute were used throughout. The animals were given intraperitoneally 0.1 ml/100 g body weight of *Salmonella typhi* endotoxin extracted according to BOIVIN and MESROBEANU [9]. This caused the death of about 10 per cent of the animals. Forty-eight hours after toxin administration the rats were killed by decapitation and their organs to be examined were removed. Innervated cells were studied in specimens of kidney and small intestinal mucosa. Aerobic glycolysis of non-innervated cells was studied on thymocytes, granulocytes and macrophages. To obtain granulocytes, 10 ml/100 g of sterile broth was injected intraperitoneally 8 hours prior to the experiment. Granulocytes obtained from the peritoneal cavity were washed with physiological Na-citrate solution and suspended in Krebs' phosphate buffer, pH 7.4. Macrophages were obtained by injecting sterile broth intraperitoneally 72 hours prior to the experiment. Oxygen uptake and lactic acid production were measured after 1 hour incubation at 37° C, using the traditional WARBURG and the DIESCHE—LÁSZLÓ method, respectively [10].

Results

The examinations revealed no notable influence of endotoxin on the oxygen uptake of non-innervated cells, except for granulocytes where a significant difference has been found. In contrast, distinct differences in lactic acid production in the presence and absence of oxygen have been observed with granulocytes and macrophages. With thymocytes, there was no demonstrable increase in lactic acid production.

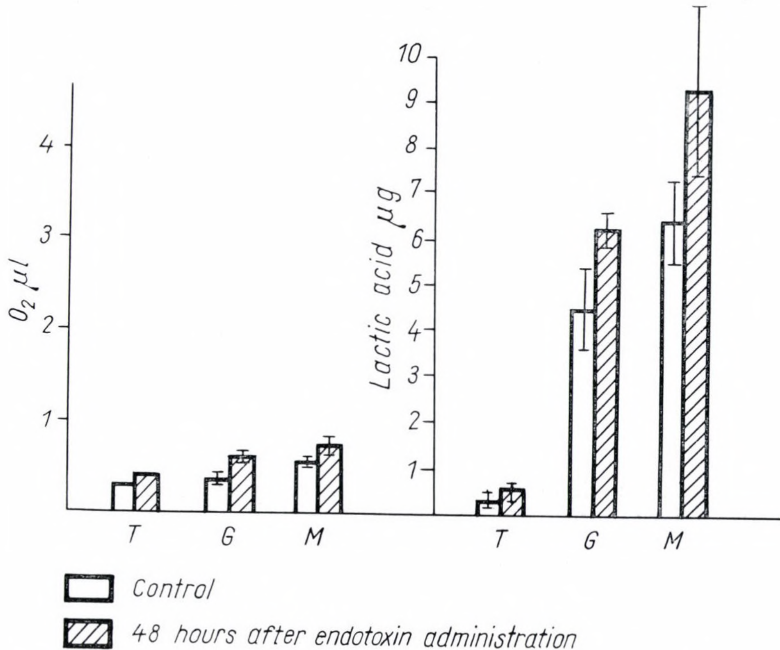

Fig. 1. O_2-uptake and aerobic glycolysis in non-innervated cells, in terms of $\mu l/10^6$ cells/hour and $\mu g/10^7$ cells/hour, respectively. Abbreviations: T = thymocytes; G = granulocytes; M = macrophages

Previous injection of endotoxin did not enhance aerobic glycolysis in the small intestinal mucosa, although it is known to have a very distinct glycolytic activity also normally. Enhancement of aerobic glycolysis was bordering significance in the kidney. Simultaneously, no notable oxygen uptake alteration took place in the intestinal epithelium and kidney specimens.

In a subsequent series of experiments the left kidney of albino rats was denervated surgically. Denervation was further ensured by phenol treatment of the renal hilum. Four or five days later LD_{10} toxin was injected into the animals and 48 hours later the respiration and aerobic glycolysis of the innervated and denervated kidneys was compared. No notable difference was found.

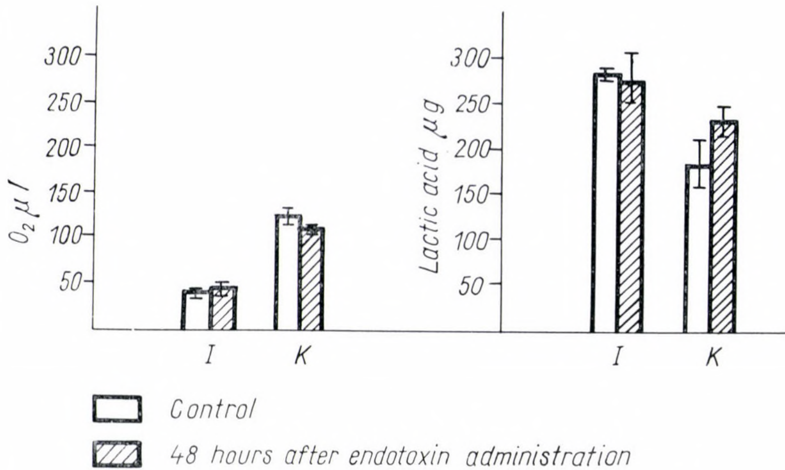

Fig. 2. O_2-uptake and aerobic glycolysis in innervated cells, in terms of μl/100 mg wet weight hour, and μg/100 mg wet weight/hour, respectively. Abbreviations: I = small intestina/ mucosa; K = kidney specimens

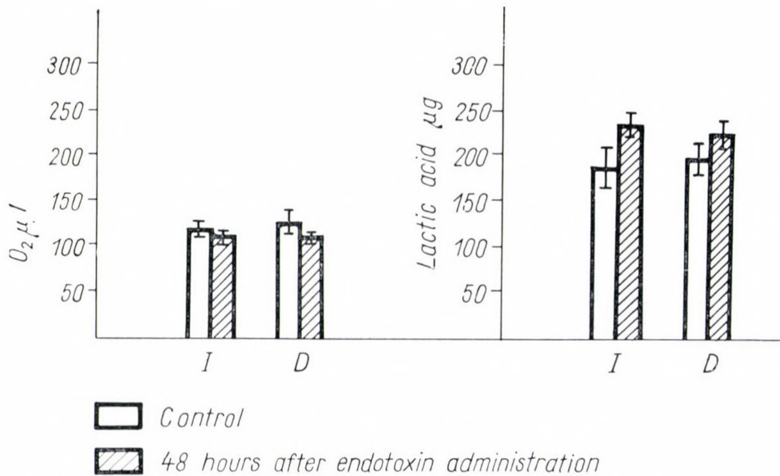

Fig. 3. O_2-uptake and aerobic glycolysis in denervated and innervated cells, in terms of μl/100 mg wet weight/hour and μg/100 mg wet weight/hour, respectively. Abbreviations I = innervated kidney; D = denervated kidney

Discussion

The experiments imply with great certainty that the endotoxin induced alteration of the cells' aerobic glycolysis depends not so much on their nerve supply as on their metabolic type. Out of the examined non-innervated cells, thymocytes showed no increase in oxygen uptake or lactic acid production

in the presence of endotoxin. Increased lactic acid production was observed with granulocytes and macrophages. The former even showed an increased oxygen uptake. Similar contradictions were observed also with the innervated cells. Lactic acid production of kidney specimens increased moderately, while that of the intestinal mucosa was not affected. This difference cannot be ascribed to an eventual storage of endotoxin in the cells, since according to recent data not even granulocytes, showing the most distinct metabolic alterations, are capable of storing endotoxin [11]. The similarity of metabolic activity in innervated and denervated cells also shows that a nerve supply is not indispensable for the development of endotoxin action. This, however, is not exclusive of an action of endotoxin on the nervous system. The presence of target cells in the central nervous system has been described recently [12—14]. All we wish to note is, therefore, that the present study failed to supply evidence of the passage of endotoxin along the neurons as well as of any definite, indispensable role of the nervous system in endotoxin action. Accordingly, the results have not been supportive of MANSFELD's theory.

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DATA ON THE CYTOTOXICITY OF ANTI-TUMOUR SERA

By

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(Received April 14, 1966)

Summary. Immune sera to NK/Ly mouse ascites tumour were prepared in rats. Such sera contain incomplete antibodies producing a cytotoxic effect only in association with complement. Attempts were made to adapt for quantitative assay a cytotoxicity test involving the neutralization of tumour cells with tumour immune serum in the presence of complement, and administration of this mixture to animals.

A similar, though less cytotoxic antiserum was obtained with spleen homogenate from normal mice. On adsorption to mouse spleen cells the cytotoxic effect disappeared. When examined at a concentration of 800,000 ascites tumour cells/ml, the adsorbed anti-tumour serum contained no cytotoxic antibody capable of ensuring the survival of experimental animals.

The adsorbed anti-tumour serum, however, prolonged the animals' survival with a few days when the cytotoxicity test was carried out with the lowest cell count sufficient for the tumour to take. Further studies are required to elucidate whether or not this effect has been brought about by the action of a tumour specific antigen induced antibody.

The purpose of the present experiments was to examine whether NK/Ly [1] ascites tumour contained a tumour-specific antigen and whether an antiserum, containing antibodies exclusively to that antigen might be obtained. In this report experiments based on the cytotoxic effect of heteroantibodies are discussed.

As a rule, anti-tumour serum is produced in an animal species not susceptible to that particular tumour. Since NK/Ly ascites lymphoma was found to take readily in many inbred mouse strains [2] as well as in random bred albino mice, the use of mouse immune serum (isoimmune serum) was beyond consideration.

Isoimmune serum should be preferred for the demonstration of tumour antigen [3], as heteroimmune sera contain various antibodies (agglutinin, lysin) [4, 5] most of which are not tumour-specific, being formed rather against normal tissues or cells. Therefore of the available combinations of experimental animals we have chosen the mouse-rat system. This, although a heterologous system, seemed to be more suitable for our purposes than the rabbit — mouse system. The mouse and rat belong to the same taxonomic order (Rodentia) and family (Muridae), being phylogenetically more closely related than the mouse and the rabbit, rabbits being classified into a different order (Lagomorpha) [6]. Our supposition seemed to be supported by the fact that in rats

immune tolerance could be induced to mouse erythrocytes [7] duly considered a very complex mouse antigen.

Materials and methods

Antigen. As tumour antigen, we used freshly harvested 10-days old NK/Ly ascites from the 50th to 60th passage in Swiss mice. Of 10-day ascites cells only 8—10 per cent stained with a 1 per cent solution of trypan blue.

Normal mouse tissue specimens (spleen, liver, lung, kidney) used for immunization or immune serum adsorption were excised from the killed mice under sterile conditions. The specimens were minced with scissors and suspended in saline in a Potter all-glass homogenizer. Although cells are impaired by homogenization (15 per cent of the suspended spleen cells stained with trypan blue), no notable loss of antigen was demonstrable. The homogenate was filtered through two sheets of gauze, centrifuged and diluted in saline or serum, depending on its use for immunization or adsorption purposes. The sediment of the homogenate was suspended to contain 80—120 million nucleated cells per ml.

Immune serum. Two groups, each containing forty CB Wistar rats were immunized with 10-day NK/Ly ascites and normal mouse tissue homogenates (spleen, liver, lung, kidney), respectively. The rats have been in kin breed for about 10 years. Immunization with tumour cells was done intraperitoneally three times weekly for three weeks, using 0.5 ml of tumour cell suspension (about 40 million cells) per animal on each occasion.

On the first occasion, the antigen was mixed with adjuvant prepared as follows. One ml of fluid culture of *Mycobacterium butyricum* in Sauton broth was centrifuged, the sediment suspended in 1 ml sterile paraffin oil and mixed with 100 mg of aluminium stearate. The antigen and the adjuvant were mixed for 30 minutes prior to inoculation [8]. The adjuvant used for second inoculation did not contain mycobacteria and further inoculations were made without adjuvant. One week after last immunization all animals were exsanguinated through the cervical vein under sterile conditions. Sera of each group of animals were pooled. The antibodies obtained were identified by agglutination, lysis and complement fixation tests carried out with TAKÁTSY's micro-method [9]. Agar gel diffusion precipitation was performed according to OUCHTERLONY [10].

Evaluations were based on the fact that in mice, 8—10 million intraperitoneally administered NK/Ly tumour cells gave rise to ascites in 6—8 days, and killed the animals in about 15 days. A part of the tumour cells that had contacted immune serum and complement was destroyed and in animals inoculated with such cells tumour development was protracted. The inoculated animals were examined three times weekly for half a year. Dead animals were dissected. As complement and control, serum pools of fresh sera from at least 10 normal rats were used.

Results

Results of serological tests and agar gel diffusion precipitation tests performed with antitumour serum are shown in Table I.

As shown in Table I, antibodies were present in the sera of the immunized animals. An agglutination reaction took place only when the test was performed in macromolecular medium. This is typical of incomplete agglutinins.

Next, the cytotoxic effect of anti-tumour serum was studied. Ten-day NK/Ly ascitic fluid was centrifuged and the sedimented cells were resuspended in immune serum, corresponding in volume to the ascitic sample's fluid phase.

The suspension was incubated at 37°C under gentle shaking. Samples were taken every 30 minutes and inoculated into Swiss mice. As a control, tumour cells suspended in normal rat serum were used under the same experimental conditions. Results of repeated examinations are shown in Table II.

Table I

Serological reactions with anti-tumour serum

Test	Antigen	Antibody	Titre	No. of assays
Complement fixation	Washed NK/Ly ascites tumour, at 10 million cells/ml concentration	Pool of immune sera inactivated at 56 °C for 30 minutes	32—64	3
Lysis	2.5 per cent mouse erythrocyte suspension	Pool of fresh immune sera	2—16	5
Agglutination	Suspension of washed NK/Ly ascites tumour cells	Pool of heat inactivated immune sera	0	6
	2.5 per cent mouse erythrocyte suspension	Pool of heat inactivated immune sera	0	3
Agglutination in a medium containing 5 per cent human albumin and made up with 1/20 vol. of Plasmodex solution	2.5 per cent mouse erythrocyte suspension	Pool of heat inactivated immune sera	8—16	3
Agar gel diffusion	Filtrate of NK/Ly ascites tumour cells homogenized in dist. water; ascitic fluid (7% protein)	Pool of fresh immune sera undiluted or diluted 1 : 2, 1 : 4	No precipitation line	3

When the cells had been incubated with the antitumour serum for 90 minutes, no ascites tumour developed until the 10th day, whereas in the controls it had regularly appeared by that time. Average survival of the animals was 15 days.

The phenomenon seemed to be due to a decrease of live cell count in the immune serum. This was indicated by the observation that, unlike the controls, the cells kept in immune serum for 10 minutes and then diluted tenfold did not induce tumour in ten days. The immune serum treated cells were examined electronmicroscopically [16]. The correlation between mortality rate and the number of inoculated tumour cells will be discussed elsewhere.

The cytotoxic titre of the immune serum was expressed in terms of the survival of animals inoculated with immune serum treated tumour cells. Eighty million NK/Ly cells were suspended in 1 ml serum dilution and incubated for 90 minutes at 37 °C. From this mixture individual doses of 0.2 ml were

Table II

Growth of implanted tumour cells in dependence on the period of incubation with immune serum

Duration of treatment with immune serum at 37°C (minutes)	Number of mice injected intraperitoneally with 0.2 ml of treated tumour cells	Number of mice developing ascites one week later
30	10	10
60	10	3
90	10	0
10 then inoculum diluted tenfold with saline	10	0
90 exposed to control serum	10	10
10 in control serum, then inoculum diluted tenfold with saline	10	10

inoculated intraperitoneally into mice. Fig. 1 shows the survival of these animals.

Fig. 1 shows that 50 per cent of the control animals died by the 15th day. Incubation of tumour cells with undiluted immune serum prolonged the survival time. With 1 : 2 diluted immune serum, the 50 per cent survival time

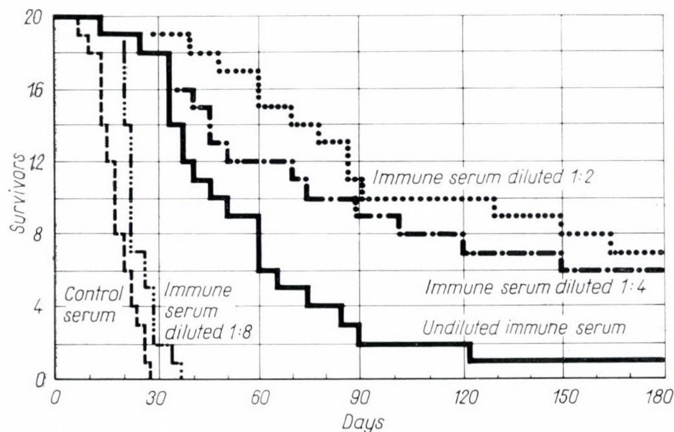

Fig. 1. Survival of mice inoculated with NK/Ly cells treated with different dilutions of tumour immune serum (Incubation mixture: 80 million cell per ml serum. Inoculum: 0.2 ml of incubation mixture)

was 90 days. At dilution of 1 : 8, the immune serum had hardly any effect. The relatively low protective activity of undiluted immune serum seemed to be due to its toxicity [11]; this however, remains to be investigated.

Preliminary experiments have shown that the intraperitoneal administration of a reduced number of tumour cells would result in a delayed development of ascites, prolonged survival time and a higher incidence of solid tumours than of ascitic ones.

Dead mice were subjected to post mortem examination. The nature of gross lesions is shown in Table III. Part of the mice survived for a long time. Five per cent of them developed leukaemia (enlargement of spleen and lymph nodes) in about 1 month. Five to ten per cent developed a solid tumour in the abdomen. In more than 50 per cent ascites was conspicuous after one month only.

In these cases, too, the cell count in the ascitic fluid was low and tumour nodules appeared in the pancreas and on the peritoneum. Thus part of the intraperitoneally inoculated cells had apparently survived the 90-minute antiserum treatment and it was in only about one third of the animals that ascites failed to appear during a long observation period.

Fresh anti-tumour serum loses its cytotoxicity by inactivation at 56 °C for 30 minutes. This effect of heat inactivation is, however, reversible by the

Table III

Gross lesions in mice died after the injection of immune serum treated ascitic cells

Inoculum: 80 million tumour cells per ml immune serum, treated for 90 min.	No. of Swiss mice given 0.2 ml intraperitoneally of the inoculum	Post mortem findings				
		Ascites tumour	Solid tumour	Leukaemia	No lesions	
					for half year	after half a year
Undiluted serum	110	57	10	6	18	19
Diluted 1 : 2	30	18	1	2	2	7
Diluted 1 : 4	30	19	2	1	2	6

Table IV

Role of thermolabile complement factors in cytotoxicity assay

Incubation at 37 °C for 90 minutes; cell count 80 million	Number of mice inoculated with 0.2 ml	Number of mice with ascites in 10 days
Heat inactivated immune serum	20	20
Heat inactivated immune serum + fresh normal rat serum v/v	20	0

addition of fresh normal serum. Since heat treatment is known to destroy mainly the complement, the latter's presence seems to be required for the development of the antibody's cytotoxic action.

Anti-tumour antibodies may be adsorbed with NK/Ly ascites cells (Table V). For this purpose, the immune serum is incubated for a short time with tumour cells, and centrifuged. This treatment deprives the rat immune serum of the cytotoxic principle. The adsorption procedure was repeated twice with each immune serum. The NK/Ly ascites tumour being a lymphoma, we used normal mouse spleen tissue as the control. Lymph nodes would have been more suitable for this particular purpose, but their collection in adequate amount would have been too difficult. The centrifuged spleen sediment was suspended in immune serum to make a concentration of about 80—120 million nucleated cells per ml. After incubation for 90 minutes at 37°C, the suspension was centrifuged and the serum adsorbed again with spleen cells. The adsorbed serum was tested for cytotoxicity in the presence of fresh complement and tumour cells.

Table V

Cytotoxicity assay with adsorbed anti-tumour serum

Incubated at 37°C for 90 minutes; cell count 80 million per ml of serum	Number of mice inoculated with 0.2 ml	Number of mice developing ascites in 10 days
Serum adsorbed with NK/Ly cells	20	20
Serum adsorbed with mouse spleen cells	20	18

The cytotoxic principle was equally adsorbed by two cycles of treatment with mouse spleen homogenate. Except for a few animals developing ascites only by the 15th to 18th day after inoculation by the 20th day they all died.

Thus, mouse spleen cells were capable of binding the cytotoxic antibodies present in anti-tumour serum. It was supposed that if spleen cells and tumour cells have a common antigen, a cytotoxic serum might be prepared also with mouse spleen cells.

For this purpose, 20 Wistar rats were immunized with mouse spleen homogenate in the same way as in the case of tumour cells.

The action of 1 : 2 diluted fresh immune serum was assayed on tumour cells (Table VI).

As shown in Table VI and Fig. 2, the cytotoxic activity of anti-spleen serum was weaker than that of the anti-tumour serum, the former causing a

Table VI

Cytotoxicity assay with sera from rats immunized with mouse spleen and mouse tumour homogenates

Incubated at 37 °C for 90 minutes; cell count 80 mill. per ml of serum	Number of animals inoculated with 0.2 ml	Number of mice developing ascites tumour in 10 days
In twofold diluted tumour immune serum	20	0
In twofold diluted spleen immune serum	20	10

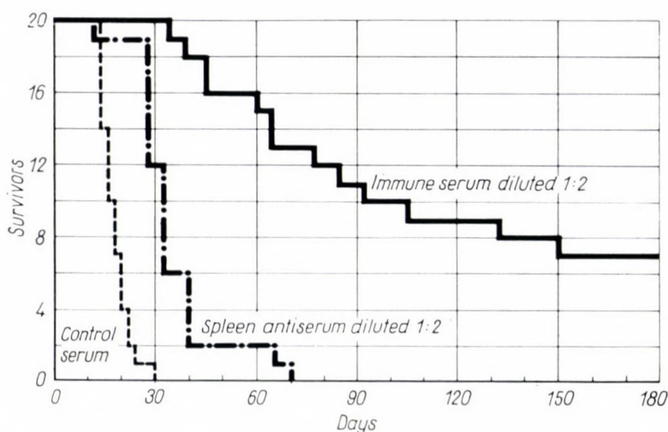

Fig. 2. Survival of mice inoculated with tumour cells treated with tumour serum and anti-spleen serum (Incubation mixture: 80 million cells per ml serum; inoculum: 0.2 ml of incubation mixture)

hardly twofold prolongation of survival. The effect of anti-liver, anti-lung and anti-kidney sera was even less.

Accordingly, anti-spleen serum, though being cytotoxic for tumour cells, was less active than anti-tumour serum. In contrast to the above described adsorption experiment, this observation suggested the dissimilarity of lymphoid and lymphoma cell antigens.

Therefore, in further assays lower numbers of tumour cells were used to test the cytotoxicity of anti-tumour sera adsorbed with spleen cells.

Eight million or 800,000 tumour cells per ml were incubated for 90 minutes in normal and spleen adsorbed anti-tumour sera with complement. After incubation, the materials were injected intraperitoneally into mice in 0.2 ml individual doses. Thus the inoculated cell count per mouse in the first case was 1.6 million and in the second case 160,000. Among the animals inoculated with the lower dilution, deaths occurred simultaneously in the experimental

and control groups. The development of tumour cells treated with normal respectively adsorbed immune serum differed only in the group inoculated with the higher dilution. Animals having received a higher dilution of the tumour-cell immune serum complex developed tumour and died, but the presence of immune serum caused a delayed growth of the tumour (Fig. 3). The anti-tumour serum did not kill all tumour cells. In fact, tumour cells damaged by specific antibody treatment were still capable of inducing tumour growth in mice and killed them with 20 days delay within a period of 90 days.

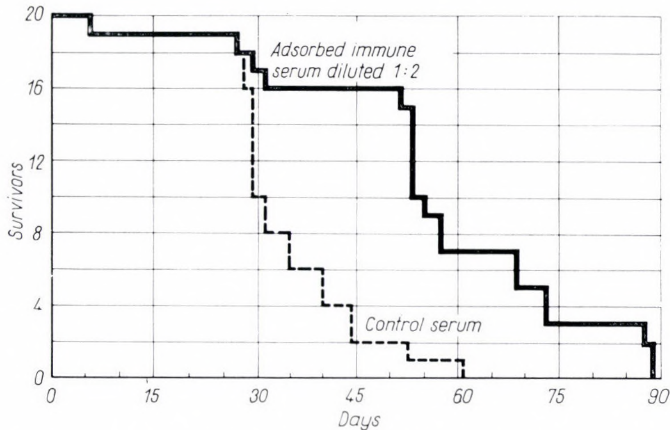

Fig. 3. Survival of mice inoculated with tumour cells incubated with tumour antiserum, previously adsorbed with normal spleen (Incubation mixture: 800 thousand cells per ml serum; inoculum: 0.2 ml of incubation mixture 160,000 cells)

According to further studies, 3-month old Swiss mice survive the intraperitoneal inoculation of 16,000 to 20,000 tumour cells by more than six months. Part of these animals fail to develop tumour. This allows the conclusion that treatment with anti-tumour serum had been survived by more than 20,000 of the 160,000 tumour cells used for inoculation.

Discussion

According to DULANEY and ARNESEN [12], sera from rabbits immunized with mouse tumour and normal mouse organs differ only in the degree of their cytotoxic effect on tumour cells. From tumour antisera produced in rabbits the cytotoxic principle could be adsorbed with homogenates of normal organs. The specific antigen of murine lymphoid tumour was, however, demonstrable in isoimmune system [3, 17]. Thus the animal species used for antiserum production should carefully be chosen. In the case of inadequate choice, a small proportion of the antibodies in the antiserum will only be tumour-specific, being surpassed by the large amount of specific antigens [13].

We, too, have found that rat antisera to mouse spleen and mouse tumour differ in titre values. Rat antiserum to mouse tumour had lost the bulk of its cytotoxic activity after adsorption with normal mouse spleen. Yet when the cytotoxic test was set up with a low tumour cell count, the adsorbed immune serum brought about a slight but distinct prolongation of survival in comparison to the controls. This means that for the differentiation of mouse antigens in the cytotoxic test, rat antisera are more efficient than rabbit antisera.

After adsorption with normal organ homogenates of isoimmune sera to lymphoid tumours Gorer, Maureen and Batchelor, too, failed to observe a tumouricide activity and registered merely the survival, or prolongation of survival of part of the animals [14].

In order to conclude to the presence of a specific tumour antigen under our experimental conditions, first of all it is necessary to adsorb the control sera in the cytotoxic test with spleen homogenate. Although it is hardly possible that the factor responsible for the prolongation of survival should originate from the spleen, this type of control test is indispensable in view of the tumour cell impairing action of spleen extracts [15]. Finally, the sensitivity of the neutralization test should also be increased, as under the given conditions only a slight difference in the lower titre range was demonstrable between adsorbed immune serum and normal serum.

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EFFECT ON INFLUENZA VIRUS OF A MODIFIED FRANCIS INHIBITOR AND ITS ACETONE-SOLUBLE FRACTION

II. STUDIES ON THE MODE OF ACTION IN DE-EMBRYONATED EGGS

By

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(Received June 20, 1966)

Summary. Francis inhibitor (K_0), its diethyl-p-phenylene-diamine-diazonium-salt derivative (modified Francis inhibitor or *8m*) and the acetone-soluble fractions of these substances (K_0ac and *8ac*) were examined for inhibitory action on the multiplication of the Budapest 4/49 strain of influenza A—1 virus in the cells of the chorioallantoic membrane of de-embryonated eggs.

(i) Twenty mg per ml K_0ac or 5 mg per ml *8ac* added to the medium half an hour after infection completely prevented the reproduction of infective virus, whereas 20 mg per ml of K_0 or *8m* caused no inhibition.

(ii) Twenty mg per ml *8ac*, administered 1 hour after infection stopped virus multiplication. The same dose given at 5 hours acted also immediately, considerably reducing the yield of the cycle in progress and preventing further cycles. Its administration at 10 hours prevented further cycles.

(iii) The duration of the events sensitive to *8ac* depends, among others, on the dose of the inhibitor: those (or that) sensitive to 5 mg per ml take(s) place from 30 minutes to 2 hours; those sensitive to 7.5 or 10 mg per ml start between 30 and 120 minutes and last until the end of the eclipse. It appears likely that some viral constituents which have not stabilized until 6 hours remain sensitive to large doses of *8ac*. The progress of the inhibitor-sensitive process of virus synthesis runs parallel with the appearance of infective virus in untreated controls. After removal of *8ac* virus synthesis regenerates and after a very short lag phase proceeds at the same rate as in the control membranes.

Previous studies [1, 11] have shown that the acetone soluble fraction *(8ac)* of our *8m* preparation (Francis inhibitor coupled with diethyl-p-phenylene-diamine-diazonium chloride) significantly inhibits the multiplication of influenza virus in the roller drum as well as in de-embryonated eggs. Virus growth was measured by titration of the haemagglutinating (HA) activity of the medium. The degree of inhibition depended on the inhibitor concentration and, not always significantly, on the size of the inoculum.

In the present work two questions have been studied in the de-embryonated egg, (i) the correlations between inhibitor concentration and degree of inhibition, considering the infective titre of the membrane homogenate at a given time to represent the index of virus multiplication; (ii) to determine the inhibitor-sensitive period of the reproduction cycle.

Materials and methods

Follow-up of the reproduction cycle. The strain Budapest 4/49 of influenza A—1 virus was used throughout. The procedure of de-embryonation has been published [1].

The inoculum was placed in each DEE in 3 ml medium. Then the DEEs were rolled at 37° C for 30 or 60 minutes and rinsed twice. Subsequently 10 ml medium, with or without inhibitor, was added. At intervals, samples of 0.1 ml volume were taken from each DEE and those taken from the same group of eggs were pooled. At the same times three chorio-allantoic membranes (CAM) were removed from each group. These were rinsed separately three times each in a Petri dish. The fluid on the surface of the CAMs was removed by repeated pressing between sterile blotting papers; the CAMs were ground with quartz sand suspended in 10 ml saline each. After centrifuging at 3000 r. p. m. for 10 minutes the supernatants were sucked off and signed as 10° dilution of the CAM extract.

Both the pooled medium and the CAM extract were titrated in the roller drum [2] in 3 hours after preparation, using $10^{-0.5}$ fold dilution series and 4 ampoules for each dilution. After the drums had rolled for 72 hours at 37°C, the HA titres of the maintenance fluids were determined [3]. The infectivity titre was expressed in ID_{50} per 0.1 ml units [4]. The volume of both the maintenance fluid and the CAM extract being 10 ml, the total amount of virus produced was 10^2 times the value obtained.

To control the reproducibility of the titres, we titrated a stock virus preparation 10 times within a period of 5 weeks. The standard deviation of the ID_{50} per ml values was $10^{0.18}$.

Change of medium. The medium of the DEE was sucked off with a syringe and cannula through a hole made in the plastic cap of the DEE. After rinsing the membrane twice with the medium, 10 ml fresh medium was added.

Designation of the inhibitor preparations. K_0 = Francis inhibitor prepared from human serum [11]; *8m* = diethyl-p-phenylene-diamine-diazonium-chloride derivative of K_0; *8ac* = acetone-soluble fraction of *8m*; K_0ac = acetone-soluble fraction of K_0.

Results

Correlation between the dose of inhibitor and the inhibition of virus multiplication in the CAM of the DEE. Eighty-eight DEEs were infected with 10^8 ID_{50} of virus each, rolled for 30 minutes and rinsed. Then 8 DEEs were filled with inhibitor-free medium, whereas the remaining DEEs were divided into 20 equal groups, each of which received inhibitor at a given concentration. Thus each of the four preparations were tested at concentrations of 20, 10, 5, 2.5 and 1.25 mg per ml. Four of the control DEEs were opened after 4-hour incubation in the roller apparatus, *i. e.* still in the eclipse phase; the remaining eggs were opened and used for virus titration after 16 hours.

In the period from 4 hours to 16 hours the infectivity of the control CAMs rose from $10^{3.5}$ to $10^{5.0}$. In the presence of K_0 or *8m* at the above concentrations, the infective titre of the CAM pools attained the control level, indicating that these preparations had not inhibited virus multiplication. In the presence of 20 mg per ml K_0ac the titre remained at the 4-hour level, indicating a complete or almost complete inhibition of virus multiplication, whereas 10 mg per ml of the same preparation showed no inhibition. Of *8ac* even 5 mg per ml caused 100 per cent inhibition, whereas in the presence of 2.5 mg or 1.25 mg per ml medium the infectivity increased to $10^{4.1}$ and $10^{5.0}$, respectively, indicating 87 and 0 per cent inhibiton.

From this experiment two conclusions can be drawn, *viz.* (i) only the acetone-soluble preparations inhibited the rise of the intracellular virus titre when such a large virus inoculum was used; (ii) the dose-response curves for the two effective substances are steep.

The results of this experiment are consistent with the experience published in the first report of this series [1]; then the same conclusion was based indirectly on the HA titre of the medium.

Attempts to determine the inhibitor-sensitive period of the reproduction cycle.

(a) Follow-up of virus multiplication and release (Table I, Fig. 1). DEEs were infected with $10^{6.9}$ ID_{50} each. After 1 hour adsorption period the seed virus was removed by rinsing the CAMs. Samples were taken 1, 3, 5, 7, 12, 24 and 32 hours after infection.

Table I

Multiplication of influenza virus in de-embryonated eggs (DEE) in the presence and absence of 8 ac

Strain: A—1 Budapest 4/49 Inoculum: $10^{6.9}$ ID_{50}/DEE								
Adsorption ratio: 76%								
8ac added at	Specimen	Infectivity titres* of samples taken						
		1	3	5	7	12	24	32
		hours after inoculation						
—	membrane	3.3	2.2	2.2	2.2	4.0	4.3	4.3
	medium	—	3.5	3.3	3.0	5.0	7.0	6.5
1 hour	membrane		3.0	3.0	3.0	3.0	3.0	1.0
	medium		1.0	1.0	3.0	2.3	3.0	2.3
5 hours	membrane				1.2	2.0	2.0	1.7
	medium				1.0	1.0	3.4	1.5
10 hours	membrane					3.3	3.0	3.0
	medium					2.0	1.0	1.3

*log ID_{50} in 0.1 ml

Titration of the pooled maintenance fluid at one hour showed that about 76 per cent ($10^{6.78}$ ID_{50}) of the seed virus had been adsorbed to cells; from the difference between this and the three-hour CAM titres it was established that $10^{6.77}$ ID_{50} per CAM had turned into eclipse.

Fig. 1A shows the dynamics of virus infectivity in the control CAM and medium pools. The infectivity of the CAM rose after the 7th hour only, to attain $10^{4.0}$ by the 12th, and $10^{4.3}$ by the 24th hour. Then it remained at the same level. The titre of the medium too began to rise after 7 hours; it reached

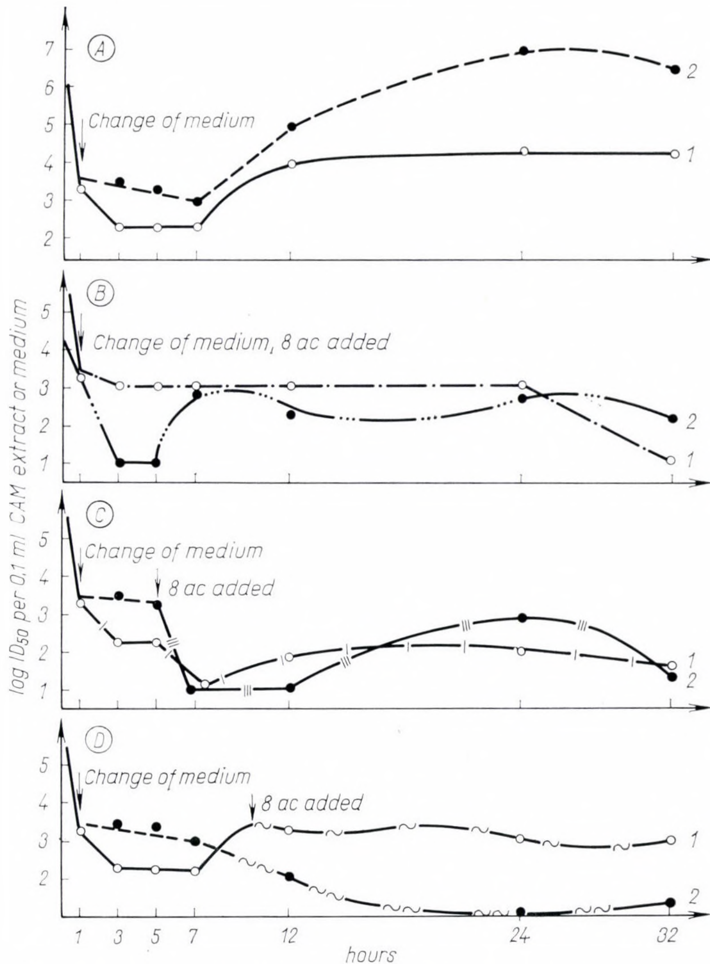

Fig. 1. The virus-reproduction-inhibiting effect of *8ac* when added to DEE at different times after inoculation with influenza virus. Strain: A—1 Budapest 4/49, inoculum: $10^{6.9}$ ID_{50}; adsorption time: 1 hour; virus turned into eclipse: $10^{6.77}$ ID_{50}; dose of inhibitor: 20 mg per ml medium. A: infectivity curves without inhibitor (virus control); B, C, D: infectivity curves when inhibitor was added at 1, 5 and 10 hours, respectively. 1: titre in CAM; 2: titre in medium

10^5 and 10^7 by 12 and 24 hours, respectively. Such a behaviour speaks for a multicyclic multiplication.

Fig. 1B shows the effect of 20 mg per ml *8ac* added to the system one hour after infection. Rise in the CAM's infectivity could not be demonstrated, in

spite of frequent samplings. The slight rise in the titre of the medium between 5 hours and 7 hours and its stagnation at the same level in the presence of the virucidal *8ac* shows that some virus was nevertheless released into the medium. The peak at the level of $10^{3 \cdot 0}$ was, however, not comparable with the 10^7 titre of the control medium.

Another difference between the control membranes and those treated with *8ac* was that in the former the titre fell from $10^{3 \cdot 3}$ to $10^{2 \cdot 2}$ during the first two hours after infection, presumably as a result of a continuous turning of virus into eclipse: a similar drop could not be observed in the CAMs that had received *8ac* at 1 hour, suggesting that *8ac* may cause a transitory disturbance in penetration or in turning into eclipse.

In the third part of the experiment (Fig. 1C) *8ac* was added in the second third of the reproduction cycle, at 5 hours. Subsequently, the CAM's infective titre dropped from $10^{2 \cdot 2}$ to $10^{1 \cdot 2}$, then rose to $10^{2 \cdot 0}$ and remained at this level till the end of the experiment. The further course of the curve did not differ significantly from that seen in Fig. 1B. The reduction in the CAM's infectivity might be explained by a forced release of virions which had been adsorbed but had not penetrated.

In the fourth part of the same experiment *8ac* was added at 10 hours. Here, too, there was no further rise in the titres after addition of *8ac*. The declining titre of the medium after 10 hours showed that further cycles had failed to proceed.

(b). Attempts to determine the inhibitor-sensitive period of the reproduction cycle by varying the time of adding or of removing 8ac, and titrating CAM infectivity at a constant time.

Since the virucidal effect of *8ac* prevents the analysis of its effect on virus adsorption, inhibition of intracellular virus multiplication was analyzed by combining two methods.

(i) Under experimental conditions resulting in an approximately one-step reproduction *8ac* was added to the system at different times and left in the medium until the end of the experiment. The latest time at which the addition of *8ac* still caused 100 per cent inhibition was considered to indicate the beginning of the inhibitor-sensitive processes. The earliest time at which the addition failed to result in inhibition was considered to indicate the end of the sensitive processes.

(ii) *8ac* was added immediately after the end of the adsorption period and the time of its removal was varied. The virus yield was estimated at a constant point of time (11 or 16 hours after infection in different experiments). The latest time at which the removal of *8ac* was still followed by an approximately 100 per cent yield was considered to indicate the onset of the sensitive processes, while the earliest removal which was followed by the lowest yield was considered to indicate the end of the same processes. On the basis of ex-

periments of this type we attempted to estimate the lag phase of the regeneration of virus reproduction from the difference in time needed for producing the same yield by the treated CAMs after removing the inhibitor and by the control CAMs after infection.

DEEs were infected with $10^{7.4}$ ID_{50} of virus each and rolled for 30 minutes. Then the medium was sucked off and the membranes were washed. From the titre of the medium the amount of infective virus that had been adsorbed to a CAM was estimated at $10^{6.9}$ ID_{50}. Infectivity of control CAMs was

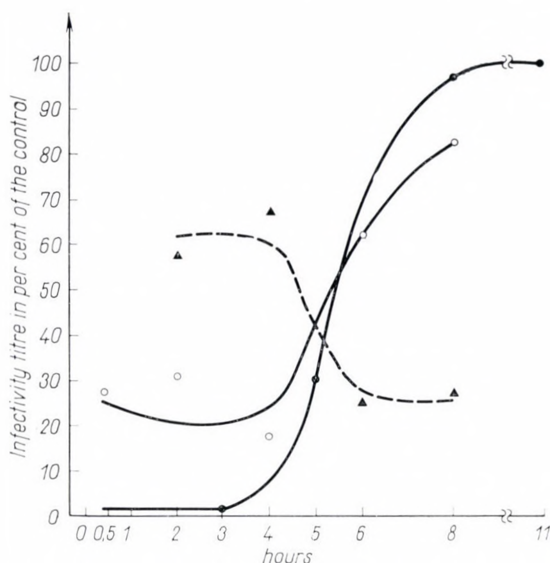

Fig. 2. Correlation between the time of addition or removal of *8ac* and the inhibition of virus multiplication in the CAM of the DEE. Virus: A—1 Budapest 4/49; inoculum: $10^{7.4}$ ID_{50} per CAM; adsorption period: 30 minutes; dose of *8ac:* 10 mg per ml. Titration of infectivity at 11 hours, in control CAMs at the indicated times. Virus titre expressed in per cent of the titre of the control CAM extract at 11 hours

Explanation: Each empty-circle (○———○) in the curve indicates the virus yield obtained at 11 hours when *8ac* was added at the time indicated by the position of the empty circle. Each full triangle (▲———▲) in the curve indicates the virus yield obtained at 11 hours when *8ac* was added at 30 minutes after infection, and removed at the time indicated by the position of the full triangle

titrated at 3, 5, 8 and 11 hours after infection. The titre at 3 hours was considered as 0 per cent multiplication, that at 11 hours as 100 per cent. The curve in the right of Fig. 2 shows the dynamics of multiplication in the control system.

The medium of groups 1—5 was changed for a medium containing 10 mg per ml *8ac* at 30 minutes, 2, 4, 6 and 8 hours, after infection (see the empty circles in Fig. 2). At 11 hours, *i.e.* when the first cycle had practically come to an end, the groups of CAM were titrated. Taking the 11-hour titre of the con-

trol group as 100 per cent, those for groups 1—5 were 28, 31, 18, 62 and 82 in the order of the time of adding the inhibitor.

Four further groups of DEE were given *8ac* in the 30th minute after infection. The inhibitor was changed for inhibitor-free medium at 2, 4, 6 and 8 hours after infection (see the filled triangles in Fig. 2). All the CAM homogenates were titrated at 11 hours. In these cases the virus titre at 11 hours was 57, 67, 25 and 28 per cent of the 11-hour value of the control, in the order of the times of removing the inhibitor.

According to these data, neither 100 per cent inhibition nor 100 per cent multiplication (*i.e.* regeneration of the multiplication) occurred in the CAMs once treated with 10 mg *8ac* per ml. If 50 per cent multiplication is accepted as practically unimpaired, the sensitive process may be considered to occur before 6 hours after infection.

Although a reproduction cycle in this system does not last longer than 11 or 12 hours, it was nevertheless supposed that a prolongation of the incubation might be favourable for approaching 100 per cent multiplication even after a period of treatment with *8ac*.

DEEs were infected with 10^8 ID_{50} of virus each and rinsed after 30-minute rolling. Then six groups of DEE were given 5 mg per ml *8ac*, whereas seven groups received the inhibitor-free medium. CAM homogenates obtained from the control group were titrated 4, 6, 8, 12 and 16 hours after infection. The 4-hour level was considered 0 per cent, the 16-hour level 100 per cent, multiplication. In Fig. 3 the virus titres of the control membrane homogenates are indicated by a solid line and full circles. The empty circles indicate the addition of 5 mg per ml *8ac* at 2 or 4 hours. Filled triangles indicate removal of the inhibitor and substitution with the inhibitor-free medium (five groups). Infectivity was titrated at 16 hours in all groups.

Five mg per ml *8ac* added after 30-minute incubation with the virus caused 100 per cent inhibition. The same concentration, however, failed to inhibit virus multiplication when added at 2 or 4 hours.

According to this experiment the process which is most sensitive to such a minimum concentration of *8ac* takes place between 30 minutes and 2 hours after virus had been added to the membranes. Regeneration ensues without any demonstrable lag phase or reduction in the final titre.

The experimental conditions and the symbols in Fig. 4 are the same as for the previous experiment and Fig. 3. The only difference was in the concentration of *8ac*, this having been twice higher, *i.e.* 10 mg per ml. Five groups of DEE were given inhibitor at 0.5, 2, 6, 8, and 12 hours, respectively. Infectivity of all these groups was titrated at 16 hours, whereas inhibitor-free groups were titrated at 4, 8, 12 and 16 hours.

This large dose of inhibitor caused 100 per cent inhibition even when added to the system after 4-hour incubation with the virus. Addition of the

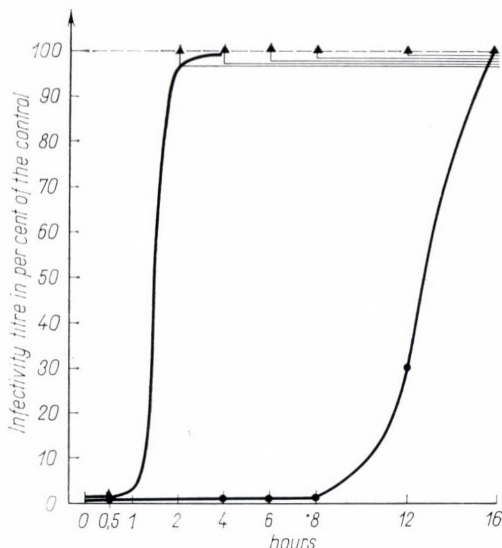

Fig. 3. Correlation between the time of addition or removal of *8ac* and the inhibition of virus multiplication in the CAM of the DEE. Virus strain: A—1 Budapest 4/49; inoculum: 10^8 ID_{50} per CAM; adsorption period: 30 minutes; concentration of *8ac:* 5 mg per ml. Infectivity titration at 16 hours and in control CAMs at the indicated times. Virus titre expressed in per cent of the infectivity of the control CAM extract estimated at 16 hours. Explanation: See under Fig. 2

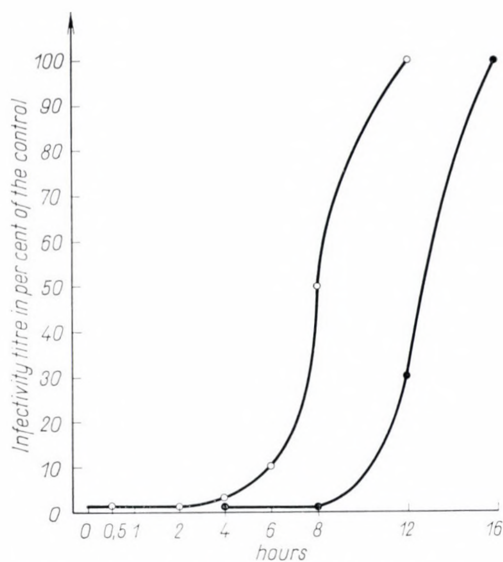

Fig. 4. Correlation between the time of addition of *8ac* and the inhibition of virus multiplication in the CAM of the DEE. Virus strain: A—1 Budapest 4/49; inoculum: 10^8 ID_{50} per CAM; adsorption period: 30 minutes; concentration of *8ac:* 10 mg per ml. Infectivity was titrated at 16 hours and in control CAMs also at the indicated times. Virus titre expressed in per cent of the titre of the control CAM extract at 16 hours. Explanation: See under Fig. 2

inhibitor at 6, 8 and 12 hours resulted in 90, 50 and 0 per cent inhibition of virus multiplication, respectively. This was consistent with the former experiment. According to the control group there was still no newly-produced infective virus in the CAM homogenate at 8 hours.

The event(s) sensitive only to this relatively large dose of inhibitor started in the 4th hour and came to an end between 8 and 12 hours, *i.e.* in the period of the appearance of new virions in the control membranes. Meanwhile the products of these events seemed to grow in amount and/or stability. Given at 12 hours, even the large dose of *8ac* failed to inhibit the further processes of reproduction. The existence of a process taking place after 12 hours is evidenced by the steep rise of the control curve between 12 and 16 hours. There is little doubt that this process was the building up of the virion from the viral components the production of which had come to an end by 12 hours. If the rise in infectivity between 12 and 16 hours had been due to a second cycle of reproduction, this — being in an early phase of the cycle — would have been inhibited by the *8ac* added at 12 hours.

The results of previous three experiments suggest that the inhibitor-sensitive phase of the reproduction cycle starts at about the 30th minute of the incubation with virus and the product(s) of the inhibitor-sensitive processes remain in an unstable state in which they cannot resist a large dose of *8ac*.

For the next experiment the virus inoculum was raised to $10^{9.5}$ ID_{50}, whereas the concentration of the inhibitor was reduced to 7.5 mg per ml. Otherwise the experiment, and also the symbols in Fig. 5, corresponded to the preceding experiment. *8ac* was added at 30 minutes and removed from the four groups at 4, 6, 8 and 12 hours, respectively.

Owing to the large inoculum, a considerable rise in the infectivity of the control CAMs was observed as soon as at 4 hours. Removal of the inhibitor at 2 hours resulted in 100 per cent multiplication, suggesting that the 14-hour period after removal was sufficient to complete the reproduction cycle. Removal at 4, 6 and 8 hours (followed by 12, 10 and 8 hours of re-incubation in inhibitor-free medium) resulted in 81, 54 and 44 per cent multiplication, respectively. Removal at 12 hours (followed by 4-hour re-incubation) was too late to allow any rise in infectivity measurable at 16 hours.

This experiment has shown that after the inhibitor had been removed CAM cells were again able to continue the interrupted cycle and produce the same infective virus yield as the untreated cells, provided there was enough time to complete a reproduction cycle until titration.

It may be concluded that the sensitive process started at about the 2nd hour and came to an end after the eclipse had been finished.

The two curves in Fig. 5, *i. e.*, the curve representing virus production and that representing the regeneration of virus reproduction after removal of

the inhibitor, were in close relation, suggesting that virus multiplication proceeds at the same rate whether or not the cells have been pretreated with *8ac*.

To visualize the expectable lag phase between the removal of *8ac* and the regeneration of the reproduction cycle, a graphic method was applied (Fig. 5). A horizontal line was drawn from each of the filled triangles representing virus production after the removal of *8ac* at the corresponding time. These horizontal lines intersect the control virus production curve (a curve drawn

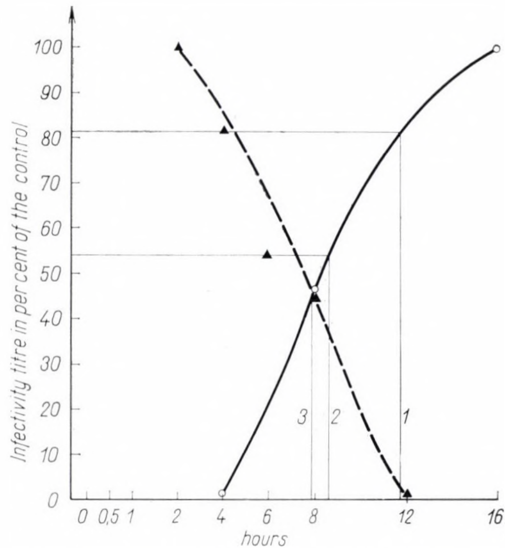

Fig. 5. Correlation between the removal of *8ac* and the inhibition of virus multiplication in the CAM of the DEE. Virus strain: A—1 Budapest 4/49; inoculum: $10^{9.5}$ ID_{50} per CAM; concentration of *8ac:* 7.5 mg per ml. Infectivity was titrated at 16 hours and in control CAMs also at the indicated times. Virus titre expressed in per cent of the titre of the control CAM extract at 16 hours. Explanation: See under Fig. 2

arbitrarily through three experimentally determined points). From each of the intersection points the perpendicular line to the time co-ordinate axis was drawn to show the period of time necessary for producing by the control membranes the same amount of infective virus which was produced until the titration time by the CAMs from which the inhibitor had been removed at the given time. Any delay in virus reproduction in the pre-treated CAMs was considered to be informative of the lag phase of regeneration. Fig. 5 shows some delays, but these correspond to very short lag phases. Production of a yield equal to that produced by the pre-treated CAMs during 12, 10 and 8 hours took approximately 11 hours 45 minutes, 8 hours 40 minutes, and 7 hours 45 minutes, respectively, in the control membranes. Thus, the estimated lag phases appear to range between 15 and 80 minutes.

Discussion

The roller drum method [2] is a sensitive procedure for titrating the inhibitors of influenza virus multiplication. The method has, however, two disadvantages: (a) the yield mostly consists in incomplete virus, which cannot be titrated but by its HA effect; (b) the inhibitor once added cannot be removed from the system. Thus, evaluation of the true effect on viral reproduction is disturbed if the inhibitor influences virus adsorption or virus HA, or both. The presence of our inhibitor inhibited both.

The DEE technique, on the other hand, has been used on a wide scale to study the reproduction of influenza virus [5—8] and the antiviral effect of chemical compounds [9, 10]. Virus production is measurable with the growth of infectivity in both the CAM cell homogenate and the fluid medium.

Using large inocula in the DEE, only two of our Francis inhibitor derivatives (K_0ac and *8ac*) have proved to inhibit viral reproduction to an extent measurable by the reduced infectivity of the CAM. Both of these derivatives are soluble in organic solvents and fail to precipitate during dialysis against distilled water. *8m*, on the other hand, which had proved more effective than K_0ac when tested in the rolling drum, had no such effect. Our earlier preparation termed No. 8 [11], which inhibited the multiplication of the influenza virus in the embryonated egg, was a mixture which contained part of the *8ac* fraction.

The investigations into the mechanism of action of *8ac* have already pointed to several characteristics. The effect of the substance appears very soon after its addition to the system, enforcing an elution of the adsorbed virions and, possibly disturbing the virus release. A disturbing effect on the penetration or on turning into the eclipse phase cannot be excluded, either.

The length of the period of virus synthesis which is sensitive to *8ac* depends on the dose of the inhibitor (and on the length of the observation). The correlation with the dose might be explained by assuming that a larger dose inhibits more processes or that the early products of viral reproduction are labile and their stabilization is inhibited by large doses of *8ac*. Accordingly, the sensitive events start between 30 minutes and 2 hours, this is followed by a stabilization period of 2 hours to 6 hours and terminates at the end of the eclipse period.

Sensitivity to large doses of inhibitor does not cease before the end of the eclipse phase. The processes in progress are slowed down by the addition of large dose of *8 ac* and then new cycles are prevented.

Virus reproduction soon regenerates after *8ac* has been removed.

Elucidation of the effect *in vivo* of *8ac* and its mode of action on the synthesis of viral constituents needs further studies.

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TICK-BORNE ENCEPHALITIS: A COMPARATIVE SEROLOGICAL SURVEY IN HUNGARY

By

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Summary. From 1037 acute neurological cases observed in Hungary in 1963—1965, serum samples were examined with the quantitative neutralization and haemagglutination inhibition tests. Neutralizing antibodies to the Hungarian KEm_1 strain of tick-borne encephalitis virus were found in 23 per cent of the cases of encephalitis and 10 per cent of those of aseptic meningitis. The haemagglutination inhibition test was positive more often than the neutralization test and the two tests gave divergent titres in the same serum samples. On the basis of the divergencies it is supposed that some other arbovirus of Casals's group B is prevalent in Hungary.

Acute neurological cases, caused by the tick-borne encephalitis (TBE) virus, occur sporadically in almost the whole area of Hungary in every year. One of us (E. M.) has examined the frequency of neutralizing antibodies to the TBE virus in the sera of such patients since 1958. Out of the cases of encephalitis and aseptic meningitis examined until the end of 1962, 25 per cent and 13 per cent, respectively, proved to be seropositive [1]. The present report describes similar serological studies for the years 1963—1965. For comparison, parallel with the neutralization test in HeLa cultures, the sera were tested for haemagglutination-inhibiting (HI) antibodies with an antigen prepared from TBE virus.

Materials and methods

Serum samples. Sera submitted to the Virus Department of this Institute from 1037 neurological cases clinically suspect of TBE or other virus infection were inactivated at 56°C for 30 minutes and kept at —7° until tested.

Virus strain. The KEm_1 strain of TBE virus was isolated in Hungary from ticks [2] and carried over numerous passages in the mouse brain before adapted to HeLa cells [3].

HeLa cell suspension. Fifteen thousand HeLa cells per ml were suspended in a medium containing 90 per cent HANKS's balanced salt solution enriched with 0.5 per cent lactalbumin hydrolysate, and 10 per cent calf serum [4].

Neutralization test. A hundred CPD_{50} of virus was mixed with an equal volume of the indicated serum dilution. The mixture was kept at 37°C for 90 minutes, then added in a test tube to the freshly prepared HeLa cell suspension. The test was described in detail elsewhere [1].

Haemagglutinating antigen. Noninfectious TBE virus antigen for the group-specific HI test was kindly supplied by the Poliomyelitis and Viral Encephalitis Research Institute of the USSR Academy of Medical Sciences, Moscow.

Haemagglutination-inhibition test. Sera were freed from nonspecific inhibitors and haemagglutinins by adsorption with 25 per cent kaolin and a 10 per cent goose erythrocyte suspension, respectively. The test was performed as recommended by CLARKE and CASALS [5].

As neuratralization and HI titre the highest serum dilution preventing the cytopathic effect and haemagglutination, respectively, was considered.

Results

In the years 1963—1965, 1500 serum samples obtained from 1037 cases were examined with virus neutralization test. The diagnosis was encephalitis in 462 cases and aseptic meningitis in 575 cases. Neutralizing serum antibodies were present in 23 per cent of the encephalitis cases and 10 per cent of the aseptic meningitis cases (Table I).

Table I

Incidence of neutralizing serum antibodies to the KEm_1 strain of TBE virus in patients with various acute neurological diseases
(1963—1965)

Year	Encephalitis			Aseptic meningitis			Total		
	No. of cases tested	Positive		No. of cases tested	Positive		No. of cases tested	Positive	
		No.	%		No.	%		No.	%
1963	106	20	19	250	28	11	356	48	13
1964	132	22	17	174	7	4	306	29	9
1965	224	65	29	151	19	13	375	84	22
1963—65	462	107	23	575	54	10	1037	161	16

Table II shows the neutralization titres of the paired sera of 294 patients: 253 cases were negative, antibodies were present in 41 convalescent-phase samples. In 9 of the seropositive cases the acute-phase sample contained high-titre antibody. In the remaining 32 cases the rise in titre was significant.

Table II

Neutralization titres of paired sera
(1963—1965)

Titres*		Convalescent samples			Total
		≦10	100	≧1000	
Acute samples	≦10	253	3	20	276
	100	0	0	9	9
	≧1000	0	1	8	9
Total		253	4	37	294

*Reciprocals

In Table III the HI titres of 221 serum pairs are presented. Out of these 76 proved to be seropositive in the convalescent phase; 33 patients had antibodies in the acute phase already.

Table III

HI titres of paired sera to the group B of arboviruses
(1964—1965)

Titres*		Convalescent sera				Total
		≤10+	40—160	320—640	≥1000	
Acute phase sera	≤ 10	145	19	6	0	170
	40—160	4	17	15	2	38
	320—640	1	5	7	0	13
Total		150	41	28	2	221

*Reciprocals

A comparison of the results of the neutralization test with those of the HI test (Table IV) has shown that out of the 1007 samples examined with both tests 124 proved to be positive (NP in the following) while 718 proved to be negative with both tests; 165 patients had only HI antibodies (HIP-NN cases). It should be emphasized that all the NP sera had group-specific HI antibodies as well.

Table IV

Comparison of TBE virus neutralization and group-specific HI titres in sera
(1964—1965)

Titres*		Neutralization titres			Total
		≤10+	100	≥1000	
HI titres	≤10	718	0	0	718
	40—320	128	35	69	232
	640—1000	37	3	17	57
Total		883	38	86	1007

* Reciprocals

Fig. 1 shows the incidence by age and sex of the NP cases. As seen, the incidence of seropositivity grew with the age, being 5, and 26 per cent in the three age groups, irrespective of sex. Of the 1037 patients 653 (63 per cent)

were males and 384 (37 per cent) females. The sex incidence among the diseased persons was about the same, 16 and 15 per cent, respectively.

Most seropositive cases occurred in the period from May to August (Fig. 2). The incidence of NP cases was the highest in June in both 1964 and 1965. The curve representing the HIP-NN cases reached its peak in July; in 1965 it remained at the same level in August (Fig. 2).

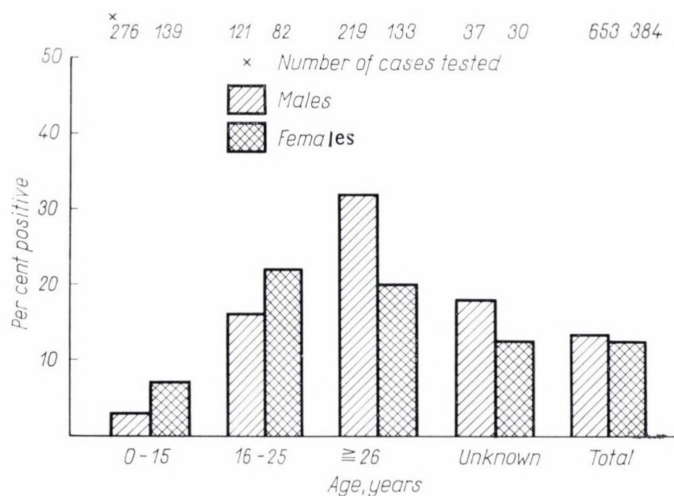

Fig. 1. Incidence of cases with neutralizing antibodies to the TBE virus, distributed by age and sex (1963—1965)

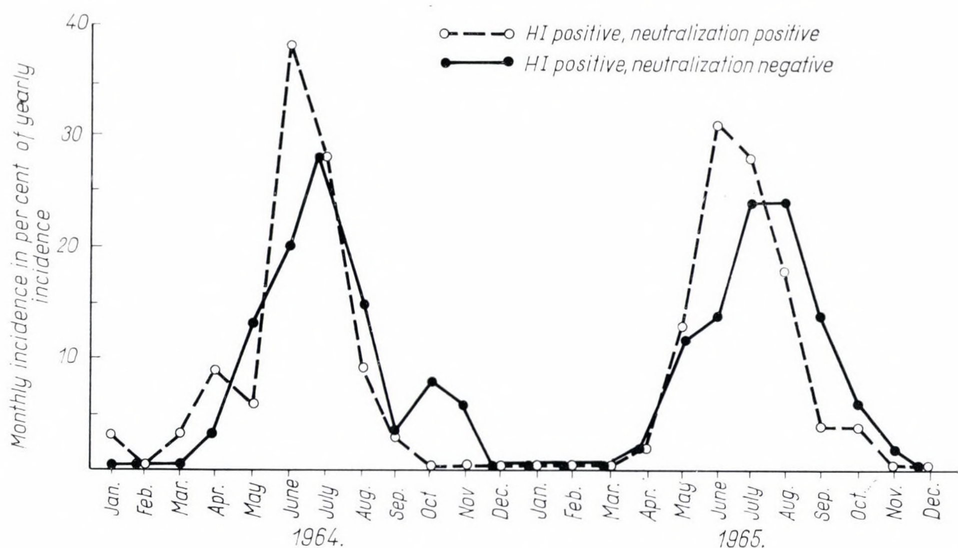

Fig. 2. Monthly incidence of seropositive TBE cases (1964 and 1965)

The geographical distribution of the NP cases observed in the years 1958—1965 is shown in Fig. 3. It is clear that the incidence was highest in the region near the western border of Hungary. Out of the 305 cases 159 (52 per cent) occurred in four counties, where on the average more than 30 per cent (in Győr-Sopron county 50 per cent) of the cases proved to be positive. In the

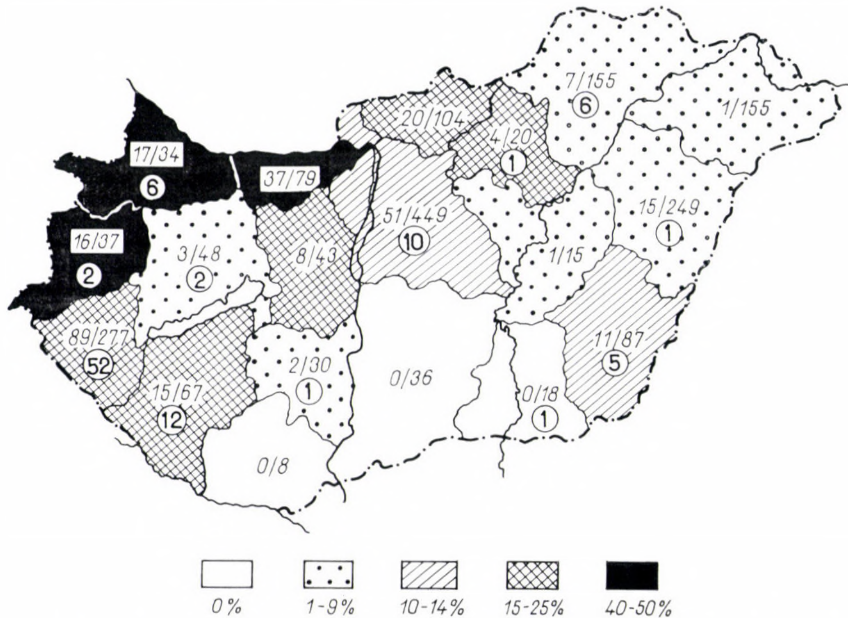

Fig. 3. Geographical distribution of seropositive patients suffering from acute diseases of the central nervous system. Numerator: cases with neutralizing antibody to TBE virus. Denominator: acute neurological cases tested. Encircled figures: cases with HI antibodies having no neutralizing antibodies

other 15 counties the average positivity was 10 per cent. Pest county and the town of Budapest are treated together in the map. Thus the 11.2 per cent positivity of neurological cases in a mostly urban population appears relatively high. Many of these patients must have had acquired the infection in the country. In three counties no NP cases were observed. From these counties, however, few (altogether 62) serum samples were submitted.

The incidence of HIP-NN cases is also shown in Fig. 3; more than 60 per cent of these cases were observed in two counties at the southwestern border.

Discussion

The clinical diagnosis of TBE presents great difficulties in the early phase, when the complaints are indistinct and thus suspection usually arises after several days' observation only, when specific serum antibodies have already appeared. Moreover the majority of patients are adult men, who usually consult a physician after they have developed neurological symptoms. In this late phase a further rise in antibody titre cannot be expected and a persistent high titre may equally indicate a current or a previous infection.

It is well-known that under the temperate climate cumulation of arbovirus infections occurs from the early summer to the autumn, depending on the blood-sucking arthropod vector. In the present study the peak of the curve representing the incidence of HIP-NN cases followed the peak of the NP curve with a delay of one or two months, suggesting that the former cases should represent infections with a group B arbovirus of different ecology. The divergencies between the HI and neutralization titres in the NP group appear to support this view.

Data suggestive of the occurrence of another group B arbovirus have been reported recently from Austria [7] and Czechoslovakia [8]. In both countries areas near the Hungarian border appear to be affected. The most affected areas of Hungary are the valleys of the rivers entering the country from the affected areas of Austria and Czechoslovakia, and now we must reckon with mosquito-borne arboviruses in those valleys.

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SEROLOGICAL PROPERTIES OF PSEUDOMONAS AERUGINOSA

I. GROUP-SPECIFIC SOMATIC ANTIGENS

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Summary. Immune sera prepared with boiled suspensions of *Ps. aeruginosa* agglutinated most definitely living cells and cultures heated first at 100°C for 2½ hours then at 130°C for 1 hour. Mild heating, alcohol, saturated sodium chloride and formalin rendered *Ps. aeruginosa* cultures O-inagglutinable. Heating at 100° to 130°C restored O-agglutinability proportionally to the time and temperature of exposure. Acid treatment exerted an effect similar to that of higher temperatures. When subjected to the above treatments, somatic antigens retained their immunogenicity and agglutinin-binding capacity.

On the basis of studies on 2197 *Ps. aeruginosa* strains isolated from various materials, an antigenic schema containing 13 O groups has been devised. By diagnostically important partial antigens serogroups 3, 4, 5, 7 and 10 were further divided into 15 subgroups. In addition to group-specific antigens most *Ps. aeruginosa* strains contained a common somatic factor. No thermolabile somatic antigens were detectable by agglutination test.

Parallel tube agglutination with heated suspensions and slide agglutination with living bacteria indicated that the two methods were equivalent for the determination of group-specific antigens. A detailed description of type strains and methods for the preparation, checking and absorption of typing sera is presented. The group and subgroup distribution of 2197 strains in various materials is discussed.

The importance of the serological differentiation of *Pseudomonas aeruginosa* is clearly shown by the fact that since JACOBSTHAL's studies in 1912 [1] at least 47 papers dealing with this problem have been published. Investigations performed until 1961, the results of which have been excellently reviewed by VERDER and EVANS [2], indicated generally that *Ps. aeruginosa* was a serologically heterogeneous species. Although the first investigators already referred to the possible presence of distinct thermostable and thermolabile antigens, later some authors claimed that unlike in the family *Enterobacteriaceae*, the somatic and flagellar antigens of *Ps. aeruginosa* cannot be distinguished serologically. Other authors suggested that the precipitation test with antigenic extracts was more helpful for the serological division of *Ps. aeruginosa*. The contradictory findings of earlier workers are explained by the fact that in lack of a knowledge of the properties of *Ps. aeruginosa* antigens, many of them performed serological studies in systems not allowing a sufficient separation of reactions given by antigens with different physicochemical properties.

More recent authors, utilizing data of the serological properties of *Enterobacteriaceae*, have made a great progress in the elucidation of the nature

of *Ps. aeruginosa* antigens. HABS [3] in 1957 by the use of heated antigens and immune sera prepared against them devised an antigenic schema suitable for the practical differentiation of *Ps. aeruginosa* strains. HABS divided 70 strains by thermostable antigens into 12 groups. KLEINMAIER [4] showed that the slide agglutination technique was suitable for the differentiation of strains according to HABS' antigenic schema, as slide agglutination with living bacteria in sera prepared with heated organisms and the tube agglutination test performed as described by HABS always gave similar results. KLEINMAIER and MÜLLER [5] demonstrated that precipitation test in sera prepared with heated bacteria also gave identical results with HABS' agglutination test. MÜLLER and KLEINMAIER [6] confirmed the HABS schema with the agar gel diffusion technique.

KLEINMAIER, SCHREINER and GRAEFF [7] have recognized that reactions given by thermolabile antigens can be distinguished from those due to thermostable antigens only in sera devoid of agglutinins reacting with the thermostable antigen. Working thus with pure antibodies, they were able to demonstrate a definite difference between the granular-type agglutination of thermostable and the fluffy-type agglutination of thermolabile antigens. They also observed that identical or related thermostable antigens may be present in strains containing different thermolabile factors. These investigations gave no definite answer as to whether these thermolabile factors were somatic or flagellar components. VERDER and EVANS [2] divided 326 pyocyanin-producing *Ps. aeruginosa* strains into 10 O groups and 13 O subgroups. On the basis of 10 H factors they distinguished 29 serotypes. In the history of *Ps. aeruginosa* serology, VERDER and EVANS were the first to use the designation system known as "a—b relationship", which has recently been employed in different *Enterobacteriaceae* serological schemata.

SANDVIK [8] included the majority of 87 strains of animal origin into his antigenic schema containing 7 O groups. He compared his type strains with those of HABS and thus first attempted to coordinate two independently elaborated *Ps. aeruginosa* antigenic schemata. GOULD and McLEOD [9] examining 240 *Ps. aeruginosa* strains concluded that at least 4 different antigenic components were present in their strains. They observed no difference in immunogenicity between antigens treated at lower and higher temperatures. MEITERT [10] by use of sera prepared with heated antigens and heated suspensions divided 304 strains into 10 O groups. MEITERT's 10 groups correspond in reality to 8 groups, since due to bilateral relationships, groups I and IV as well as VI and X cannot be considered distinct groups. HOBBS, CANN, GOWLAND and BYERS [11] showed by agar gel diffusion a number of different antigens in pseudomonads and other Gram negative bacteria. Of the 6 common components at least one was present in all examined *Pseudomonas*, *Achromobacter*, *Vibrio* and *Aeromonas* strains. WAHBA [12] in a study of 1961 *Ps. aeruginosa*

strains confirmed that living cultures could be typed by slide agglutination according to HABS' antigenic schema.

The purpose of the present work was to elaborate a simple, well-reproducible method for the serological typing of *Ps. aeruginosa*, which, in addition that it allows the differentiation of a relatively large number of serological units, offers valuable information to the epidemiologist. In order to elucidate contradictory findings of earlier authors, special attention has been paid to the properties of *Ps. aeruginosa* antigens.

Materials and methods

Ps. aeruginosa strains. The sources of the 2197 strains isolated during the years 1958—1965 are presented in Table X. Strains originating from non-faecal material were isolated on culture media used in routine bacteriology (nutrient, blood, chocolate, eosin-methylene blue and brilliant green agar). Part of the faecal samples was cultured on eosin-methylene blue, brilliant green, desoxycholate citrate and bismuth sulphite agar; other faecal specimens were seeded into liquid merthiolate-brilliant green enrichment medium [13]. Water and sewage samples were cultured by the membrane filter or the multiple tube dilution technique [13].

In order to obtain pure cultures, each primary isolate was streaked onto a brilliant green agar plate, then one isolated colony was transferred to an agar slant. The strains were stored in a dark place at room temperature in Liebig extract agar stab cultures stoppered with cork sterilized by heating in paraffin. Subcultures were made at 1 ½ to 2 year intervals. More important strains were maintained also by freeze drying.

Identification of Ps. aeruginosa. Strains exhibiting the following properties were regarded as *Ps. aeruginosa:* characteristic growth after 18 hours at 37°C on brilliant green agar, growth at 42°C on beef extract agar, positive oxidase test [14], oxidative decomposition of glucose, rapid liquefaction of gelatin [15], reduction of nitrate into nitrogen [16]. Pigment production was also examined, but because of a fairly frequent incidence of pyocyanin negative strains, it was not considered a differential criterion.

Preparation of immune sera. O sera were prepared with strains producing "smooth" or "mucoid" colonies. From all strains selected for serum production at least two different antigens were prepared as follows. An 18 to 20 hour nutrient agar culture of the strain was washed off with saline, then half of the suspension was heated in a 75°C water bath for 1 hour; the other half was steamed at 100°C in an Arnold sterilizer for 2½ hours. The suspension was then diluted so as to correspond approximately to the density of a standard suspension which, when diluted 1 : 10 and placed in cuvettes with 1 cm light path, gave 60 per cent transmittance in the Beckman model DU spectrophotometer set at 0.03 mm slit and 530 mμ wavelength. Rabbits were injected twice weekly with graded intravenous doses of 0.5—1.0—2.0—4.0—5.0 ml. When living antigens were used, 0.1—0.2—0.5—1.0—2.0—4.0 ml doses were given. The sera were preserved with 0.5 per cent phenol and stored at 4°C.

Slide agglutination was performed with 18 to 20 hour ox-blood agar cultures in the "working dilution" of O sera. The working dilution corresponded to a dilution of the serum in which the homologous culture and antigenically identical strains agglutinated strongly in 1 to 4 seconds (++++ agglutination), but reactions due to minor antigenic relationships were weak or absent. The sera were diluted with physiological saline containing 0.5 per cent phenol. The working dilutions usually varied from 1 : 10 to 1 : 30; some sera were used in dilutions 1 : 50 to 1 : 150.

Tube agglutination. Analysis of O antigens was performed with suspensions heated first for 2 1/2 hours at 100°C then for 1 hour at 130 °C. This antigen, referred to in the following as "2 1/2 hr. 100° + 1 hr. 130°" antigen, was prepared as follows. The growth of nutrient agar plates was suspended in saline and steamed for 2 1/2 hours at 100°C in an Arnold sterilizer. The suspension was centrifuged and the bacteria were resuspended in glycerol (1 Roux flask culture in 2 to 3 ml), pipetted in 50 ml Erlenmeyer flasks which were then placed in a 130 °C hot air oven for 1 hour. After heating the antigens were left to stand at room temperature for 2 days, then adjusted photometrically with phenolized saline to the density of a standard giving 74 per cent transmittance in 1 : 10 dilution in 1 cm cuvettes of the Beckman model DU spectrophotometer set at 0.03 mm slit and 530 mμ wavelength.

Tube agglutination was performed with 0.5 ml serum dilutions and 0.05 ml bacterial suspensions. Readings were made after incubating the tubes in a water bath at 50°C for 20 hours.

Special antigens for tube agglutination. Serological properties of *Ps. aeruginosa* were examined by use of the following antigens. Bacteria grown for 18 to 20 hours on agar, blood agar, 0.3 per cent sodium desoxycholate-containing blood agar or eosin methylene blue agar prepared with 1 per cent Mavekal (anionic detergent [17]) were used as living suspensions or, after an exposure to various temperatures, as heated suspensions. Acid treatment was performed by adding 3 volumes of N hydrochloric, 0.1 N hydrochloric, or glacial acetic, acid to suspensions about two or three times as concentrated as the standard density described for the "2 1/2 hr. 100° + 1 hr. 130°" antigen. After incubation at 37 °C for 1 day the suspensions were centrifuged and the deposit was neutralized with sodium hydroxide. Alcoholized antigen was prepared by adding 1 volume of 96 per cent ethanol to the suspension, then after incubation at 37°C for 2 days the bacteria were centrifuged and finally resuspended in saline. Sodium chloride treatment was carried out as follows. The living suspension was saturated at 37°C with sodium chloride then after 2 days incubation at 37°C the salt was removed by centrifugation or dialysation against tap water. Formalinized antigen was prepared either with bacteria cultured on the above media or with broth cultures of strains passaged previously in U tubes containing semisolid agar. Formalin was added to a final concentration of 0.5 per cent, then the suspensions were incubated at 37°C for 2 days. All the above antigens were adjusted to the approximate density described for the "2 1/2 hr. 100° + 1 hr. 130°" antigen.

Absorption of agglutinins. Absorbed sera for the determination of O subgroups were prepared as follows. The growth from 18 to 20 hour Roux flask cultures (1 Roux flask contained about 130 ml nutrient agar) was suspended in saline, steamed at 100°C for 1 hour then centrifuged. The bacteria were resuspended in saline to a volume which together with the concentrated serum gave the required serum dilution. Generally, serum dilutions 3 to 4 times as concentrated as the working dilution were absorbed. The suspensions were incubated first for 2 hours at 37°C then overnight at 4°C. After centrifugation the supernatant serum dilution was preserved with phenol at 0.5 per cent final concentration. The bacteria remaining in the deposit could be used for a repeated absorption; they were resuspended, heated at 100°C for 1 hour, centrifuged and used for exhausting a fresh aliquot of unabsorbed serum. For the complete removal of all unrequired agglutinins a considerable amount of culture was needed (see Table VIII).

Results

1. *Properties of Ps. aeruginosa antigens*

Agglutinability. The properties of *Ps. aeruginosa* antigens were studied on a number of different strains. Bacterial suspensions used in the experiments were treated with heat and chemical substances, then their agglutinability, immunogenicity and agglutinin-binding capacity were examined. In Table I some characteristic data are presented. These results were obtained with strains Ps 11 and Ps 304, which contained identical H antigens but different O antigens. Table I shows the effects of various treatments on agglutination. Only titres obtained in tubes incubated at 37°C for 2 hours are presented. All tests were performed also in series incubated for 20 hours at 37°C, 2 hours at 50°C and 20 hours at 50°C. Readings differing significantly from results obtained after 2 hours at 37°C are presented in the text.

From Table I it is clear that the living antigen after a short incubation period gave a high titre agglutination in all sera. In this respect there was no significant difference between sera prepared with homologous antigens subjected to different treatments. The living suspension produced a coarse granu-

Table I

Effect of heat and chemical agents on the agglutinability and immunogenicity of Ps. aeruginosa antigens
(Reciprocal agglutination titres in tubes incubated for 2 hours at 37°C)

Antigens	Antigens used for immunization											
	Ps 11 living	Ps 11 1 hr. 60°	Ps 11 1 hr. 75°	Ps 11 1 hr. 100°	Ps 11 2 1/2 hr. 100°	Ps 11 sat. NaCl	Ps 11 50% ethanol	Ps 11 *N* HCl	Ps 11 acetic acid	Ps 11 0.5% for-malin	Ps 304 0.5% for-malin	Ps 11 0.5% form. ab-sorbed by heated Ps 11
Ps 11 living	5120	2560	2560	2560	1280	2560	2560	1280	5120	1280	320*	320*
Ps 11 1 hr. 60°	0	0	C	0	0	0	0	0	0	0	0	0
Ps 11 1 hr. 75°	0	0	0	0	0	0	0	0	0	0	0	0
Ps 11 1 hr. 100°	40	20	0	20	20	0	20	0	40	20	0	0
Ps 11 2 1/2 hr. 100°	160	80	40	40	80	20	40	20	40	80	0	0
Ps 11 2 1/2 hr. 100° + 1 hr. 130°	640	320	320	320	320	640	640	160	640	320	0	0
Ps 11 saturated NaCl	20	40	20	20	0	20	0	0	40	20	0	0
Ps 11 50% ethanol	0	0	0	0	0	0	0	0	0	0	0	0
Ps 11 *N* HCl	320	160	160	320	320	640	320	160	640	320	0	0
Ps 11 glacial acetic acid	320	160	80	160	160	640	160	80	160	320	0	0
Ps 11 0.5% formalin	640*	1280*	0	0	0	2560*	320*	0	0	1280*	2560*	640*
Ps 304 0.5% formalin	640*	1280*	0	0	0	2560*	160*	0	0	640*	5120*	640*

Strains Ps 11 and Ps 304 contained different O antigens, but identical H antigens.
Titres marked with asterisks correspond to H-type agglutinations.

lar clumping resembling the agglutination of *Escherichia* B antigens. The living antigen did not show this type of agglutination in sera absorbed with a boiled culture of the homologous strain. If serum dilutions were further incubated at 37°C for 20 hours with the living antigen, the agglutination, instead of becoming stronger and higher in titre, remained distinct only in the first tubes of the series (approximately up to a titre of 1 : 160). At higher serum dilutions the fluid became clear, the cells underwent lysis and the bacterial debris adhered to the tube wall. When the living culture was incubated at 50°C for 2 hours, the titre was similar to that of tests read after 2 hours at 37°C. After 20 hours incubation at 50°C, due to a loss of agglutinability discussed in the following, the titre was practically nil.

When prior to the tube agglutination test the bacterial suspension was exposed to mild heat (several hours at 50°C or 1 hour at 60 to 75°C), it became inagglutinable. If the antigens treated in this manner were incubated overnight at 37°C, weak agglutination occurred in the first tubes. The suspensions (especially those exposed to 75°C) became strongly viscid on heating; they could be pipetted into the agglutination tubes only after vigorous shaking. Suspensions heated at 100°C for 1 hour somewhat differed in behaviour from those exposed to mild heat; they were less viscid, and although after 2 hours incubation at 37°C they gave a readable agglutination only in the first tubes, after 20 hours at the same temperature the agglutination became higher in titre. Antigens heated at 100°C for 2 1/2 hours showed definite agglutination (1 : 40 to 1 : 160) as soon as after 2 hours; by the 20th hour they had reached titres of 1 : 320 to 1 : 640. When tubes with these antigens were incubated at 50°C, the titres rose by a further degree (Table II).

Table II

Effect of incubation time and temperature on the somatic agglutinability of Ps. aeruginosa antigens

Antigens	Reciprocal titres in serum Ps 11 prepared with "2 1/2 hr. 100°" antigen; duration and temperature of incubation			
	2 hr. 37°	20 hr. 37°	2 hr. 50°	20 hr. 50°
Ps 11 living	1280	lysis	2560	slime production
Ps 11 2 1/2 hr. 100°	80	640	160	640
Ps 11 2 1/2 hr. 100° + 1 hr. 130°	320	1280	640	2560

The titres were even higher if "2 1/2 hr. 100° + 1 hr. 130°" antigens were used. As these antigens gave the highest titres and were the most sensitive detectors of serological relationships among somatic factors, they were used throughout this study for the serological analysis of O antigens. As compared to living cells, "2 1/2 hr. 100° + 1 hr. 130°" antigens showed a finer type of

granular clumping, which resembled the agglutination of *Salmonella* O antigens. Temperatures over 130°C had a deleterious effect on the antigen and caused spontaneous lability. Even the "2 1/2 hr. 100° + 1 hr. 130°" antigen was satisfactory only when the suspension reached 130°C slowly (in a hot air oven). Suspensions heated in a 130°C oil bath were unstable.

Antigens treated with saturated sodium chloride or ethanol were similar in behaviour to suspensions exposed to mild heat. These antigens were practically inagglutinable within 2 hours. When incubated for 20 hours they agglutinated only in the first tubes.

Suspensions treated with hydrochloric or glacial acetic acid showed a reaction somewhat lower in titre than, but similar in appearance to, the agglutination of "2 1/2 hr. 100° + 1 hr. 130°" antigen. Among acid treated antigens that exposed to *N* hydrochloric acid showed the best agglutination. Acetic acid-treated cells reacted in somewhat lower titres. Bacteria treated with 0.1 *N* hydrochloric acid gave titres corresponding only about to titres obtained with suspensions treated at 100°C for 2 1/2 hours.

The lower rows of Table I show the agglutination of formalinized suspensions of motile cultures of strains Ps 11 and Ps 304. Titres marked with asterisks indicate a reaction different from the aforementioned types of agglutination: after 2 hours incubation at 37°C this reaction appeared as a finely granular clumping of bacteria. After prolonged incubation at 37°C the titre became higher and the reaction was not unlike the agglutination of peritrichate cells, although no such large loose clumps were observed. In contrast to somatic agglutination, the H agglutination of *Ps. aeruginosa* was characterized by a definite zone phenomenon. H agglutinins were detectable also with living suspensions when H sera not reacting with the O antigens of the culture were used (right hand columns of Table I: serum Ps 304 or Ps 11 absorbed by heated Ps 11 culture).

The action of formalin on O-agglutinability was similar to the effect of mild heat, alcohol or saturation with salt, as especially after short incubation periods the formalinized suspension was practically inagglutinable in O serum. There was, however, an essential difference between the above-mentioned and formalinized suspensions, in that formalin did not inhibit H agglutination.

When the suspensions were first treated with chemical substances then exposed to 75°C for 1 hour the following results were obtained. As expected, suspensions which had lost their agglutinability as a result of alcohol or salt treatment, remained inagglutinable after heating at 75°C. The formalinized suspension also remained inagglutinable in O serum and, owing to the heating, lost also its H-agglutinability. In contrast to the others, suspensions treated previously with *N* hydrochloric acid, retained their agglutinability after exposure to 75°C. Suspensions treated with glacial acetic acid showed a slight decrease in agglutinability after heating at the same temperature.

Table III

Effect of heat and chemical agents on the agglutinin-binding capacity of Ps. aeruginosa antigens
(Reciprocal agglutinin titres in tubes incubated for 2 hours at 37°C)

Antigens	Antigens used for absorption of serum Ps 11 prepared with living antigen										
	Ps 11 living	Ps 11 1 hr. 60°	Ps 11 1 hr. 75°	Ps 11 1 hr. 100°	Ps 11 2 1/2 hr. 100°	Ps 11 sat. NaCl	Ps 11 50% ethanol	Ps 11 *N* HCl	Ps 11 acetic acid	Ps 11 0.5% formalin	Ps 304 0.5% formalin
Ps 11 living	0	0	320*	320*	320*	0	0	160*	160*	0	5120
Ps 11 2 1/2 hr. 100° +1 hr. 130°	0	0	0	0	0	0	0	0	0	0	640
Ps 11 0.5% formalin	0	0	640*	640*	640*	0	traces*	640*	640*	0	0
Ps 304 0.5% formalin	0	0	640*	640*	640*	0	traces*	640*	640*	0	0

Strains Ps 11 and Ps 304 contained different O antigens, but identical H antigens.
Titres marked with asterisks correspond to H-type agglutinations.

In order to exclude that reactions attributed to flagellar antigens were caused by thermolabile somatic factors, some strains were cultured on media inhibiting the development of flagella (blood agar containing sodium desoxycholate and eosin methylene blue agar containing anionic detergent). Although there was a marked difference in H agglutination between such suspensions and motile cultures, the development of flagella was never suppressed completely. Immunization with bacteria grown on the inhibitory media yielded fairly good H antibodies and, when applied in sufficient amounts, these cultures removed H agglutinins.

Immunogenicity. As it follows from the reactions of strains Ps 11 and Ps 304 which contained different O but identical H antigens, sera prepared with living, formalinized and sodium chloride-saturated antigens contained both O and H antibodies. Bacteria treated with alcohol or heated at 60°C still gave rise to lower titre flagellar agglutinins. It is clear from Table I that suspensions heated at 75°C or higher temperatures as well as acid-treated bacteria failed to produce H antibodies. Good O titres were obtained with suspensions treated for 1 to 2 1/2 hours between 75°C and 100°C. Heating at 130°C or over impaired the agglutinogenic capacity of the antigen.

Agglutinin-binding capacity. Antibody absorption by antigens exposed to the action of various physical and chemical agents is shown in Table III. It is clear that O agglutinins were removed by all antigens. H agglutinins were left unabsorbed by bacteria heated at 75°C or over and also by acid-treated bacteria. Living, formalinized, salt-treated organisms and even bacteria heated

Table IV

Properties of group specific antigens of Ps. aeruginosa

Antigen	Agglutina-bility*	Immuno-genicity	Agglutinin-binding capacity**
Living	++++	++++	++++
1 hr. 60—75°C	—	++++	++++
1 hr. 100°C	+	++++	++++
2 1/2 hr. 100°C	++	++++	++++
2 1/2 hr. 100°C + 1 hr. 130°C	+++	++	++++
Saturated NaCl	—	++++	++++
50% ethanol	—	++++	++++
N HCl	+++	++++	++++
0.5 % formalin	—	++++	++++

* In tubes incubated for 2 hours at 37°C. After prolonged incubation negative reactions are characterized by low titre agglutinations.
** Absorption of antibodies from sera prepared with heated antigens

at 60°C removed H agglutinins. Absorption experiments with alcoholized suspensions were difficult to evaluate, as the highly viscid antigen could not sufficiently be separated from the serum.

Table IV summarizes the agglutinability, immunogenicity and agglutinin-binding capacity of *Ps. aeruginosa* O antigens after exposure to various physical and chemical agents.

2. Somatic antigens of Ps. aeruginosa

Group specific antigens and intra-species antigenic relationships. From each type strain representing an antigenic group or subgroup at least two different sera were prepared (one with bacteria heated for 1 hour at 75°C and one with bacteria heated for 2 1/2 hours at 100°C). All sera and all type strains were examined in cross agglutination tests performed with "2 1/2 hr. 100° + 1 hr. 130°", "2 1/2 hr. 100°" and living antigens. The heated antigens were examined with tube agglutination tests incubated at 50°C for 20 hours. Tests with living antigens were read after 2 hours incubation at 37°C.

Some cross agglutinations obtained with "2 1/2 hr. 100° + 1 hr. 130°" antigens are presented in Table V. It is seen that the homologous strain gave a high titre agglutination in each corresponding serum. This reaction was demonstrated also with living bacteria and suspensions heated for 2 1/2 hours at 100°C. The latter antigens agglutinated in much lower titres than antigens "2 1/2 hr. 100° + 1 hr. 130°" or living bacteria (see Table II). A definite agglutination of the homologous culture was observed also with the slide method. Antigens responsible for this type of agglutination were regarded as group specific factors constituting the basis of serological classification.

From Table V it is also evident that almost all strains showed a more or less marked cross agglutination. As compared to the homologous strain, heterologous cultures reacted in a lower titre and showed a much finer granular clumping. This type of cross agglutination between heterologous strains was not observed when living or "2 1/2 hr. 100°" suspensions were tested. These findings indicated that, in addition to the group specific antigens, *Ps. aeruginosa* strains contain certain common components. These factors produce low titre antibodies which can be detected only with bacteria exposed to high temperatures. In order to confirm this finding, the O sera were absorbed with different heterologous strains. After absorption the sera agglutinated only the homologous "2 1/2 hr. 100° + 1 hr. 130°" antigens. Sera absorbed in this manner retained their original titres also against living and "2 1/2 hr. 100°" antigens.

Certain O sera after absorption with heterologous cultures still agglutinated some *Ps. aeruginosa* strains. This reaction resembled the coarser type agglutination of the homologous strain and was observed with living and "2 1/2

Table V

Cross-agglutination reactions obtained with some Pr. aeruginosa strains
(Reciproeal agglutination titres with "2 1/ hr. 100° + 1 hr. 130°" antigens in tubes incubated for 20 hours in 50°C water bath)

Designation of O antigens	Strains	Immune sera										
		Ps 304	Ps 21	Ps 11	Ps 340	Ps 317	Ps 161	Ps 217	U 645	Ps 319	U 72/59	U 118/59
1	Ps 304	*5120*	40	40	0	0	320	20	160	160	80	20
2	Ps 21	160	*5120*	0	0	0	20	20	20	80	160	0
3a, 3b	Ps 11	20	0	*2560*	*320*	*2560*	20	40	40	40	160	0
3c	Ps 340	20	20	*160*	*10 240*	*320*	40	40	320	40	40	320
3a, 3d	Ps 317	20	160	*1280*	*40*	*2560*	20	40	160	160	40	40
4a, 4b	Ps 161	320	160	40	80	20	*5120*	*1280*	640	320	320	320
4a, 4c	Ps 217	20	160	320	320	320	*5120*	*10 240*	320	80	80	160
5a, 5b, 5c	U 645	0	80	0	20	20	160	40	*1280*	*1280*	160	160
5a, 5b, 5d	Ps 319	320	160	160	20	0	640	40	*640*	*10 240*	160	160
6	U 72/59	40	0	20	0	0	20	40	320	80	*5120*	80
7a, 7b	U 118/59	0	0	0	0	0	160	20	80	80	160	*10 240*

Figures printed in italics designate reactions detectable also with living or "2 1/2 hr. 100°" antigens and after an absorption of the serum by strains of different O groups. Nonitalicized titres were demonstrated only with "2 1/2 hr. 100° + 1 hr. 130°" antigens and the corresponding antibodies were removed practically by any heterologous culture.

hr. 100°" as well as with "2 1/2 hr. 100° + 1 hr. 130°" suspensions. These strains were therefore regarded as containing group specific (O) antigens identical with, or related to, the O antigens of the strain used for the preparation of the serum. In Table V figures printed in italics show titres due to group specific antibodies which cannot be absorbed with strains belonging to other O antigenic groups. Titres indicating inter-group agglutinins, which are removed practically by any heterologous strain, are shown by non-italicized figures.

Cross-absorption experiments with strains belonging to the same O group showed that certain groups were constituted of two or more subgroups. An example of an antigenic analysis is presented in Table VI.

The antigenic schema elaborated in this manner and cross-agglutination titres obtained with tube agglutination in sera depleted of intergroup antibodies are presented in Table VIIa.

Table VIIb presents the slide agglutination of living strains in the working dilutions of unabsorbed O sera. When comparing Tables VIIa and VIIb it is evident that the same cross reactions are revealed with heated and living antigens. It should be noted that the presented antigenic schema contains only major somatic antigens which are useful for the practical differentiation of strains. Minor antigens which were often absent or gave unilateral reactions were omitted from the schema.

Several hundred strains belonging to various O groups were compared by tube agglutination with "2 1/2 hr. 100° + 1 hr. 130°" antigens and by slide agglutination with living cultures. As the results were always identical, slide agglutination was regarded satisfactory for routine O grouping. As to the result of slide agglutination there was no major difference between 18 to 20 hour cultures grown on common dye and indicator-free solid media. As yeast agar containing 5 per cent ox-blood yielded the most readily suspendable cultures, this medium was used throughout the study.

The above experiments were performed in parallel with sera prepared with cultures heated at 75°C for 1 hour and at 100°C for 2 1/2 hours. Agglutination with living and heated antigens and cross-absorption tests gave identical results in both kinds of sera. Unrequired cross-agglutinations were somewhat less marked in sera prepared with antigens heated at 75°C than in sera prepared with boiled antigens, therefore the former were used for slide agglutination.

Antigenic variation. Formulae shown in the antigenic schema represent the O antigens of the majority of strains. In addition to differences in minor antigens omitted from the schema, not unfrequently there was a variation in diagnostically important antigens. A smaller part of strains characterized by O antigens "3c" and "3d, 3f" showed a more or less developed antigen "3a". From Table VI it is seen that the type strains of these subgroups (Ps 340 and Ps 48), although they agglutinated in very low titres in serum 3a, 3b, were

Table VI

Cross-absorption experiments with 0 group 3 strains

(Reciprocal agglutination titres with "2 1/2 hr. 100° + 1 hr. 130°" antigens in tubes incubated for 20 hours in 50°C water bath)

Strain	O serum Ps 11 absorbed by					O serum Ps 340 absorbed by				
	—	Ps 340	Ps 317	Ps 469	Ps 48	—	Ps 11	Ps 317	Ps 469	Ps 48
Ps 11	2560	320	320	320	320	320	0	0	80	0
Ps 340	160	0	0	0	0	10 240	320	320	320	640
Ps 317	1280	0	0	0	0	40	0	0	0	0
Ps 469	640	0	0	0	0	320	0	0	0	0
Ps 48	160	0	0	0	0	40	0	0	0	0
Agglutinins	3a, 3b	3b	3b	3b	3b	(3a), 3c, . . .	3c	3c	3c, . . .	3c

Strain	O serum Ps 317 absorbed by					O serum Ps 469 absorbed by				
	—	Ps 11	Ps 340	Ps 469	Ps 48	—	Ps 11	Ps 340	Ps 317	Ps 48
Ps 11	80	0	0	0	0	2560	0	0	0	0
Ps 340	40	0	0	0	0	320	0	0	40	20
Ps 317	2560	320	160	0	0	2560	160	160	0	0
Ps 469	320	160	160	0	0	10240	320	160	160	160
Ps 48	320	160	160	0	0	1280	160	160	0	0
Agglutinins	3a, 3d	3d	3d	—	—	3a, 3d, 3e, . . .	3d, 3e	3d, 3e	3e, . . .	3e, . . .

Strain	O serum Ps 48 absorbed by				
	—	Ps 11	Ps 340	Ps 317	Ps 469
Ps 11	160	0	0	0	0
Ps 340	20	0	0	0	0
Ps 317	640	80	160	0	0
Ps 469	160	20	20	0	0
Ps 48	2560	640	640	640	640
Agglutinins	(3a) 3d, 3f	3d, 3f	3d, 3f	3f	3f

Result of antigenic analysis:

Ps 11 = 3a, 3b
Ps 340 = (3a), 3c
Ps 317 = 3a, 3d
Ps 469 = 3a, 3d, 3e
Ps 48 = (3a), 3d, 3f

table to absorb agglutinin 3a. This finding indicated that "3c" and "3d, 3f" strains which did not contain antigen "3a" in detectable amounts, should be placed in O group 3. Many "4a, 4d" cultures occurred in which antigen "4a" was slightly developed.

An interesting antigenic variation has been observed among strains isolated in the László hospital. In two wards of the hospital O group 7 strains were commonly encountered. One strain from one ward and 6 strains from the other were characterized by the antigenic formula "7a, 7c", whereas the majority of strains from both units contained antigens "7a, 7b". Among the latter some strains produced colonies agglutinating in absorbed serum "7c". Thus, although antigen "7c" was a distinct partial factor and antigen "7b" was never present in "7a, 7c" strains, it was supposed that strains "7a, 7c" developed from strains "7a, 7b".

Mucoid variants. Part of the examined cultures produced mucoid colonies. Such mucoid forms were observed in most antigenic groups. Moreover, in materials derived from one hospital unit, members of the same antigenic group often appeared as mucoid and non-mucoid forms. These findings indicate that mucoid and non-mucoid strains have a common origin. The two forms behaved similarly in tube agglutination with heated bacteria. With slide agglutination mucoid strains reacted less readily than their non-mucoid counterparts; however, determination of the O antigens of mucoid strains was as exact as that of non-mucoid variants. The mucoid substance probably exerts some inhibitory effect on the agglutination of living cultures. None of our results indicated that the mucoid substance was antigenic.

Polyagglutinable and unstable strains. Due to agglutination in several (but not all) sera, certain *Ps. aeruginosa* strains could not be included in any of the antigenic groups. This agglutination occurred independently of the treatment of the antigen used for the preparation of the serum. The receptor responsible for this reaction was probably identical or related in all such strains as, although with different intensity, all strains agglutinated in the same sera. These strains were not "unstable" variants since, in addition to their producing smooth colonies, they showed no agglutination in 3 per cent sodium chloride solution. Provisionally these bacteria were named polyagglutinable strains.

Unstable strains were characterized by agglutination in 3 per cent sodium chloride and spontaneous lability of heated suspensions.

3. *Routine determination of O antigens*

Preparation, checking and absorption of sera. It follows from the results of this study that immune sera prepared with cultures heated in the range 75° to 100°C for 1 to 2 1/2 hours are suitable for the determination of O antigens. Checking of the serum and routine grouping of unknown strains are most

Table VIIa

Antigenic relationships between Ps. aeruginosa O groups

(Reciprocal agglutination titres with "2 $^{1}/_{2}$ hr. 100° + 1 hr. 130°" antigens in tubes incubated for 20 hours in 50°C water bath)

Sero-group	O antigens	Type strain	Titre of homologous serum	Cross-agglutination titres in O sera absorbed by strain Ps 10 (01) or Ps 21 (02)			
1	1	Ps 304	5120	—			
2	2	Ps 21	5120	—			
3	3a, 3b	Ps 11	2560	3c: 160	3a, 3d: 1280	3a, 3d, 3e: 640	3d, 3f: 160
	3c	Ps 340	10240	3a, 3b: 320	3a, 3d: 40	3a, 3d, 3e: 320	3d, 3f: 40
	3a, 3d	Ps 317	2560	3a, 3b: 80	3c: 40	3a, 3d, 3e: 320	3d, 3f: 320
	3a, 3d, 3e	Ps 469	10240	3a, 3b: 2560	3c: 320	3a, 3d: 2560	3d, 3f: 1280
	3d, 3f	Ps 48	2560	3a, 3b: 160	3c: 20	3a, 3d: 640	3a, 3d, 3e: 160
4	4a, 4b	Ps 161	5120	4a, 4c: 5120	4a, 4d: 640	10a, 10b: 80	
	4a, 4c	Ps 217	10240	4a, 4b: 1280	4a, 4d: 640		
	4a, 4d	Ps 323	10240	4a, 4b: 160	4a, 4c: 320		
5	5a, 5b, 5c	U 645	1280	5a, 5b, 5d: 640	5a, 5d: 320		
	5a, 5b, 5d	Ps 319	10240	5a, 5b, 5c: 1280	5a, 5d: 2560		
	5a, 5d	Ps 194	2560	5a, 5b, 5c: 1280	5a, 5b, 5d: 2560		
6	6	U 72/59	5120	—			
7	7a, 7b	U 118/59	10240	7a, 7c: 640			
	7a, 7c	B 415	5120	7a, 7b: 5120	11: 320		
8	8	U 900/60	2560	—			
9	9	Ps 910	2560	—			
10	10a	Ps 196	2560	10a, 10b: 2560			
	10a, 10b	Ps 275	10240	10a: 1280			
11	11	Ps 898	5120	7a, 7c: 160			
12	12	L 83	10240	—			
13	13	V 142a	2560	—			

Table VIIb

Antigenic relationships between Ps. aeruginosa O groups
(Slide agglutination with blood agar cultures in working dilutions of sera)

Sero-group	O antigens	Type strain	Homologous serum	Cross agglutination in unabsorbed O sera				
1	1	Ps 304	++++	4a, 4c: ±				
2	2	Ps 21	++++	—				
3	3a, 3b	Ps 11	++++	3c: ++	3a, 3d: +++	3a, 3d, 3e: ++	3d, 3f: ±	
	3c	Ps 340	++++	3a, 3b: ++	3a, 3d: ±	3a, 3d, 3e: ++	3d, 3f: ±	11: ±
	3a, 3d	Ps 317	++++	3a, 3b: ++	3a, 3d, 3e: ++++	3d, 3f: +++	11: ±	
	3a, 3d, 3e	Ps 469	++++	3a, 3b: ++	3c: +	3a, 3d: +++	3d, 3f: ++++	
	3d, 3f	Ps 48	++++	3a, 3b: +	3a, 3d: ++	3a, 3d, 3e: +++	11: ±	
4	4a, 4b	Ps 161	++++	4a, 4c: ++++	4a, 4d: +++			
	4a, 4c	Ps 217	++++	4a, 4b: ++++	4a, 4d: ++	8: ±		
	4a, 4d	Ps 323	++++	4a, 4b: +	4a, 4c: ++			
5	5a, 5b, 5c	U 645	++++	5a, 5b, 5d: +++	5a, 5d: ++	8: ±	10a, 10b: +	
	5a, 5b, 5d	Ps 319	++++	5a, 5b, 5c: ++++	5a, 5d: ++++			
	5a, 5d	Ps 194	++++	5a, 5b, 5c: +++	5a, 5b, 5d: +++			
6	6	U 72/59	++++	—				
7	7a, 7b	U 118/59	++++	7a, 7c: ++++				
	7a, 7c	B 415	++++	7a, 7b: ++				
8	8	U 900/60	++++	7a, 7c: +				
9	9	Ps 910	++++	8: ++				
10	10a	Ps 196	++++	10a, 10b: +++				
	10a, 10b	Ps 275	++++	10a: ++				
11	11	Ps 898	++++	7a, 7c: ++				
12	12	L 83	++++	—				
13	13	V 142a	++++	—				

Table VIII

Determination of the O antigens of Ps. aeruginosa

O antigens	Agglutination in	No. of Roux flasks*
1	Unabsorbed serum Ps 304 (1)	—
2	Unabsorbed serum Ps 21 (2)	—
3a	Unabsorbed serum Ps 11 (3a, 3b) and unabsorbed serum Ps 317 (3a, 3d)	—
3b	Serum Ps 11 (3a, 3b) absorbed by Ps 317 (3a, 3d)	15
3c	Serum Ps 340 (3c) absorbed by Ps 469 (3a, 3d, 3e) + Ps 11 (3a, 3b)	20+10
3d	Serum Ps 317 (3a, 3d) absorbed by Ps 11 (3a, 3b)	30
3e	Serum Ps 469 (3a, 3d, 3e) absorbed by Ps 317 (3a, 3d) + Ps 340 (3c)	25+20
3f	Serum Ps 48 (3d, 3f) absorbed by Ps 317 (3a, 3d)	20
4a	Unabsorbed serum Ps 161 (4a, 4b) and unabsorbed serum Ps 217 (4a, 4c)	—
4b	Serum Ps 161 (4a, 4b) absorbed by Ps 217 (4a, 4c)	20
4c	Serum Ps 217 (4a, 4c) absorbed by Ps 161 (4a, 4b)	30
4d	Serum Ps 323 (4a, 4d) absorbed by Ps 217 (4a, 4c) + Ps 831 (4a, . . .)	20+10
5a	Unabsorbed serum U 645 (5a, 5b, 5c) and unabsorbed serum Ps 194 (5a, 5d)	—
5b	Serum Ps 319 (5a, 5b, 5d) absorbed by Ps 194 (5a, 5d)	30
5c	Serum U 645 (5a, 5b, 5c) absorbed by Ps 319 (5a, 5b, 5d)	30
5d	Serum Ps 319 (5a, 5b, 5d) absorbed by U 645 (5a, 5b, 5c)	30
6	Unabsorbed serum U 72/59 (6)	—
7a	Unabsorbed serum U 118/59 (7a, 7b) and unabsorbed serum B 415 (7a, 7c)	—
7b	Serum U 118/59 (7a, 7b) absorbed by B 415 (7a, 7c)	30
7c	Serum B 415 (7a, 7c) absorbed by U 118/59 (7a, 7b)	30
8	Unabsorbed serum U 900/60 (8)	—
9	Unabsorbed serum Ps 910 (9)	—
10a	Unabsorbed serum Ps 196 (10a)	—
10b	Serum Ps 275 (10a, 10b) absorbed by Ps 196 (10a)	20
11	Unabsorbed serum Ps 898 (11)	—
12	Unabsorbed serum L 83 (12)	—
13	Unabsorbed serum V 142a (13)	—

*Amount of bacteria needed for the absorption of 10 ml diluted serum. Serum dilutions used for absorption should be about 3 to 4 times as concentrated as the working dilution.

conveniently carried out by slide agglutination using 18 to 20 hour blood agar cultures. A serum is regarded suitable when it gives cross reactions not differing significantly from the pattern presented in Table VIIb.

Differentiation of subgroups by partial antigens can be performed only by use of absorbed sera. Preparation of absorbed sera and determination of partial antigens are shown in Table VIII.

Polyvalent sera. When dealing with larger numbers of strains it is advisable to use polyvalent sera. In preparing polyvalent sera minor antigenic relationships omitted from the schema should also be considered, as the combined effect of related antibodies may cause unrequired cross-reactions. In this study always pooled sera were used. In view of antigenic relationships it is often unnecessary or even disadvantageous to incorporate in the pooled serum the whole amount of monovalent serum calculated on the basis of the working dilution. Table IX presents a guide to corrections in the amount of unabsorbed sera necessary for the preparation of polyvalent sera I, II, III and IV. In calculating the needed amount of one monovalent serum constituent, other monovalent sera of the same pool should, of course, be regarded as a diluent. It should be emphasized that prescriptions for the preparation of absorbed (Table

Table IX

Monovalent ingredients of pooled sera

Ingredients		Correction factor*	Ingredients		Correction factor*
Polyvalent I			*Polyvalent III*		
1	O serum Ps 304	0.5	3a, 3b	O serum Ps 11	0.5
4a, 4b	O serum Ps 161	0.25	3c	O serum Ps 340	0.5
4a, 4c	O serum Ps 217	0.25	3a, 3d, 3e	O serum Ps 469	1.0
4a, 4d	O serum Ps 323	0.5	3d, 3f	O serum Ps 48	0.5
8	O serum U 900/60	0.5			
9	O serum Ps 910	1.0			
10a	O serum Ps 196	0.75			
10a, 10b	O serum Ps 275	0.6			
11	O serum Ps 898	0.75			
Polyvalent II			*Polyvalent IV*		
7a, 7b	O serum U 118/59	1.0	2	O serum Ps 21	1.0
12	O serum L 83	1.0	5a, 5b, 5d	O serum Ps 319	0.5
13	O serum V 142a	1.0	5a, 5d	O serum Ps 194	1.0
			6	O serum U 72/59	1.0

*The needed amount of each serum is calculated from the corresponding working dilution and the result is multiplied by the given correction factor. In the serum pool the corrected amount of serum should be incorporated. *E. g.* If the previously determined working dilutions of sera 3a, 3b; 3c; 3a, 3d, 3e; 3d, 3f are 1 : 20; 1 : 10; 1 : 50; 1 : 40, respectively, the necessary amount of each for the preparation of 10 ml polyvalent serum III will be: 3a, 3b, 0.5 ml × 0.5 = = 0.25 ml; 3c, 1.0 ml × 0.5 = 0.5 ml; 3a, 3d, 3e, 0.2 ml × 1.0 = 0.2 ml; 3d, 3f, 0.25 ml × 0.5 = = 0.12 ml. The corrected amounts of concentrated sera are mixed and the volume is made up with phenolized saline to 10.0 ml.

VIII) and pooled sera (Table IX) have been elaborated on the basis of results obtained with sera used in this study. The amounts of bacteria suggested for absorption and serum quantities recommended for the preparation of polyvalent sera are approximate and may differ with each serum batch.

Polyvalent sera prepared as shown in Table IX were found to be an excellent help in grouping 2197 strains by slide agglutination. Major cross-reactions between the pooled sera causing diagnostic difficulties were rarely observed. As each pooled serum contained at least one monovalent component reacting with "polyagglutinable" strains, these cultures were easily recognized by their agglutination in all polyvalent sera.

4. Distribution of Ps. aeruginosa serogroups

Table X summarizes the serogroup distribution of 2197 *Ps. aeruginosa* strains. In order to avoid erroneous conclusions, strains originating in larger numbers from one hospital unit are shown independently of the material from which they had been isolated.

Table X indicates that more prevalent serogroups or subgroups of *Ps. aeruginosa* have been encountered in all kinds of materials. However, the serogroup distribution is not quite uniform. In miscellaneous non-faecal pathological materials taken from hospitalized individuals or out-patients (columns 1 to 5 of Table X), strains belonging to serogroup 3 occurred particularly frequently (306 out of 827 strains = 37.0 per cent). The incidence of O group 1, 4 and 5 strains in this material was 9.4, 14.1 and 12.9 per cent, respectively. In miscellaneous faecal specimens (columns 6 to 8 of Table X) the members of O group 3 occurred considerably less frequently than in non-faecal material (82 out of 403 strains = 20.3 per cent). The incidence of O1, O4 and O5 strains in faeces was 6.9, 28.8 and 10.2 per cent, respectively. Thus in faeces O4 strains were the commonest. This holds true also for water and sewage specimens, in which O4 strains comprised 26.7 per cent of all *Ps. aeruginosa* strains.

The above results should be evaluated separately from those of strains derived in large numbers from certain hospital units. In these materials members of otherwise less frequent serogroups or subgroups tended to predominate. This was due partly to repeated isolations of the same organism from the same patient, partly to the "nosocomial" character of the serogroup.

Faecal and non-faecal strains isolated on several subsequent occasions from the same patient usually belonged to the same serological unit. Accordingly, these examinations indicated the epidemiological reliability of the method. These results will be discussed elsewhere.

Table X

Distribution of 2197 Ps. aeruginosa strains

O antigens	Non-faecal materials					Faeces					Miscellaneous					Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
1	26	21	12	3	16	5	19	4	—	3	2	1	4	19	—	135
2	4	—	1	—	—	1	2	4	—	—	1	17	—	13	—	43
3a, . . .	—	—	—	—	—	—	—	3	—	—	—	—	1	—	—	4
3a, 3b	53	16	9	13	29	13	9	28	4	23	2	—	—	2	2	203
3c	8	14	3	4	10	4	—	1	1	3	3	—	—	—	2	53
3a, 3d	15	8	30	4	5	3	2	3	—	4	6	49	—	5	4	138
3a, 3d, 3e	6	2	4	2	4	2	1	—	—	1	1	13	27	2	5	70
3d, 3f	27	7	7	10	16	6	3	4	—	11	10	—	—	3	—	104
4a, . . .	—	—	—	—	—	—	1	—	—	—	—	—	—	4	1	6
4a, 4b	13	—	1	—	—	5	6	5	—	1	—	—	—	7	1	39
4a, 4c	27	2	3	2	3	4	13	21	1	2	2	—	—	18	—	98
4a, 4d	46	1	5	7	7	10	5	46	10	56	15	88	8	23	—	327
5a, 5b, 5c	7	1	1	—	—	1	—	—	8	—	1	—	—	1	—	20
5a, 5b, 5d	37	7	6	2	5	8	6	11	—	1	1	8	1	4	—	97
5a, 5d	20	7	2	7	5	2	5	8	—	4	1	1	—	12	—	74
6	16	5	1	1	6	5	13	4	3	—	3	—	1	39	—	97
7a, 7b	33	6	6	7	7	1	7	21	1	78	19	95	11	9	—	301
7a, 7c	—	—	—	—	—	—	—	—	—	—	—	1	6	—	—	7
8	1	—	—	—	—	—	—	3	—	—	1	—	—	—	—	5
9	1	3	—	—	—	—	1	—	—	—	—	—	—	1	—	6
10a	13	1	1	2	1	1	5	3	—	—	—	2	—	13	—	42
10a, 10b	1	—	—	—	—	—	4	—	—	—	—	—	—	2	—	7
11	12	—	—	—	—	1	5	9	—	6	—	1	—	9	6	49
12	—	—	—	—	—	—	1	—	—	—	—	3	2	1	1	8
13	—	—	—	—	—	—	—	—	—	—	—	—	—	2	—	2
Unstable	—	—	3	—	3	—	3	3	—	4	2	2	1	4	1	26
Polyagglutinable	42	2	14	10	16	5	7	27	5	12	13	71	6	2	4	236
Total no. of strains	408	103	109	74	133	77	118	208	33	209	83	352	68	195	27	2197

1 = ear; 2 = wounds; 3 = urine; 4 = other non-faecal materials (blood, CSF, duodenal juice, etc.); 6 = miscellaneous faecal samples; 7 = faeces from healthy adults; 8 = faeces from infants treated in various hospitals; 9 = infants' faecal samples from Apáthy Hospital; 10 = infants' faecal samples from Árpád Hospital; 11 = non-faecal samples from Árpád Hospital; 12 = miscellaneous materials I, László Hospital; 13 = miscellaneous materials II, László Hospital; 14 = water and sewage; 15 = miscellaneous strains obtained from other laboratories.

Discussion

The present studies have confirmed that successful serological analyses can be performed only in systems allowing the separate study of antigens with different physicochemical properties. This conception has been well known from studies on the serology of *Enterobacteriaceae* and has recently been employed by some authors for *Ps. aeruginosa.* The group specific antigens of *Ps. aeruginosa* can be determined in sera containing both O and H antibodies, if the tests are performed with bacteria not reacting with H agglutinins. Such antigens can be prepared by heating the suspensions at 100° C for 2 1/2 hours [2, 3, 7, 10]. If a more sensitive method suitable for the demonstration of minor antigenic relationships is required, the "2 1/2 hr. 100° + 1 hr. 130°" antigens used in this study may be employed. On the other hand, O antigens can be determined simply with slide agglutination of living cultures if sera devoid of H agglutinins are prepared [4, 8, 12].

From the observation that *Ps. aeruginosa* suspensions become inagglutinable after exposure to mild heat, many authors have suggested that different antigens are responsible for the agglutination of living and boiled cells. Thus it has been supposed that thermolabile somatic antigens cause inagglutinability by masking O antigens. A well-defined differentiation of reactions given by thermostable and thermolabile antigens of *Ps. aeruginosa* was first made by Kleinmaier, Schreiner and Graeff [7]. Although at first they were unable to demonstrate whether the agglutination obtained in sera absorbed by heated bacteria was due to somatic or flagellar factors, it seems probable that their thermolabile components were H antigens. Later Kleinmaier, Schreil and Quincke [18] demonstrated by morphological examinations that this type of reaction corresponded most probably to a flagellar agglutination. Verder and Evans [2] allowing that *Ps. aeruginosa* may possess thermolabile somatic antigens, suggested that agglutination of formalinized suspensions in sera absorbed with heated bacteria was due to H antigens.

The present studies indicate that *Ps. aeruginosa* contains no thermolabile somatic antigens. In agreement with findings of other investigators [4, 8, 12] it has been revealed that immune sera prepared with heated antigens agglutinate living bacteria in high titres and group antigen determinations performed with living and heated suspensions always gave identical results. It has also been demonstrated that homologous cells treated with various physical and chemical agents completely adsorb antibodies responsible for the agglutination of both living and heated cells. H sera which have been depleted of their O agglutinin content by absorption with heated cultures strongly agglutinate bacteria with well developed flagella (broth cultures passaged previously in semisolid medium). The same serum agglutinates agar plate cultures less definitely and organisms grown on media inhibiting the development of flagella,

poorly. This type of agglutination definitely differs in appearance from O agglutination. These findings correspond to our knowledge of the nature of *Enterobacteriaceae* H antigens. Thus the thermolabile factors of *Ps. aeruginosa* are probably flagellar and not somatic antigens.

If the agglutination of living and heated cells of *Ps. aeruginosa* in O serum is attributable to the same somatic factor, how can we explain the inagglutinability of bacteria treated with mild heat, alcohol, formalin and saturated sodium chloride? The phenomenon of reduced agglutinability as an effect of mild heating has been observed also with other bacteria. FELIX [19] showed that heating at 75°C rendered *S. typhi* and *S. paratyphi-C* completely Vi-inagglutinable and partly O-inagglutinable; exposure to 100°C restored full O-agglutinability of all strains. VAN DEN ENDE [20] observing that alcohol reduced the agglutinability of *Ps. aeruginosa*, concluded that the masking of specific O agglutination was due to a precipitation of nucleoprotein which appeared to be a relatively inert antigen on the surface of the cell. According to TCHERNOMORDIK [21] who has also shown the loss of agglutinability of *Ps. aeruginosa* heated at 60°C, this organism contains a non-immunogenic component which inhibits the reaction of antibodies with more deeply situated antigens. This substance, forming a thin envelope, masks somatic agglutination but does not affect flagellar agglutination. It was therefore concluded that the agglutination of living *Ps. aeruginosa* cells was a pure flagellar reaction.

The present studies did not confirm TCHERNOMORDIK's latter conclusion. It is evident that living strains show a strong, high titre somatic agglutination. The assumption of a non-antigenic layer or envelope is, however, probably correct. It would appear that mild heat, alcohol and saturated sodium chloride cause a swelling of the hypothetical envelope, which results in a viscidity of the suspension. Therefore not only O antigens are masked but H-agglutinability is also inhibited. Formalin, instead of inducing swelling, causes perhaps a denaturation of the envelope, and therefore it inhibits O agglutination but not H agglutination.

Ps. aeruginosa strains did not lose their O-agglutinability when the suspensions had been treated with hydrochloric acid prior to exposure to 75°C. This finding may be explained by a hydrolytic decomposition of the substance responsible for the O-inagglutinability of heated cells.

The present studies gave no answer as to whether the hypothetical envelope is composed of the same substance as the mucoid material produced in large amounts by some strains. Although living mucoid strains showed a markedly reduced O-agglutinability, they were far from being inagglutinable and determination of their O antigens with slide agglutination was as accurate as that of non-mucoid strains. The present investigations did not give any indication as to the antigenicity of either the hypothetical envelope or the mucoid

substance; at least, these components produced no antibodies detectable by agglutination.

The observation of KLEINMAIER, SCHREINER and GRAEFF [7] that in O serum living bacteria produce much larger aggregates than heated cells, which has been confirmed in the present studies, does not indicate that two different components are involved. It has been shown that O-inagglutinability caused by mild heat can be eliminated only by subsequent exposure to relatively high temperatures. Thus suspensions treated at 100°C for 2 1/2 hours give still considerably lower titres and weaker agglutination than living bacteria. Titres of living suspensions can be approached only by heating at unusually high temperatures.

The lysis of living *Ps. aeruginosa* cells in higher dilutions of immune serum on prolonged incubation at 37°C is an interesting phenomenon. It was observed by MEITERT and MEITERT in 1960 [22]. HOBBS *et al.* [11] demonstrated in electron micrographs that cell walls and flagella are broken down in the presence of antibodies.

In the present studies, in addition to group specific somatic antigens, the existence of a common thermostable factor has been observed. This antigen is present practically in all *Ps. aeruginosa* strains. Agglutinins against this factor can be demonstrated only with cells heated first at 100°C for 2 1/2 hours then at 130°C for 1 hour. This factor had no importance in the serological determination of *Ps. aeruginosa*. HOBBS *et al.* [11] revealed several components widely distributed in pseudomonads and other bacteria; at least one of these was present in all examined strains.

Serological examination of 2197 cultures isolated from various materials has shown that the antigenic schema elaborated in this study is suitable for the detection of the source and epidemiological importance of *Ps. aeruginosa* strains.

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INCIDENCE OF PSEUDOMONAS AERUGINOSA SEROGROUPS IN WATER AND HUMAN FAECES

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Summary. Bacterial examination of 9219 drinking water, 1065 river water and 419 sewage samples and faecal specimens of 7650 healthy adults and 650 hospitalized infants supplied 521 *Ps. aeruginosa* strains. The percentage frequency of *Ps. aeruginosa* was 1.6 in wells, 4.1 in springs, 0.6 in piped water, 1.8 in river and 10.0 in sewage samples. The faeces of healthy adults living in the same geographic area were positive in 1.5 per cent. Hospitalized infants harboured the organism in 32.0 per cent.

The serogroup distribution of *Ps. aeruginosa* was similar in water and adults' faecal samples. In both groups of material serogroups 1, 3, 4, 5 and 6 were the most frequent. This finding indicates that the presence of *Ps. aeruginosa* in drinking water is probably the result of faecal contamination. A comparison of the data with other studies has shown that, although in a somewhat different distribution, the above serogroups of *Ps. aeruginosa* are most commonly encountered in intestinal and extraintestinal infections. The results support the opinion that waters containing this organism are unsatisfactory for human consumption.

Bacteria of the genus *Pseudomonas* are common in surface waters. *Ps. aeruginosa*, which differs in cultural and pathogenic properties from other pseudomonads, is sometimes also isolated from different water samples. It is questionable whether *Ps. aeruginosa* found in such materials originates from humans or, as other pseudomonads, is a normal inhabitant of water.

As this problem has not been investigated extensively, it is difficult to draw final conclusions. HUNTER and ENSIGN [1] have observed that after ingestion of milk contaminated with *Ps. aeruginosa*, a number of adults developed diarrhoea or became carriers. New-born babies infected secondarily by these adults developed serious, often fatal enteritis. Bacteriological examination of water samples during the outbreak revealed the presence of *Ps. aeruginosa* in some wells. RINGEN and DRAKE [2] by the use of an effective enrichment medium isolated *Ps. aeruginosa* from human faecal samples in 11.0 and from raw and clarified sewage samples in 90 per cent. They were unable to recover this organism from sludge, soil, animal faeces or surface waters and wells free from faecal pollution. HOADLEY and McCOY [3] found that 3 per cent of glucose-oxidizing Gram negative bacteria isolated from river specimens belonged to *Ps. aeruginosa*. This organism occurred with great frequency at a sampling point situated near to a sewage inflow. More than two thirds of oxidase positive strains isolated by SELENKA [4] from surface waters were identified as *Ps. aeruginosa*.

Although in view of these data it would appear that *Ps. aeruginosa* occurring in water originates from human faeces, only some authors regard such waters as inadequate for human consumption. According to SELENKA [4] water samples containing *Ps. aeruginosa* may represent a danger to the health of the community and therefore on the basis of the German Food Sanitation Law, they must not be used for human consumption. The Hungarian National Standard for the Bacteriological Examination of Water [5] prescribes definitely that the presence of *Ps. aeruginosa* indicates pollution and therefore any drinking water that contains this organism is of unsatisfactory quality.

The following investigation has been performed in order to collect further evidence by serological typing concerning the association between *Ps. aeruginosa* strains isolated from drinking water and human excreta.

Materials and methods

Water specimens. A total of 9219 drinking water (5293 well, 170 spring and 3756 piped water specimens), 1065 river water and 419 sewage and sludge samples were examined. The overwhelming majority of samples were taken for laboratory examination from villages and smaller towns of 3 neighbouring counties (Pest, Nógrád and Fejér) comprising an area of 13,310 square kilometres and an approximate population of 1,400,000.

Faecal specimens. One group of the examined samples originated from 7650 healthy adults (mainly food handlers) living in the rural area and smaller towns of Pest and Nógrád counties (8937 square kilometres, approximately 1,000,000 population). Other faecal samples were obtained from infants under one year of age hospitalized with intestinal and extraintestinal diseases; the 650 specimens were sent for bacteriological examination mainly by Budapest hospitals.

Bacteriological examination of water and sewage samples. Coliform bacteria in drinking water samples were counted by the membrane filter technique described in the Hungarian National Standard for the Bacteriological Examination of Water [5]. Fifty ml of each sample was passed through a Co 5 filter (Membranfiltergesellschaft, Göttingen) boiled previously in twice-changed distilled water. After filtration of the sample, the membrane was placed over special Endo agar and incubated for 24 hours at 37°C. All lactose positive (purplish) colonies morphologically characteristic of *Enterobacteriaceae*, were counted as coliform organism. Part of river water specimens was also examined by the membrane filter technique. Other river water samples were examined with the multiple tube fermentation technique by inoculating 1 ml aliquots of each appropriate dilution into 5 tubes containing lactose-phenol red broth. After incubation for 48 hours the growth in each tube was subcultured on normal Endo agar. All purplish (lactose-fermenting) colonies morphologically characteristic of *Enterobacteriaceae* were considered members of the coliform group. Suspected colonies of *Ps. aeruginosa* on membrane filters or on Endo plates were subcultured on agar plates then transferred to agar slants. In order to obtain pure cultures, the growth from each agar slant was streaked onto a brilliant green agar plate, then an isolated colony was inoculated on agar slant. The strains were preserved by making Liebig meat extract agar stab cultures.

Bacteriological examination of faecal samples. About 0.5 to 1 g amounts of faeces were inoculated into tubes containing 6 to 8 ml merthiolate-brilliant green broth. After incubation at 37°C for 18 hours the cultures were streaked with glass rods onto brilliant green agar plates. The subcultures were incubated at 37°C for 18 hours, then pseudomonad colonies were streaked again on brilliant green agar. Then one isolated colony from each plate was transferred on an agar slant. The strains were maintained in Liebig extract agar stab cultures.

Culture media. The special Endo agar for membrane filter technique contained beef extract, 15 g; peptone (Richter), 10 g; agar, 10 g; NaCl, 3 g; $Na_2HPO_4 \cdot 12\ H_2O$, 4 g; tap water, 1000 ml; after adjusting to pH 8.4 and autoclaving, 10 g lactose, 5 ml alcoholic basic fuchsin and 1.25 g Na_2SO_3 were added.

The normal Endo agar contained the same ingredients, except that it was solidified with 1.5 to 1.7 per cent agar.

Lactose phenol red broth: yeast supernatant (1000 g baker's yeast cake mixed with 1200 ml water and autoclaved at 121°C for 30 minutes), 10 ml; peptone (Richter), 5 g; lactose, 5 g; 0.02 per cent aqueous phenol red solution, 25 ml; tap water, 1000 ml; pH 7.2.

Brilliant green agar: Tripkazin (pancreatic digest of casein, Human, Budapest), 6.6 g; peptone (Richter), 1.1 g; yeast supernatant, 15 ml; concentrated yeast suspension, 0.75 ml; agar, 15 to 17 g; NaCl, 5 g; tap water, 1000 ml; after adjusting to pH 7.4 and autoclaving, the following ingredients were added: Andrade indicator, 30 ml; lactose, 10 g; sucrose, 1 g; glucose, 0.5 g, 0.1 per cent aqueous brilliant green solution, 4 ml.

Merthiolate-brilliant green enrichment medium: beef extract, 15 g; peptone (Richter), 10 g; NaCl, 3 g; $Na_2HPO_4 \cdot 12 H_2O$, 4 g; tap water, 1000 ml; after adjusting to pH 7.4 and autoclaving, 0.5 ml 0.1 per cent aqueous brilliant green solution and 0.5 ml 0.2 per cent aqueous merthiolate solution were added.

Identification of Ps. aeruginosa. Strains showing the following cultural and biochemical properties were regarded as *Ps. aeruginosa:* characteristic growth after 18 hours at 37 °C on brilliant green agar; growth at 42°C on beef extract agar; positive oxidase test [6]; oxidative decomposition of glucose; rapid liquefaction of gelatin [7]; reduction of nitrate into nitrogen [8]. Determination of O antigens was performed according to LÁNYI's antigenic schema by slide agglutination with ox-blood agar cultures [9].

Results

Cultivation of Ps. aeruginosa. Examination of a 50 ml portion of the sample by the membrane filter technique seemed suitable for an effective isolation of *Ps. aeruginosa* from drinking water. These specimens usually contained low numbers of coliform bacteria (0 to 10^2/100 ml), thus pseudomonad colonies grown on membranes placed over Endo plates were recognized without difficulty. Isolation of *Ps. aeruginosa* from lactose-phenol red broth used for the examination of river water and sewage samples, was less effective. Although the coliform content of sewage (10^4 to 10^5/ml) was considerably lower than the coliform counts of faeces, due to the overgrowth of these organisms, samples containing *Ps. aeruginosa* in large numbers yielded positive results only.

In view of the high coliform and the generally low *Ps. aeruginosa* counts of the faeces of adults, faecal samples were inoculated into liquid enrichment medium. For the selective cultivation of *Ps. aeruginosa* LOWBURY [10] devised a medium containing cetylmethylammonium chloride. For the same purpose GOULD and MCLEOD [11] recommended brilliant green as an inhibitor of other organism. In our experiments 3 kinds of enrichment medium were compared: the selenite-brilliant green broth elaborated by STOKES and OSBORNE [12] for salmonellae, a brilliant green-cetylpyridinium bromide, and a brilliant green-merthiolate broth. Model experiments with beef extract broth showed that in the presence of 1 : 2,000,000 brilliant green + 1 : 200 Sterogenol (10 per cent alcoholic solution of cetylpyridinium bromide) or in the presence of 1 : 2,000,000 brilliant green + 1 : 1,000,000 merthiolate even 10 to 20 *Ps. aeruginosa* cells were able to multiply freely when inoculated in a dense suspension of faeces. As the merthiolate-brilliant green broth seemed to yield more constant results, this medium was used throughout the investigations. The salmonella enrichment medium was also suitable for the selective culturing of *Ps. aeruginosa:*

Table I

Distribution of Ps. aeruginosa strains

O antigens	5293 well samples		170 spring samples		3756 piped water samples		1065 river samples		419 sewage samples		7650 adults' faecal samples		650 infants' faecal samples	
1	9		—		1		4		5		19		4	
2	10		1		—		—		2		2		4	
3a, . . .	—		—		—		—		—		—		3	
3a, 3b	1		—		—		1		—		9		28	
3c	—	6	—		—		—	3	—	3	—	15	1	39
3a, 3d	2		—		—		1		2		2		3	
3a, 3d, 3e	1		—		—		1		—		1		—	
3d, 3f	2		—		—		—		1		3		4	
4a, . . .	2		—		1		—		1		1		—	
4a, 4b	4	20	2	3	—	10	1	6	—	13	6	25	5	72
4a, 4c	5		—		7		1		5		13		21	
4a, 4d	9		1		2		4		7		5		46	
5a, 5b, 5c	—		—		1		—		—		—		—	
5a, 5b, 5d	1	6	—		2	6	—	3	1	2	6	11	11	19
5a, 5d	5		—		3		3		1		5		8	
6	16		1		5		2		15		13		4	
7a, 7b	2		—		—		3		4		7		21	
8	—		—		—		—		—		—		3	
9	—		—		—		—		1		1		—	
10a	6	7	1	1	1	2	1	1	4	4	5	9	3	3
10a, 10b	1		—		1		—		—		4		—	
11	6		—		—		1		2		5		9	
12	1		—		—		—		—		1		—	
13	—		—		—		—		2		—		—	
Ungroupable	2		1		—		—		1		3		3	
Polyagglutinable	—		—		—		—		2		7		27	
Number of strains	85		7		24		23		56		118		208	
Positive samples	85 = 1.6%		7 = 4.1%		24 = 0.6%		19* = 1.8%		42** = 10.0%		118 = 1.5%		208 = 32.0%	

*4 samples contained 2 different serogroups; **8 samples contained 2 and 3 samples contained 3 different serogroups.

Table II

*Incidence of Ps. aeruginosa in drinking water samples qualified satisfactory and unsatisfactory on the basis of coliform counts**

O antigens	5293 well samples		170 spring samples		3756 piped water samples	
	2519 unsatisfactory samples	2774 satisfactory samples	47 unsatisfactory samples	123 satisfactory samples	971 unsatisfactory samples	2785 satisfactory samples
1	5	4	—	—	1	—
2	5	5	—	1	—	—
3a, 3b	—	1	—	—	—	—
3a, 3d	2	—	—	—	—	—
3a, 3d, 3e	1	—	—	—	—	—
3d, 3f	2	—	—	—	—	—
4a, . . .	1	1	—	—	—	1
4a, 4b	2	2	1	1	—	—
4a, 4c	4	1	—	—	2	5
4a, 4d	5	4	—	1	1	1
5a, 5b, 5c	—	—	—	—	1	—
5a, 5b, 5d	1	—	—	—	2	—
5a, 5d	1	4	—	—	1	2
6	12	4	—	1	4	1
7a, 7b	1	1	—	—	—	—
10a	6	—	1	—	1	—
10a, 10b	—	1	—	—	1	—
11	5	1	—	—	—	—
12	1	—	—	—	—	—
Ungroupable	—	2	1	—	—	—
Samples containing Ps. aeruginosa	54 =2.1%	31 =1.1%	3 =6.4%	4 =3.3%	14 =1.4%	10 =0.4%

*Maximum number of coliform organisms allowable per 100 ml of water: dug wells, 20; driven wells, 4; springs, qualified according to type of cover and use of water; non-chlorinated piped water, 2; chlorinated piped water, 0.4.

this medium, however, gave positive results only when larger inocula were used. Cultures obtained in the enrichment medium were transferred to brilliant green agar. This inhibited the growth of *Escherichia* and *Proteus* cultures effectively, but, as shown by a long experience, it allowed a free and characteristic growth of *Ps. aeruginosa.*

Incidence of Ps. aeruginosa in water and in faeces. From Table I it is clear that *Ps. aeruginosa* strains occurring in various water and sewage specimens belonged to the same serogroups as strains isolated from human faeces.

There was a striking similarity as to the relative frequency of serological groups and subgroups: water, sewage and adults' faeces usually contained group 1, 3, 4, 5 and 6 strains. As regards subgroup distribution, among O group 3 strains members of subgroup 3a, 3b were fairly frequent in faeces. The frequency of O group 4 strains in water and sewage as well as in faeces was remarkable: about one fourth of all strains belonged to this group. There was no great difference between water, sewage and faecal samples in the subgroup distribition of O4 strains.

Ps. aeruginosa occurred in drinking and river water samples with about the same frequency as in the intestinal tract of the the healthy adult population (0.6 to 1.8 versus 1.5 per cent). In sewage this bacterium was more common, 10.0 per cent, in spite of a less effective, nonselective culturing. In the incidence of *Ps. aeruginosa*, drinking water and adults' faecal samples considerably differed from faecal specimens of hospitalized infants, which gave positive results in 32.0 per cent. Although in predominating serogroups the latter material was similar to specimens of other origin, there was some difference in the group distribution: in infants' faeces members of groups 1 and 6 were relatively unfrequent, while the incidence of polyagglutinable strains was higher.

Table II compares the distribution of *Ps. aeruginosa* serogroups in drinking water samples qualified satisfactory and unsatisfactory on the basis of coliform counts. It is evident that the serogroup distribution of *Ps. aeruginosa* was similar in both kinds of samples. However, unsatisfactory specimens contained this organism about twice as often as satisfactory specimens.

Discussion

The present study has shown that the incidence of *Ps. aeruginosa* is similar in drinking water and in the faeces of healthy individuals and the distribution of serogroups is also similar in these materials. From this finding it would appear that the presence of *Ps. aeruginosa* in drinking water is due to faecal contamination.

Since water samples and faecal specimens from healthy adults were obtained from the same geographic area, the question arises whether *Ps. aeruginosa* appeared in water as a faecal contaminant, or, on the contrary, healthy individuals became transient carriers of this organism as a result of waterborne infection. That *Ps. aeruginosa* is not widely distributed in nature is indicated by the findings of RINGEN and DRAKE [2] who showed that natural water supplies presumably not contaminated with waste materials of human origin were free from *Ps. aeruginosa*. On the other hand, the fact that *Ps. aeruginosa*, when introduced as a faecal contaminant, may disappear rapidly from surface waters, has been observed by HOADLEY and McCOY [3]. These

authors were unable to recover *Ps. aeruginosa* from river water specimens taken at sampling points situated lower than an inflow of sewage containing this bacterium.

Our examinations have shown that drinking water samples qualified satisfactory according to coliform counts contained *Ps. aeruginosa* in 0.4 to 3.3 per cent. If we accept *Ps. aeruginosa* as a faecal contaminant, the said finding indicates that the organism survives in water for longer periods than coliform bacteria do.

There was a striking difference between healthy adults and hospitalized infants in the incidence of *Ps. aeruginosa* carriers. Since faecal specimens of infants were obtained from several different hospitals, it may be concluded that the majority of infants had acquired their *Ps. aeruginosa* strains in the hospital. It should be noted that a considerable part of infants harbouring *Ps. aeruginosa* had no enteric symptoms and the present examinations supplied no data as to the association of the organism with infantile enteritis.

Ujváry *et al.* [13] found mainly *Ps. aeruginosa* O3, O4 and O5 strains in the faeces of infants suffering from intestinal and extraintestinal diseases. In faecal materials of different origin the same serogroups were found to predominate [9]. From non-faecal pathological specimens (urine, discharge from ear and wounds, etc.) O3, O4 and O5 strains were also frequently isolated (37.0, 14.1 and 12.9 per cent of all *Ps. aeruginosa* strains, respectively). These materials frequently contained members of subgroup 3a, 3b [9].

In infants' faeces examined in the present work O group 4 cultures constituted 34.6 per cent of all strains; members of O group 3 were also common (18.7 per cent).

Thus it may be concluded that, although in a somewhat different distribution, the same serogroups of *Ps. aeruginosa* occur in water, sewage, faeces and even in non-faecal pathological materials. In serogroup distribution water samples stand more closely to adults' faeces than to other materials: serogroup 1, 3, 4, 5 and 6 strains occurred in water and sewage in 9.7, 6.2, 26.7, 8.7 and 20.0, in adults' faeces in 16.1, 12.7, 21.2, 9.3 and 11.0 per cent, respectively. The presented data indicate that in water, sewage and adults' faeces O group 4 strains predominate, while in non-faecal pathological materials O group 3 strains are the most common. As regards the incidence of O group 3, infants' faecal specimens occupy an intermediary position between the above two groups of material.

As the causative agents of water-borne infectious diseases originate primarily from the human intestine, the main purpose of bacteriological examination of water is the detection of faecal pollution. In the evaluation of coliform counts as an indicator of faecal pollution there are two conceptions. Part of the authors divide lactose-fermenting *Enterobacteriaceae* into strictly intestinal organisms present in faeces in large numbers ("coli type 1") and into

bacteria more common in nature *(Klebsiella, Enterobacter)*. Other authors claim that organisms of the latter group, though in smaller numbers, are also normal inhabitants of the human intestine and, as in routine examinations they cannot reliably be differentiated from strictly intestinal bacteria, their presence should also be regarded as an indicator of faecal pollution. For the Hungarian National Standard for the Bacteriological Examination of Water the latter conception has been adopted, that is, above given counts, the presence of any *Enterobacteriaceae* strains fermenting lactose promptly *(Escherichia, Klebsiella, Enterobacter, Citrobacter)* is regarded as an index of pollution [5].

According to this principle any other easily cultivable bacteria which are present in the human intestinal tract and do not survive long in water, should be regarded as evidence of faecal pollution. Although the incidence of *Ps. aeruginosa* in faecal samples of normal adults was low, the similarity of serogroup distribution in water and faecal samples indicate that the presence of this organism in drinking water may be a result of faecal contamination. A comparison of the present results with other studies on the incidence of *Ps. aeruginosa* in non-faecal clinical material has shown that, although in a somewhat different distribution, the same *Ps. aeruginosa* serogroups predominate in water and in intestinal and extra-intestinal infections. These findings support the opinion that waters containing this organism are unsatisfactory for human consumption.

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INDUCTION AND MULTIPLICATION OF λ-PHAGE

I. THE EFFECT OF CHLOROMYCETIN

By

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Summary. With cells in shift down state the total infective centre count immediately increases on the addition of mitomycin; the increase is not affected by chloromycetin and is inhibited by trypaflavin. In the presence of chloromycetin the dissociated prophage retains primary activity for long periods.

Without shift down, the dissociated prophage is inactivated in the presence of chloromycetin.

A group of anticancer substances induces the λ-phage production of *E. coli* [1], and there is a good correlation between the inductive and anticancer effect of these substances [2]. This method and its modification suitable for mass-examinations [3] have been routinely used in our laboratory for the detection of anticancer substances. In the course of these experiments several observations have indicated that by the aid of antibiotics further information can be obtained concerning phage induction and multiplication, and concerning the effect on multiplication of the host cell's metabolism. This latter possibility is especially facilitated by the fact that the *E. coli* strain K_{12} λ-28 used in our laboratory is inhibited by valine [4], and in the shift down state also by leucine and other amino acids [5].

The present paper will deal with the action of chloromycetin (CM) which, due to its inhibitory effect on protein synthesis, inhibits the multiplication of phage. We have shown that in the shift down state on the addition of mitomycin the prophage dissociates and the dissociated prophage maintains its primary activity for a long time.

Materials and methods

Strains. E. coli K_{12} λ-28 was used as lysogenic strain. Phage-determinations were carried out with *E. coli* C 600 strain.

Nutrient media. Bouillon was used as complete medium, while as minimal medium that of Davis and Mingioli [6] was used. In some cases the latter was complemented with "Difco" casein hydrolysate.

Experimental conditions. Preparation of culture in shift down state: a 16-hour-old bouillon culture is inoculated in 1 : 10 proportion to the same medium and after incubating for two hours, the cells are washed twice and suspended in minimal medium at an average

concentration of 10^6 cells/ml. With cultures without shift down, the same media were used in all the three steps.

The cultures were incubated in the upright position, generally at 37°C.

The concentration of mitomycin C used for induction was 1 μg/ml on minimal medium and 5 μg/ml in bouillon.

After induction the viable cell count was determined, and the count of total infective centre (CTIC) was also assayed at 28° by mixing the induced strain with the indicator strain. After treatment with 2 drops of chloroform/ml culture, at least for 5 minutes at 2°C, the plaque count was determined at 28°C and regarded as the relative number of free phages. Without induction the free phage number of the culture is low, thus the CTIC-value can be directly used for the estimation of the number of induced cells.

Results

In Fig. 1 the inductive effect of mitomycin C is shown in the shift down state. It can be seen that the CTIC-value increased sharply after the addition of mitomycin without a lag-phase. Between 60 and 90 minutes the induced cell-count did not change appreciably. CM at a concentration of 20 μg/ml did not affect the shape and the peak of the curve attained in 90 minutes. The free phage number began to rise after a long lag-phase, but before the first cell-division in the control culture had taken place. Free phage production was completely inhibited by CM.

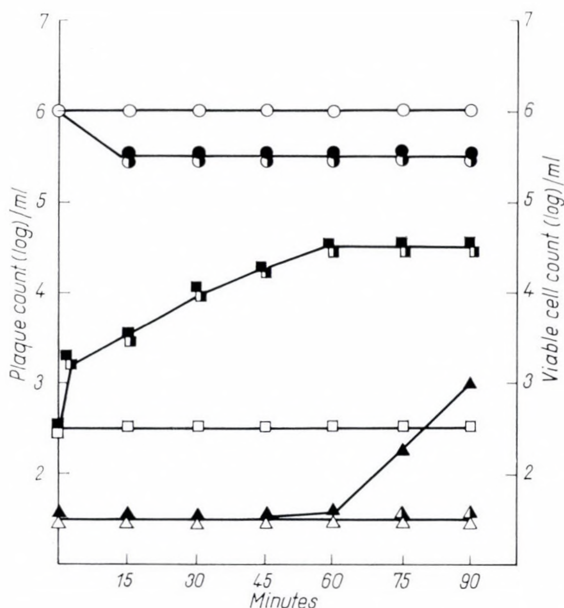

Fig. 1. Effect of CM on induction in the shift down state. Symbols: circles: viable cell count; squares: CTIC value; triangles: free phage number. Empty symbols: not induced culture; filled symbols: culture induced with 1 μg/ml of mitomycin C; half-filled symbols: culture induced with 1 μg/ml of mitomycin C in the presence of 20 μg/ml of CM

If under conditions identical to those described above, the cells grown on complete or minimal medium were induced in a medium identical to that of the inoculum (Fig. 2a and 2b), in the presence of CM the CTIC-value increased only slightly and transiently as compared to the control.

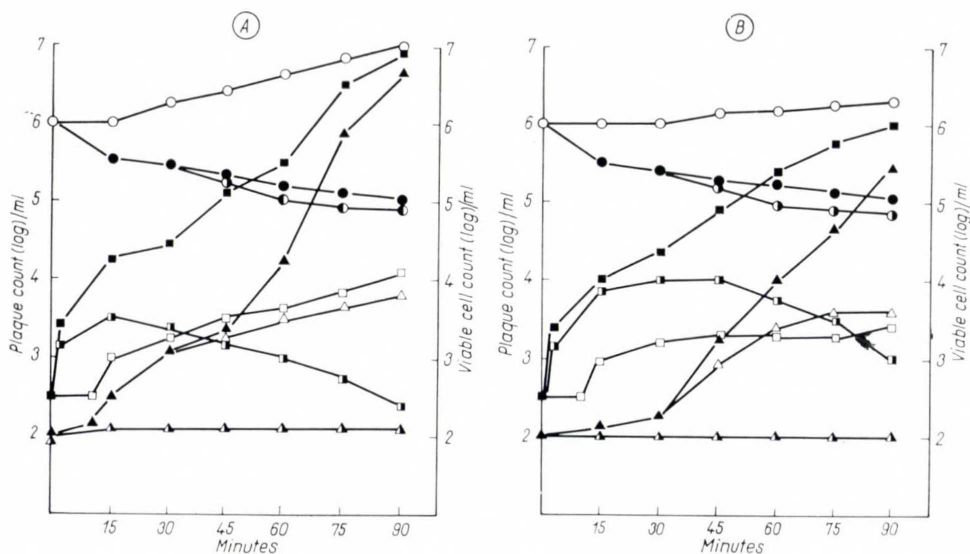

Fig. 2. Effect of CM on induction in media identical with that of the inoculum. A: complete medium; B: minimal medium. Symbols: circles: viable cell count; squares: CTIC-value; triangles: free phage number. Empty symbols: not induced culture; filled symbols: culture induced with 5 or 1 μg/ml of mitomycin C, respectively; half-filled symbols: culture induced with 5 or 1 μg/ml of mitomycin C, respectively in the presence of 20 μg/ml of CM

If the shift down state was partly suspended by the addition of casein hydrolysate (Fig. 3), the concentration of the latter failed to affect the viable cell count in the induced and not-induced cultures within the experimental period. In the absence of CM, minimal (0.005 per cent) amounts of casein hydrolysate already increased the rise in CTIC-number as compared to the shift down state, and this increasing effect was of the same extent at different concentrations of the hydrolysate. If different amounts of casein hydrolysate were added to the induced cells in the presence of CM, 0.005 per cent did not influence the rise of the CTIC-value. At very high concentrations, after a transient increase the CTIC-value decreased.

Since acridine derivatives such as acriflavine are especially damaging episomes in the unintegrated state [7, 8], it was assumed that the sensitivity of the prophage also increases during induction.

If 30 μg/ml acriflavine was added together with mitomycin to a shift down culture, the CTIC-value failed to increase. At lower concentrations acriflavine caused partial inhibition (Fig. 4). Acriflavine at the concentrations applied

decreased only slightly the CTIC value of not-induced cultures. 15 μg/ml did not affect the CTIC value, 30 μg/ml caused an 80 per cent decrease. When acriflavine was added to induced cells, it took longer for the CTIC value to decrease to the level of uninduced cultures (Fig. 5).

The effect of acriflavine is irreversible. If induction with mitomycin was performed at +2°C, CTIC increased rapidly by about one order of magnitude and persisted at that level for prolonged periods. If the culture was diluted

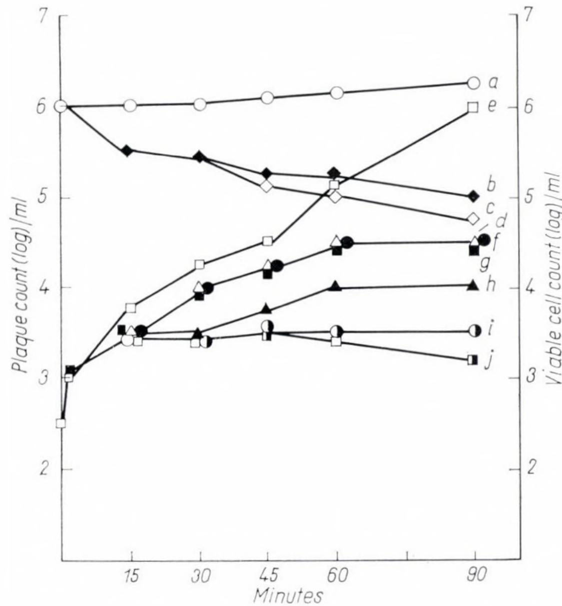

Fig. 3. Effect of CM on induction in the case of partial suspension of the shift down state. Symbols: Viable cell counts on minimal medium completed with 0.005—0.25 per cent casein hydrolysate: a: not induced; b: induced; c: induced in the presence of 20 μg/ml of CM. CTIC values in the induced cultures: d: in shift down; e: in minimal medium complemented with 0.005—0.25 per cent casein hydrolysate. CTIC values in cultures induced in the presence of 20 μg/ml of CM: f: in shift down; in minimal medium complemented with g: 0.005, h: 0.02, i: 0.1, j: 0.25 per cent casein hydrolysate

200fold with a medium containing 1 μg/ml of mitomycin at +2° C, the CTIC value calculated for the initial volume did not change for a long time. If this experiment was carried out in the presence of 20 μg/ml of acriflavine with or without mitomycin, CTIC failed to rise, and if diluted 200fold with a medium containing 1 μg/ml of mitomycin, the CTIC value remained at the original level (Fig. 6).

The extent of induction by mitomycin at +2°C agreed in every experiment with the value yielded by immediate determination at higher temperatures. This finding might mean that the cells must be in a certain state (in a

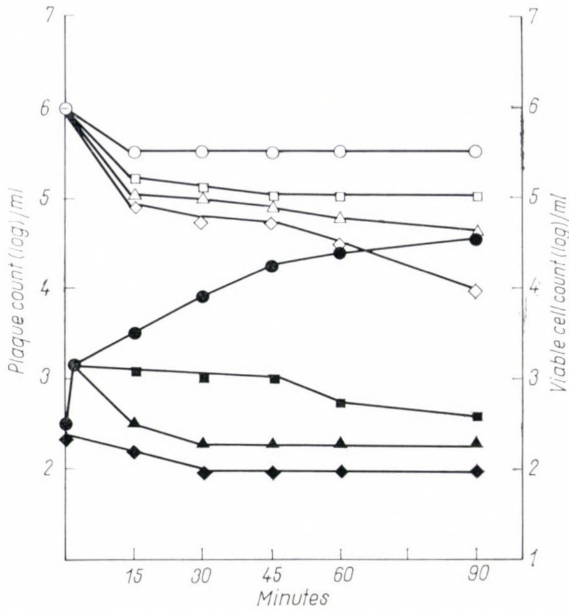

Fig. 4. Effect of acriflavine on induction in the shift down state. The cultures were induced with 1 μg/ml of mitomycin C. Symbols: circles: acriflavine-free control; squares, triangles, and rhombi: 15, 20, and 30 μg/ml of acriflavine, respectively, added to the culture at 0 time. Empty symbols: viable cell count; filled symbols: CTIC value

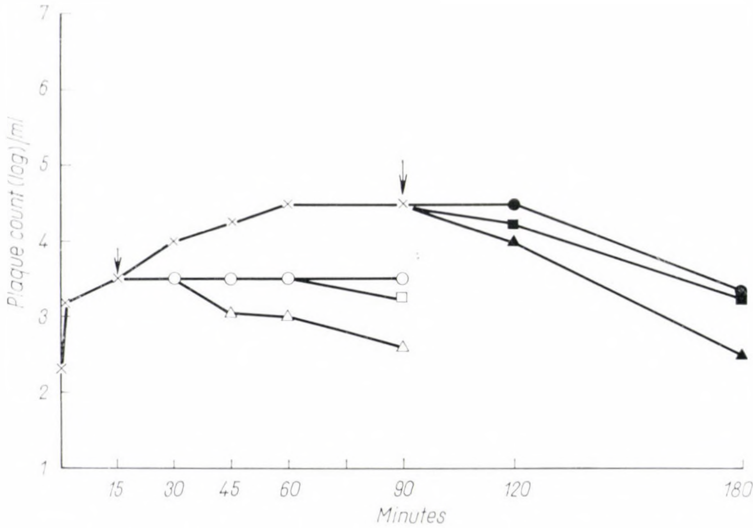

Fig. 5. Effect of acriflavine added at various times to induced cultures in the shift down state. The cultures were induced with 1 μg/ml of mitomycin C. Symbols: ×——×: CTIC value in culture induced without acriflavine. Circles, squares, and triangles: addition of 15, 20, and 30 μg/ml of acriflavine at 15 minutes (empty symbols) or at 90 minutes (filled symbols)

certain phase of DNA replication?) to allow the prophage to dissociate. Investigations into this problem are in progress.

The above described effect of acriflavine was exerted also in case of induction in the presence of CM.

These experiments suggest that the prophage, dissociated in the shift down state and detectable in the presence of CM for a prolonged time, is in-

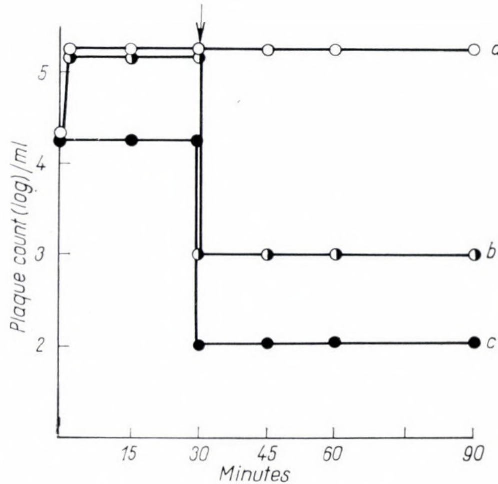

Fig. 6. Effect of acriflavine on induction at +2°C in the shift down state. The cultures were induced with 1 μg/ml of mitomycin. a: CTIC values in cultures induced in the absence of acriflavine, b: the same as before but at 30 minutes the culture was diluted 200fold at unchanged mitomycin concentration. c: at 0 time 20 μg/ml of acriflavine was added together with, or without, mitomycin C, then at 30 minutes it was removed by diluting 200fold with the maintenance of the mitomycin C concentration. Inoculum concentration in all three cases 10^8 cells/ml

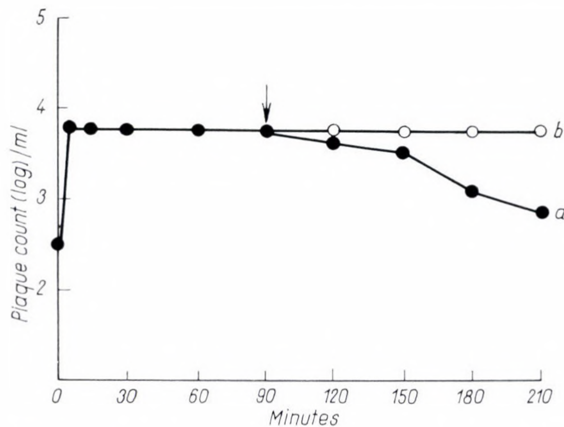

Fig. 7. Decrease of CTIC value at temperatures favourable to metabolism. a: the bouillon culture was induced with 5 μg/ml of mitomycin C at +2°C in the presence of 20 μg/ml of CM, then after 90 minutes it was further induced at 37°C, b: induction under unchanged conditions

activated by acriflavine. Without shift down, the CTIC value after a transient increase decreased in the presence of CM; this means that under such conditions the prophage is inactivated by aspecific enzymes. This hypothesis is supported by the observation that if induction is carried out at 2° C in CM containing buillon, a rapid rise of CTIC value occurs which otherwise would have been stable at that low temperature. If the culture is allowed to stand in the presence of CM at 37°C, the CTIC value decreases (Fig. 7).

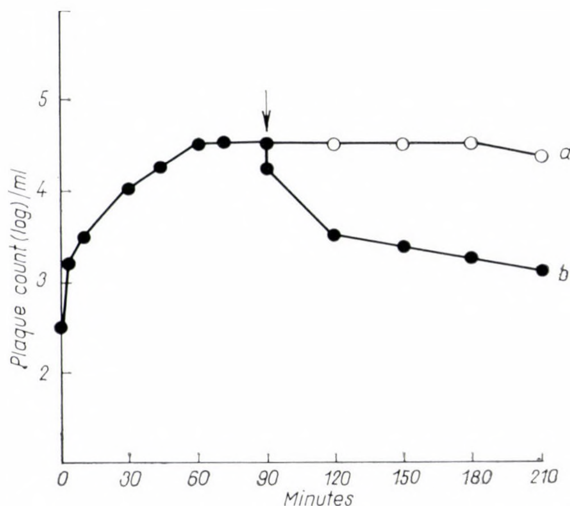

Fig. 8. Decrease of CTIC value in media favourable to metabolism. a: the culture was induced with 1 μg/ml of mitomycin C at 37°C in the presence of 20 μg/ml of CM in the shift down state, then after 90 minutes it was diluted two-fold with bouillon with maintenance of the original CM concentration; b: induction under unchanged conditions

Essentially the same result is obtained if induction is performed in the shift down sate in the presence of CM and then bouillon is added to the culture (Fig. 8).

Discussion

Numerous papers have been published concerning the effect of CM on the multiplication of phage. The view has been formed that both with infection [9, 10] and induction [11] an early stage of the vegetative cycle requires protein synthesis, therefore the inhibitory effect of CM is the more pronounced the earlier it is applied. According to earlier data [12, 13], in UV induction protein synthesis is required also for the dissociation of the phage. Marcovich [14] has shown that UV irradiation interferes with the replication of the bacterial chromosome thus resulting in the dissociation of the prophage. In

this respect the UV irradiation would act through the same mechanism as alkylating agents and thymine-deprivation, consequently protein synthesis may be required also in the latter cases.

This question is, however, still not clear. KORN and WEISSBACH [15] and MELECHEN and SKAAR [16] observed the inhibitory effect of CM in the case on induction by thymine-deprivation. Later, however, MELECHEN [17], SENO and MELECHEN [18] have modified their conclusion, stating that the initiation of induction is independent of protein synthesis. Their experimental approach, which is based upon the assay of induced cell-number by plating, does not exclude the possibility that protein synthesis taking place after plating could play a role in the induction.

It was to answer this question that we have applied acriflavine which, in addition to its slight effect on the spontaneous CTIC value, inhibited the induction by mitomycin. The irreversible nature of this effect compared to the effect of acriflavine added after the induction shows that in the latter case the dissociated prophages were inactivated by the acriflavine. However, if the dissociation of the prophage had taken place only after plating, independently of the time of application, the gradual decrease of CTIC value could not be caused by the acriflavine.

Consequently, the prophage dissociates already in the shift down culture. Since the occurrence of some slight protein synthesis at the expense of the amino acid pool cannot be excluded even under these conditions, the independence of induction from protein synthesis is proved convincingly only by the fact that the rise of CTIC in the shift down state takes place in the presence of CM in the same way as in the control, and acriflavine has the same effect as in the case of induction without CM.

Without shift down at 37° C the dissociated prophage is inactivated to an extent depending upon the composition of the medium. It is, however, seen from our experiments, too, that the shift down state is very suitable for the prolonged preservation of the primer activity of the dissociated prophage since, if the prophage can dissociate from the bacterial chromosome in spite of the inhibition of protein synthesis, and the replication of DNA cannot begin, the induced cells retain their plaque-forming ability only in the case if the prophage as a primer is not inactivated.

Completion of an induced down-shifted culture or a temperature shift from 2 to 37° C already allowed to inactivate the dissociated prophage in the presence of CM, but only to a smaller extent.

The independence of induction of protein synthesis does not exclude a preformed protein to play a role in the induction. LIEB [19] and GREEN [20] have observed CM-resistance on heat-induction with certain mutants. Both authors have assumed the presence of a protein participating actively in the induction, a protein either occurring in preformed state and liberated during

the inactivation of repressor [19], or one formed from the C_1-product itself [20]. In the case of mitomycin induction this mechanism is not conceivable since mitomycin is unable to act on the protein-like C_1-product. Consequently, mitomycin induction does not require a special protein which would be directly activated by mitomycin.

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SOME PHYSICAL CHARACTERISTICS OF INFLUENZA VIRUS DETERMINED BY ELECTRONMICROSCOPY

By

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Summary. Purified and concentrated samples of complete and incomplete Paris PL 1/49 A—1 influenzavirus particles were mixed with Dow polystyrene latex particles. The preparations were fixed with OsO_4 or left unfixed and examined either shadowed with palladium or unshadowed.

By means of the latex particles, the S value characteristic for the used microscope was calculated. The gradation of the photonegative (γ) for electron expositions of 45 KV acceleration was determined.

By means of scanning measurement, density curves were taken of individual virions. Their diameter and height were measured by a comparator.

It was demonstrated that during preparation and examination, the virions were considerably flattened. An attempt was made to calculate the virion's real diameter on the basis of data for the flattened preparations.

The virion's dry mass was calculated from the data of density measurements, and estimates were made as to the amount of OsO_4 uptake.

An attempt was made to calculate the real (wet) mass and density. It is emphasized that no particular changes are required in the method of preparation and micrography to obtain data not only concerning the particles' dimensions, but also their mass.

The requirement for some quantitative interpretation of electron microscopic data has arisen ever since the very first electron microscopic studies on viruses [1]. The introduction of shadowing techniques permitted the determination of the shapes and dimensions of the particles [2]. The addition of polystyrene latex spheroids of known and constant diameter increased the accuracy of measurement of the dimensions of the examined virions [3]. In shadowed preparations of mixtures of latex and virus particles, the electron microscopic magnification can be determined and the local angle of shadow casting can be controlled [4]. By the above methods, data were obtained on the flattening and possible other distortions of the virions during preparation and examination.

Different optical methods (interference microscopy, absorption of X-ray and UV light) have extensively been used for mass, thickness and density measurements in quantitative cytochemical studies [5, 6, 7].

The application of the above optical methods in electron microscopy represents a shift of the method's limits towards lower orders of magnitude. Marton and Schiff [8] in 1941 already used the measurement of density on photographic plates for the determination of the preparation's thickness. Essentially the same technique was used by Hall in 1955 [9] and by Amenlunxen in 1959 [10], for the examination of the effectiveness of electron stains

used to increase the contrast in virus micrographs. In 1957, HOLLÓS [11] made estimations of the structural and compositional differences of complete and incomplete influenzavirus particles from the differences measured in their densities. The mass of phage particles was measured by electron microscopy in 1960 by BURGE and SYLVESTER [12].

In the present study the density, diameter and thickness of complete and incomplete influenza particles was measured. The flattening of the particles, the diameter of non-flattened (spherical) particles and the dry mass of virions were estimated. Approximative calculations were made as to the true mass of virions, their density and OsO_4-uptake.

Materials and methods

Virus strain. The Paris PL 1/49 A—1 strain of influenzavirus, kindly supplied by the World Influenza Centre, London, was used throughout.

Complete virus. From the 10^4 dilution of the virus suspension considered complete, 0.1 ml was inoculated into the allantoic cavity of 11-day old chick embryos, incubated for 36 hours at 35°C and harvested after appropriate chilling. The ID_{50}/HA quotient was 10^6. The ID_{50} was determined by the method of HORVÁTH [13], calculated according to REED and MUENCH [14]. Haemagglutination (HA) titration was performed according to TAKÁTSY [15].

Incomplete virus was obtained as described by VON MAGNUS [16]. Three subsequent passages were made with freshly harvested undiluted allantoic fluid inoculated in 0.3 ml volume into the allantoic cavity of 11-day old chick embryos. Incubation and harvesting were made as described above. The ID_{50}/HA quotient of the virus from the 3rd passage was 10^4.

Purification of the virus. Both complete and incomplete virus suspensions were clarified by centrifugation at 3000 r.p.m. for 1 hour. The material was then adsorbed to washed chick red blood cells at +4°C and washed with saline. Elution took place at 37°C in physiological saline, in one tenth of the initial volume. The eluate was centrifuged for clarification. The supernatant was centrifuged at 16 000 r.p.m. for 1 hour. The sediment was resuspended in physiological saline, in 1/50 of the initial volume. The suspension was centrifuged for one hour for clarification. The supernatant obtained was used as purified concentrated virus suspension.

Electron microscopy. To the purified and concentrated virus suspensions appropriate amount of Dow latex particles L_3 (1880 Å $\pm$ 70 Å) were added. The virus-latex mixed suspension was carried by means of a micro-loop onto micro-screens with Formvar films. The preparations were either unfixed [1] or fixed [2] with 1 per cent OsO_4 buffered solution according to PALADE [17] at +4°C for 20 minutes. Crystalloids were removed from the micro-drops by repeated immersion into distilled water. The preparations were then dried in air. Part of the preparations was shadowed with palladium.

Electron micrographs were made with EM-3 1951 USSR electron microscope, at 45 KV at 11,000fold direct magnification at 5×10^{-3} radian objective aperture angle, using Agfa document film. Illumination, exposition and development of films was made in a standard way throughout.

Measurement of density. The apparatus for density measurement was constructed from an optical microscope equipped with a cross table endowed with a nonius scale and a high sensitivity densitometer (Orion EMG Magnefot Type 2212). Scanning of the original electron micrographs was made with a light beam 0.2 mm in diameter, obtained by a special illumination system. The disturbance possibly caused by the dispersed light was eliminated by a diaphragm placed before the densitometer's photocell and constructed in accordance with the testing light spot and the magnification of the optical microscope. The relative diameter of the scanning light spot was 19 mμ, considering the 11,000fold direct magnification of the electron micrograph. The zero point of the apparatus was adjusted to the density of the unexposed film and density measurements were taken at 19 mμ steps along the diameter of the virion or latex particles and thereafter on the background. Two series of measurements were performed per particles, along its two perpendicular diameters. From these measurements density value s

were obtained for support microfilm (D_1) and for the examined particle (D_2). The distribution of mass in the individual particles was characterized by the individual (D_1-D_2) values.

The γ-curve of the photographic material was measured under constant conditions of development, after exposure to constant intensity 45 KV electron beam for 0.1 to 22 seconds. Blackening of the film was exponentially related to the exposition time in the region $D = 0.35-0.9$. The γ-value of the photographic material was 0.88.

Of the micrographs only those were used which displayed densities of both the supporting microfilm and the particles within the linear region of the γ-curve.

Density distribution was established along the diameters of at least 24 virions per preparation, 114 in all, and a total of 47 L_8 latex particles, in uniform distribution.

The D_1-D_2 values obtained in individual measurements were plotted. The values for the identical points plotted against the diameters were summed and their mathematical means were calculated. The density curves along the diameter of an average latex or virus particle were constructed from the above mean values.

Determination of the diameter. The diameter of the particles was determined during density measurement, by graphical interpolation and, in shadowed preparations, perpendicular to the direction of shadow casting, by means of an Abbe comparator (Zeiss).

In the Tables data obtained by both methods are presented. Since measurements of shadowed preparations allowed a direct determination of the diameters these data were given preference in further calculations.

Determination of height. The height of the particles was determined in shadowed preparations. In each evaluated preparation there were 2 to 5 latex particles allowing calculation of the local angle of shadow casting, tg α.

$$\operatorname{tg} \alpha = \frac{d}{(d/2) + h} \tag{1}$$

where d is the diameter of the latex particle and h the length of the shadow. Measuring the length of the shadow of virus particles, their height can be determined on the basis of the local tg α. When measuring the shadow length, full shadows were only considered

In density measurement, only the mass-thickness of the particle $\varrho \times t$, could be determined (ϱ = density of the particle, t = the particle's height measured parallel to the electron beam). For the determination of density (ϱ), the data on the mass obtained by density measurements and those for the volume obtained by measurements of shadowed preparations, were used. The density (ϱ) of the particle being known, its height could be calculated from data of density measurements.

Statistical methods. Arithmethic means ($\bar{x}$), standard deviation (S), and standard error of the mean ($S_{\bar{x}}$) were computed.

Results

In Table I data on the measurement of Dow latex L_8 are shown.

In the first part of Table I, the density values of 47 latex particles, measured along two perpendicular diameters and expressed as differences, are presented. The averaged points of measurement were at the five sixths, two thirds, one third, and middle of the diameter. The percentual error of the average density values was the highest at the edges of the particles and decreased towards the centre. Thus, the percentual standard error of the mean tended to decrease (± 8 per cent; ± 4.7 per cent; ± 1.4 per cent; ± 1.8 per cent).

In Fig. 1, the average density curve of the latex particle is presented. On the axis $r(x)$, the diameter and its fractions, whereas on the axis $D_1 - D_2(y)$ the corresponding density values are shown. The points approximate well a parabola, characterized by the second degree equation, $y = 0.274\, x^2$. The normal value $x = 1$ corresponds to 9.1×10^{-6} centimeters.

Table I

Measurements of L_8 Dow latex particles

Density measurement in grades $D_1 - D_2$	N	$\bar{x}$	S	$S_{\bar{x}}$
At 5/6 of the diameter	94	0.055	0.043	0.005
At 2/3 of the diameter	94	0.164	0.074	0.008
At 1/3 of the diameter	94	0.283	0.038	0.004
At middle of the diameter	47	0.307	0.037	0.006
Diameter in mμ				
Diameter from density measurement	47	187	62	3
Measured diameter	32	185	37	6.5

$$\bar{x} = \frac{\Sigma x}{n} \qquad S = \sqrt{\frac{\Sigma (x - \bar{x})^2}{n - 1}} \qquad S_{\bar{x}} = \sqrt{\frac{\Sigma (x - \bar{x})^2}{n (n - 1)}}$$

In the second part of Table I, statistically evaluated data of measurements on the diameter of a total of 79 shadowed and unshadowed latex particles are shown. The given diameter of 188 ± 7.6 mμ was satisfactorily approximated by both methods of measurement. With density and direct diameter measurements, the values obtained were 187 ± 3 mμ and 185 ± 6.5 mμ, respectively.

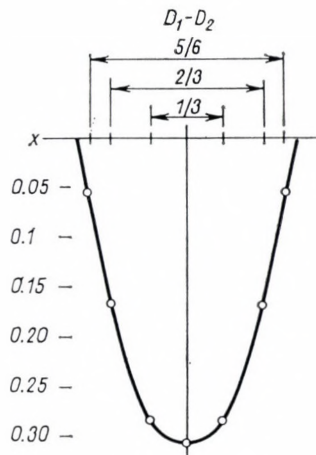

Fig. 1. Average density curve of L_8 Dow Latex particles

Determination of the mass constant (S) of the electron microscope. The electron beam deviated by a particle of "M" mass, resulting in differences of darkening of the photographic film is related to the mass of the particle.

$$S \times M = \int_A \left(\ln \frac{I_1}{I_2} \right) dA \,, \tag{2}$$

where A is the projection of the particle along the electron beam, I_1 the electron beam reaching the particle, I_2 the electron beam traversing the particle and reaching the photographic film, and S a relation factor.

The value of "S" depends hardly on the particle's material; it is determined chiefly by the adjustment of the electron microscope. Its dimension is $cm^2 \times g^{-1}$.

When referred to particles of rotational symmetry with R radius, formula (2) is

$$S = \frac{1}{M} \int_0^{\pi R^2} (\ln I_1 - \ln I_2)_A \, dA \,. \tag{3}$$

Since in the case described, the grade of darkening of the photographic material is related to the natural logarithm of the electron beam's intensity, from formula (3)

$$S = \frac{1}{M} \gamma \frac{1}{\log e} \int_0^{\pi R^2} (D_1 - D_2)_A \, dA, \tag{4}$$

where γ is the darkening factor of the photographic material, $(D_1 - D_2)_A$ the difference in darkening measurable in relation to the background (site dependent).

The S value characteristic of the electron microscope was computed from the known and measured values of the polystyrene latex particles. The average density curve of the latex particles (Table I) is well approximated by an analytically expressed $D = 0.274x^2$ parabola ($x = 1$ normal value $= 9.1 \times \times 10^{-6}$ cm). By the aid of the approximating parabola can be computed the integral in (4), from which can be derived the value of "S". Under the conditions prevailing in this experiment, the electron microscope used is characterized by

$$S = 2.4 \times 10^4 \pm 0.4 \times 10^4 \text{ cm}^2 \cdot \text{g}^{-1} .$$

The standard deviation of the "S" value was derived from the given fluctuation (± 7.6 mμ) of the latex diameter and from the standard error of the mean densities.

Density measurements of virions are summarized in Table II, as related to complete, incomplete or fixed, unfixed preparations. Presentation of the virions' densities along the diameter was made in the same manner as with the latex particles, *viz.* at two thirds, one third, and middle of the diameter. Fig. 2 shows the average density curves of virions per preparation. The standard error of the mean tended to decrease towards the centre also with the complete unfixed virus, yielding $\pm$4.6, 2.6 and 2.4 per cent at the above points.

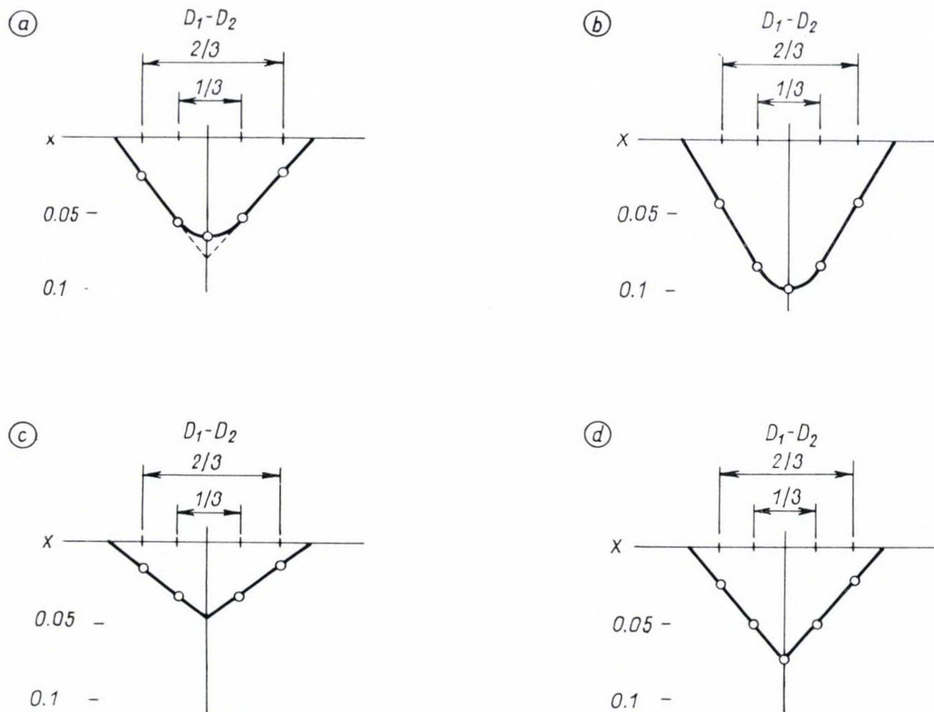

Fig. 2. Average density curve of the virions. a: complete unfixed virions; b: complete OsO_4-fixed virions; c: incomplete unfixed virions; d: incomplete OsO_4-fixed virions

With the fixed preparation, the standard error of the mean was higher, being $\pm$5.3, 2.8 and 4 per cent. The standard error of the mean was unproportionally high with the incomplete virions. The reason for this should not be sought in the lesser number of measurements, neither in the greater probability of subjective error at the adjustment and reading of the lower absolute values, but rather in the more mixed population of the incomplete preparations. Therefore, the standard error of the mean of the unfixed preparation was $\pm$12.8, 7.5 and 2.5 per cent, in the above sequence and also for the fixed preparations it was higher than that of complete preparations, being $\pm$7.3, 4.4 and 3.9 per cent. These deviations show that the measurements were of satis-

Table II

Density of virions

Preparation	D_1—D_2	N	$\bar{x}$	S	$S_{\bar{x}}$
Complete unfixed	At 2/3 ⌀*	80	0.027	0.011	0.001
	At 1/3 ⌀	80	0.054	0.013	0.001
	At middle	40	0.067	0.010	0.002
Complete fixed with OsO_4	At 2/3 ⌀	84	0.036	0.018	0.002
	At 1/3 ⌀	84	0.080	0.020	0.002
	At middle	42	0.107	0.028	0.004
Incomplete unfixed	At 2/3 ⌀	48	0.013	0.012	0.002
	At 1/3 ⌀	48	0.030	0.015	0.002
	At middle	24	0.048	0.019	0.001
Incomplete fixed with OsO_4	At 2/3 ⌀	70	0.024	0.015	0.002
	At 1/3 ⌀	70	0.053	0.020	0.002
	At middle	35	0.074	0.017	0.003

$$\bar{x} = \frac{\Sigma x}{n} \qquad S = \sqrt{\frac{(\Sigma x - \bar{x})^2}{n-1}} \qquad S_{\bar{x}} = \sqrt{\frac{\Sigma (x-\bar{x})^2}{n\,(n-1)}}$$

* ⌀ = diameter

factory accuracy as compared to the usual error of electron microscopic measurements.

The viruses' average density curve approximated a perpendicular section along the axis of a cone or a truncated cone, thus the value of the integral in formula (5) (see later), readily could be computed.

Diameter and height of virions. Pertaining results are presented in Table III. In shadowed preparations, the diameter of non-fixed and fixed complete viruses was 125 mμ and 121 mμ, respectively. The cause of this moderate, but significant discrepancy was due to data for height, *i.e.* 47 mμ for non-fixed, and 56 mμ for the fixed viruses. The respective M/d quotients were 0.376 and 0.46. Thus, the cause of the moderate difference of the diameters was the more marked flattening of the unfixed preparation, as compared to the fixed ones. It is clear, moreover, that independently of the measured difference, a more or less pronounced flattening of the viruses occurred during the preparation procedure in both types of preparation.

The diameter of unfixed incomplete virions was 133 mμ and their height was 40 mμ. The corresponding data for fixed preparations were 127 and 43 mμ.

Table III

Height and diameter of virions

Preparation	a) Measured in shadowed preparation							
	Diameter (mμ)				Height (mμ)			
	N	$\bar{x}$	S	$S_{\bar{x}}$	N	$\bar{x}$	S	$S_{\bar{x}}$
Complete unfixed	50	125	16	2	50	47	8	1
Complete OsO_4-fixed	50	121	11	2	50	56	11	2
Incomplete unfixed	50	133	18	3	50	40	12	2
Incomplete OsO_4-fixed	50	127	17	2	50	43	11	2
	b) Measured in unshadowed preparation by densitometry							
Complete unfixed	40	124	20	3	40	60	—	—
Complete OsO_4-fixed	41	117	17	3	20	70	—	—
Incomplete unfixed	24	126	21	4	24	63	—	—
Incomplete OsO_4-fixed	22	118	21	5	35	72	—	—

$$\bar{x} = \frac{\Sigma x}{n} \qquad S = \sqrt{\frac{\Sigma (x - x^{-})^2}{n - 1}} \qquad S_{\bar{x}} = \sqrt{\frac{\Sigma (x - \bar{x})^2}{n (n - 1)}}$$

The M/d quotient, showing the degree of flattening, was 0.30 and 0.34, respectively.

Diameters measured parallel to density assessment agreed well with those obtained in shadowed preparations. Height in these preparations was not measured directly, but derived by computation. The error of these data was not calculated, but it should be noted that it comprises a sum of the errors of S values and of diameter and density measurements. Thus, these data were considered less reliable than those obtained by direct measurement.

Three conclusions were drawn from the above data, *viz.* (*i*) all preparations were flattened; (ii) complete virions were less flattened than the incomplete ones; (iii) flattening was less distinct in fixed than in unfixed preparations.

Attempt to compute the true diameter from data obtained with flattened virions. Our reasoning was based on the following considerations. (1) The virion is a perfect sphere; (2) flattening results in the formation of another solid figure, a rotational ellipsoid; (3) flattening is not accompanied by disintegration; (4) the original and the flattened solid figures are identical in volume. Since the virion is not a perfect sphere, nor is the flattened virion a regular solid figure, the computed volume is an approximative value and so is the computed diameter of a non-flattened virion.

Assuming that the volume of the rotational ellipsoid and that of the original sphere are identical, and expressing the radius of the latter, we obtain

$$V_e = \frac{4}{3} a^2 b = V_{\text{sph}} = \frac{4}{3} r^3, \qquad r^3 = a^2 b$$

$$r = \frac{d}{2}, \; d_{\text{sph}} = d_e^2 \cdot M_e$$

where d_{sph} is the sphere diameter, d_e the diameter of the rotational ellipsoid (identical with the measured diameter of the virion), M_e the height of the rotational ellipsoid (identical with the measured height of the virion).

Table IV

Diameter of the spherical virion

Preparation	on the basis of diameter measurement (mμ)	on the basis of density measurement (mμ)
Complete unfixed	90	97
Complete OsO_4-fixed	93	99
Incomplete unfixed	89	100
Incomplete OsO_4-fixed	89	100

The computed diameters are shown in Table IV. The values calculated by direct measurement on shadowed preparations are in particularly good correlations, the virion diameters vary from 89 to 93 mμ. Density measurements uniformly show that differences in the diameters (97—100 mμ) result exclusively from the varying extent of flattening. The fitting of the results obtained by the two different methods was much better than expected, especially in view of the difficulties involved. In further work therefore, 90 mμ was taken for the diameter of the non-flattened virions.

The virion's mass. Values of A and S in formula (5) can be derived from formula (4):

$$M_{virion} = \frac{\gamma}{S \cdot \log e} \int_0^{\pi R^2} (D_1 - D_2)_A \cdot dA \,. \tag{5}$$

The integral in formula (5) represents the approximation of the volume of a cone or truncated cone, the basic area of which corresponds to the virion's projection and height to the maximum density value.

The first column in Table V shows the masses of virions computed by means of formula (5), together with the S values and the limits of error involved in the diameter determination. Considering the technical conditions of preparation, the mass obtained represents the dry mass of the virion. Taking into account the limits of error, the dry mass of complete virions exceeded that of incomplete ones. The mass of fixed virions exceeded that of unfixed ones, obviously as a result of OsO_4 uptake.

Table V

Data obtained by electron microscopy (EM) and ultracentrifugation (UC)

Preparation	Dry mass ($\times 10^{-16}$g)	Wet mass EM dry mass + +UC water content ($\times 10^{-16}$ g)	Wet mass EM volume $\times$ $\times$ UC density ($\times 10^{-16}$ g)	Electron microscopic density $\frac{\text{EM wet mass}}{\text{EM volume}}$ ($\times 10^{-16}$g)
Complete unfixed	2.8 (3.3–2.6)	5.5 (6.4–5.0)	4.2	1.4 (1.7–1.3)
Incomplete unfixed	1.9 (1.95–1.8)	4.1 (4.3–3.9)	4.1	1.0 (1.1–1.0)
Complete OsO_4-fixed	4.3 (4.7–3.9)	—	—	—
Incomplete OsO_4-fixed	2.5 (2.8–1.9)	—	—	—

The computed amount of OsO_4 fixed by the complete and incomplete virions was 1.451×10^{-10} μg and 0.647×10^{-10} μg, respectively. Considering Avogadro's number, the above amounts represent about half a million molecules for the complete and a quarter million molecules for the incomplete virion.

Further data characteristic of the virion and derived from other data available. The computed diameter of a non-flattened virion was found to be 90 mμ (9×10^{-6} cm), and its volume, 3.8×10^{-16} cm^3.

If our hypothesis on the flattening of the virion is correct, the wet and dry mass of the virion is comprised in an identical volume. According to TAKÁTSY's ultracentrifugal analysis [18], the water content of complete and incomplete particles is 48 and 56 per cent, respectively. Adding this to the electron microscopically determined dry mass of the virion, the wet mass of the virion is obtained. These values are shown in the second column of Table V, for unfixed complete and incomplete virions. Data calculated by taking into consideration the error of mass determination are also given in brackets. The

density determined by ultracentrifugation [18] was 1.104 for complete and 1.073 for incomplete virions. Multiplying these values by the average volume of a virus, we obtain data on the wet mass of a virion. Values derived by this method for the wet mass agreed well with those obtained by the method used in the present study. It should be noted that data on the complete virion yielded by electron microscopic mass determination are somewhat higher than those obtained by ultracentrifugation. As to the incomplete virion, both methods yielded identical values. Electron microscopic data for the density of the complete virion are somewhat higher than in reality, those for the density of the incomplete virion correspond to the actual values, thus the relative difference between K_0 and IK_0 is higher than the density difference found with ultracentrifugation.

Discussion

Considerable differences between the complete and incomplete influenzavirus have been demonstrated by ultracentrifugation, electron microscopy and chemical analysis [19, 20, 21]. Our aim was, therefore not to present further evidence in favour of these well-known facts, but rather to decide whether from appropriately obtained electron micrographs it was possible to derive data on certain characters of the virus, determined usually by other methods. To certain preparations latex particles were added and fixed as well as unfixed preparations were examined. Our preparations were not superior to those used by WILLIAMS in 1953 [4] for the determination of M/d quotient for unfixed, formalin fixed influenzavirus (0.3 and 0.5, respectively) and the electron micrographs were taken with the usual technique. The gradation of a given photographic material had to be determined only once. Naturally, for adequate measurements special apparatuses are needed. The choice of an appropriate method of measurement and the evaluation of the results require, however, a thorough knowledge of the physical phenomena involved. In the work reported in the present paper, the collaboration of the physician and the physicist proved to be fruitful.

It seems to be of interest to perform similar measurements on negatively stained preparations. The more so, as measurements can only be performed in an order of magnitude considerably below the resolution of the available electron microscope. Thus, for the measurement of the mass of influenzavirus, the electron microscope used in this laboratory was just the ideal one.

An apparatus with a resolving power of 10 Å, however, would allow to determine the mass of 50—60 Å elements.

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REGULATION OF VALINE AND ISOLEUCINE BIOSYNTHESIS IN STREPTOMYCES RIMOSUS

By

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Summary. In *Streptomyces rimosus*, similarly to other microorganisms, there is a transitory increase in the synthesis of enzymes participating in the biosynthesis of isoleucine and valine after inoculation. This increased synthesis is further enhanced by valine. The spontaneous increase of enzyme synthesis can be repressed by valine, leucine and isoleucine.

The previously described inductive effect of α-ketobutyric acid prevails during the whole period of cultivation; it can be repressed by the simultaneous administration of valine and leucine while valine alone causes only partial repression.

The first common enzyme of valine and isoleucine biosynthesis, acetohydroxy acid synthetase, is inhibited by valine.

In our metabolic studies with *Streptomyces rimosus*, isoleucine secretion has been observed in media containing threonine [1, 2]. Studying the secretion of isoleucine with washed mycelia we have found that α-ketobutyric acid, whether added to the medium, or formed from threonine, increased inductively the synthesis of enzymes which participate in the biosynthesis of valine and isoleucine. The acetohydroxy acid synthetase content of cells, the amount of enzyme measured in toluene-treated cells, was four times higher in the induced cells than in the control ones [2, 3]. Metabolic studies have shown that this induction affects the metabolism and oxytetracycline production of the microorganism [2, 4, 5].

The present paper presents further experiments on the regulation of valine and isoleucine metabolism in *Streptomyces rimosus*.

Materials and methods

Cultivation of Streptomyces rimosus. The experiments were carried out with *Streptomyces rimosus* BS 21/76 strain. Cultivation was performed in media containing glycine-glucose, which was complemented with substances as indicated in the Tables and Figures. The samples were inoculated with a 5 per cent, 44-hour-old vegetative inoculum [6].

Assay of enzymic activity. The cells were washed twice with distilled water by centrifugation and suspended (6—8 mg/ml dry weight) in 0.4 *M* phosphate buffer, pH 7.6, containing 0.1 *M* $MgSO_4$ and 100 μg/ml thiamine pyrophosphate. The suspension was sonicated three times for three minutes each at 0°C (Mulard sonic disintegrator, 20 000 KC, 50 soundwatt), then the crude extract thus obtained was gel-filtered on Sephadex G—50 column equilibrated with the buffer solution used for sonication at 0°C, to remove the inhibitory effectors [7]. Acetohydroxy acid synthetase activity was estimated according to UMBARGER and BROWN [8], dihydroxy acid dehydration by the method of WIXOM et al. [9], while threonine deaminase

was assayed according to SZENTIRMAI and HORVÁTH [10]. If threonine deaminase was also determined, sonication was carried out in the presence of aluminium oxide, to achieve solubilization of the enzyme [10]. Enzyme activities were assayed at 28°C, and specific activity was defined in terms of μmole products formed in 1 hour per mg of protein. Protein concentration was estimated by the method of LOWRY et al. [11], with crystalline serum albumin as the standard.

Chemicals. Commercial preparations of reagent grade were used. α-β-dihydroxy-isovaleric acid was synthesized according to the method of SJOLANDER et al. [12].

Results

In the course of cultivation acetohydroxy acid synthetase activity was estimated in the crude extracts gel-filtered on Sephadex G-25. Following inoculation the specific activity of extracts increased transitorily. A similar phe-

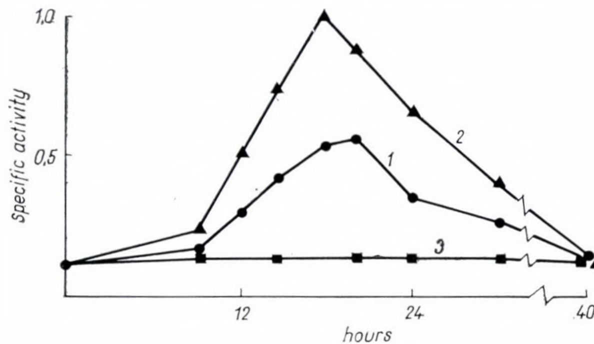

Fig. 1. Enhanced synthesis of acetohydroxy synthetase after inoculation; the effect of valine. The following additions were made to the glycine-glucose medium 1: none; 2: 8 *mM* L-valine; 3: 8 *mM* L-valine + 8 *mM* L-leucine + 8 *mM* L-isoleucine

nomenon was observed first in *E. coli* [8] and later in several other microorganisms [13, 14, 15]. This initial increased enzyme synthesis was influenced only by valine from among the branched chain amino acids. In the presence of valine there was an increase in enzyme production only during enhanced synthesis. This effect of valine varied considerably from experiment to experiment, sometimes a 50 per cent, in other cases a five-fold increase was observed. The enhanced enzyme synthesis which followed inoculation was inhibited by the simultaneous presence of all three branched chain amino acids (Fig. 1).

If α-ketobutyric acid was added to the media, the increase in enzyme synthesis was considerably more marked than in the above cases and persisted during the whole period of cultivation. This enhancing effect of α-ketobutyric acid was not influenced by isoleucine and leucine, was partially repressed by valine, while complete repression could be achieved by the simultaneous addition of both leucine and valine (Fig. 2). As to the influence of α-ketobutyric acid concentration on induction, 1 *mM* had a significant effect, while the maxi-

mum effect was achieved by 10—20 mM. Threonine and α-aminobutyric acid also had an inductive effect; the breakdown of these led to α-ketobutyric acid, but the inductive ability of the substances was weaker than that of α-ketobutyric acid (Fig. 3).

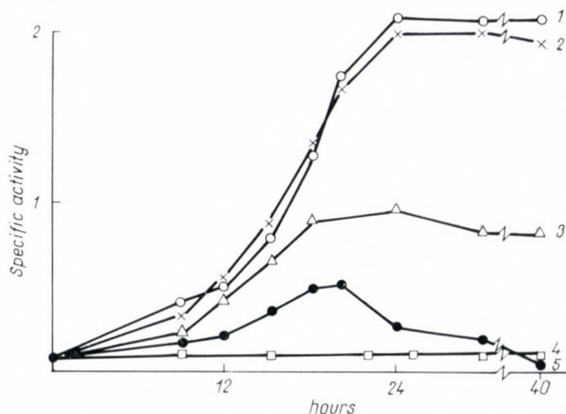

Fig. 2. Inductive effect of α-ketobutyric acid. The following additions were made to the glycine-glucose medium. 1: 20 mM α-ketobutyric acid; 2: 20 mM α-ketobutyric acid + 8 mM L-leucine; 3: 20 mM α-ketobutyric acid + 8 mM L-valine; 4: 20 mM α-ketobutyric acid + 8 mM L-valine + 8 mM L-leucine; 5: none

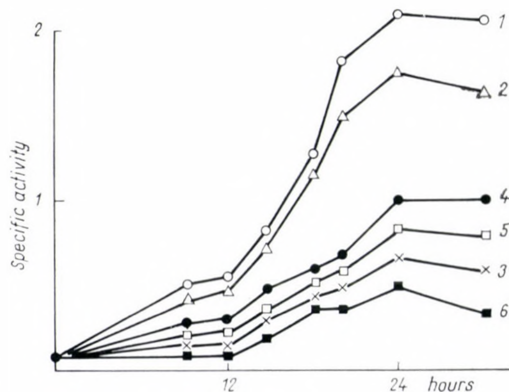

Fig. 3. Inductive effect of threonine and α-aminobutyric acid. The following additions were made to the glycine-glucose medium. 1: 20 mM α-ketobutyric acid; 2: 10 mM α-ketobutyric acid; 3: 1 mM α-ketobutyric acid; 4: 10 mM L-α-aminobutyric acid; 5: 10 mM L-threonine; 6: none

In the above experiments the effect of various substances was followed by the aid of one enzyme, α-acetohydroxy acid synthetase. In further experiments in extracts of points showing the greatest differences, threonine deaminase and dihydroxy acid dehydrase activities were also assayed, since these enzymes participate in the synthesis of isoleucine and valine. The specific ac-

tivity of threonine deaminase did not depend on cultivation conditions and additional substances [10] while dioxy acid dehydrase activity changed parallel with that of α-acetohydroxy acid synthetase (Table I).

Table I

The coordinative character of changes in enzyme levels

Addition	Threonine deaminase, μmoles of α-ketobutyric acid $hour^{-1}$ $protein^{-1}$	Acetohydroxy acid synthetase, μmoles of α-acetolactic acid $hour^{-1}$ $protein^{-1}$	Dihydroxy acid dehydrase, μmoles of α-ketoisovaleric acid $hour^{-1}$ $protein^{-1}$
—	2.75	0.58	0.15
8 *mM* L-valine	2.45	0.85	0.17
20 *mM* α-ketobutyric acid	2.55	3.30	0.50
20 *mM* α-ketobutyric acid + + 8 *mM* L-valine	2.85	1.47	0.24
20 *mM* α-ketobutyric acid + + 8 *mM* L-valine + 8 *mM* L-leucine	2.36	0.34	10.0

The extract was made from 24-hour-old cultures

It has been shown in several microorganisms that acetohydroxy acid synthetase, the enzyme catalyzing the first common step of valine-isoleucine biosynthesis, is inhibited by valine [8, 13, 14, 15, 16]. In our first experiments with toluene-treated cells valine failed to cause inhibition [2, 3]. In the present experiments, however, in extracts treated with Sephadex G—25 inhibition by valine was observable, but the extent of inhibition never exceeded 50 per cent (Fig. 4).

The degree of inhibition depended on the substrate concentration.

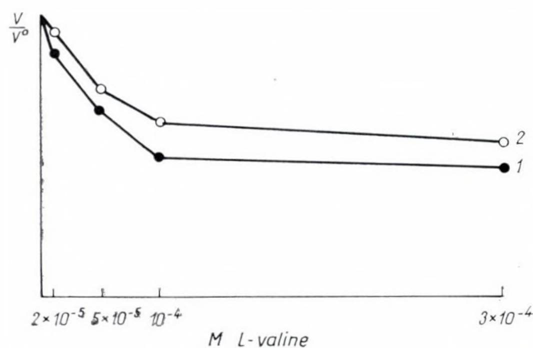

Fig. 4. Inhibition by L-valine of acetohydroxy acid synthetase. 1: substrate concentration, $3.10^{-2}M$ pyruvate; 2: substrate concentration, $10^{-1}M$ pyruvate

Discussion

It has been shown with washed mycelia of *Streptomyces rimosus* that α-ketobutyric acid caused an inductive increase in the amount of enzymes participating in the biosynthesis of valine and isoleucine. With mycelia grown in media containing glycine, chloramphenicol inhibited the secretion of isoleucine, while with mycelia grown in threonine containing media, chloramphenicol was ineffective, and production of valine from pyruvic acid could even be observed in the presence of chloramphenicol with mycelia grown in media containing threonine [2, 3]. As a continuation of these experiments we have attempted to estimate the acetohydroxy acid synthetase activity with washed toluene-treated mycelia, the results obtained were not equivocal. The filamentous growth of *Streptomyces rimosus* was the source of several errors. In the possession of source experience gained in experiments with bacteria [14, 15], we reassumed the experiments with *Streptomyces rimosus* the result of which was as follows.

The spontaneous and transitory enhancement of enzyme synthesis in *Streptomyces rimosus* following inoculation was similar to that in other microorganisms [8, 13, 14, 15]. This explains why we could not obtain reliable data in experiments with washed mycelia. The different degree of spontaneous increase was partly or completely masking the inductive effect of α-ketobutyric acid within the short experimental period. The effect of branched-chain amino acids on spontaneous increase was examined. It was found that valine increased the transitorily enhanced synthesis which varied considerably from experiment to experiment. This transitory increase in enzyme activity did not lead to isoleucine secretion even in the presence of valine, in contrast to induction with α-ketobutyric acid. A similar spontaneous increase and valine-effect could be observed with *Streptomyces fradiae* [17], which also failed to cause isoleucine secretion.

In the above spontaneous increase a decisive role must have been played by the endogenous pool of branched-chain amino acids determined by the rate of synthesis, breakdown and utilization. This hypothesis is supported by our observation that the simultaneous addition to the media of the three amino acids completely inhibited the spontaneous rise. The biosynthesis of branched-chain amino acids is known to have three common steps, and thus in the regulation of biosynthesis the regulating system should control not only the functioning of enzymes, through influencing the enzyme action or synthesis, but also the appropriate proportion of the synthesis. This multiple regulation system probably loses its equilibrium after inoculation resulting in the spontaneous increase of the enzyme level.

The enhancing effect of valine on the spontaneous increase of the enzyme level can be interpreted in light of the results obtained in our experiments with

Mycobacterium pellegrino [15]. In this microorganism the effect of valine is much more pronounced, it prevails for a longer period, and can be elicited at any stage of growth. The effect of valine can be explained by the fact that the first common enzyme of the biosynthesis is intensely inhibited by valine. In the case of *Streptomyces rimosus* the increase in enzyme synthesis can be conceived on the basis of a similar mechanism, since the first common enzyme of the biosynthesis in this case is also sensitive to valine. The extent of inhibition achieved by valine in the crude extract did not exceed 50 per cent which could be attained at low valine concentrations. This may presumably be due to the fact that the sensitivity to valine of the enzyme decreases in the course of preparation. The question obviously arises why this effect of valine is only transient. In the regulation of enzyme synthesis, in addition to the endogeneous pool determined by synthesis, breakdown and utilization, the state of the enzyme might also have a regulatory role, as it has been proved in the case of *Pseudomonas aeruginosa* [7, 18, 19]. We have shown with *Pseudomonas aeruginosa* that the enzyme is present in the cell in two states, one of which is sensitive, while the other insensitive to valine. These two states can reversibly be transformed into each other. Valine promotes the formation of the sensitive form, while pyruvate that of the insensitive form. If we deal with a similar system in *Streptomyces rimosus*, it appears probable that in the early stages of growth at a low metabolic rate the pyruvate is unable to exert any effect and the valine-sensitive form predominates, but with the increase of the metabolic rate sufficient amounts of pyruvate are produced to counteract the effect of valine.

The inductive effect of α-ketobutyric acid could be observed so far only in the case of *Streptomyces rimosus*. As to the mechanism of this induction inferences can be made from the facts that valine causes a partial, and valine + leucine a complete, repression. On this basis it may be assumed that the cause of enhanced enzyme synthesis is the lack of both valine and leucine, that brings about a derepressed state. In *Mycobacterium pellegrino* the derepressed state could be achieved by the addition of valine [15], since the action of the first enzyme of the biosynthesis is strongly inhibited by valine.

Wagner et al. [20] studied in a cell-free system of *Neurospora crassa* the synthesis of valine and isoleucine from the corresponding ketoacids. They have shown that the substrate affinity of acetohydroxy acid synthetase plays an important role in controlling the ratio of valine and isoleucine production. On this basis it may be assumed that in the case of *Streptomyces rimosus*, in one of the steps of the two-step condensation reaction the affinity of α-ketobutyric acid is so strong as compared to that of pyruvate that it results in a lack of valine and leucine.

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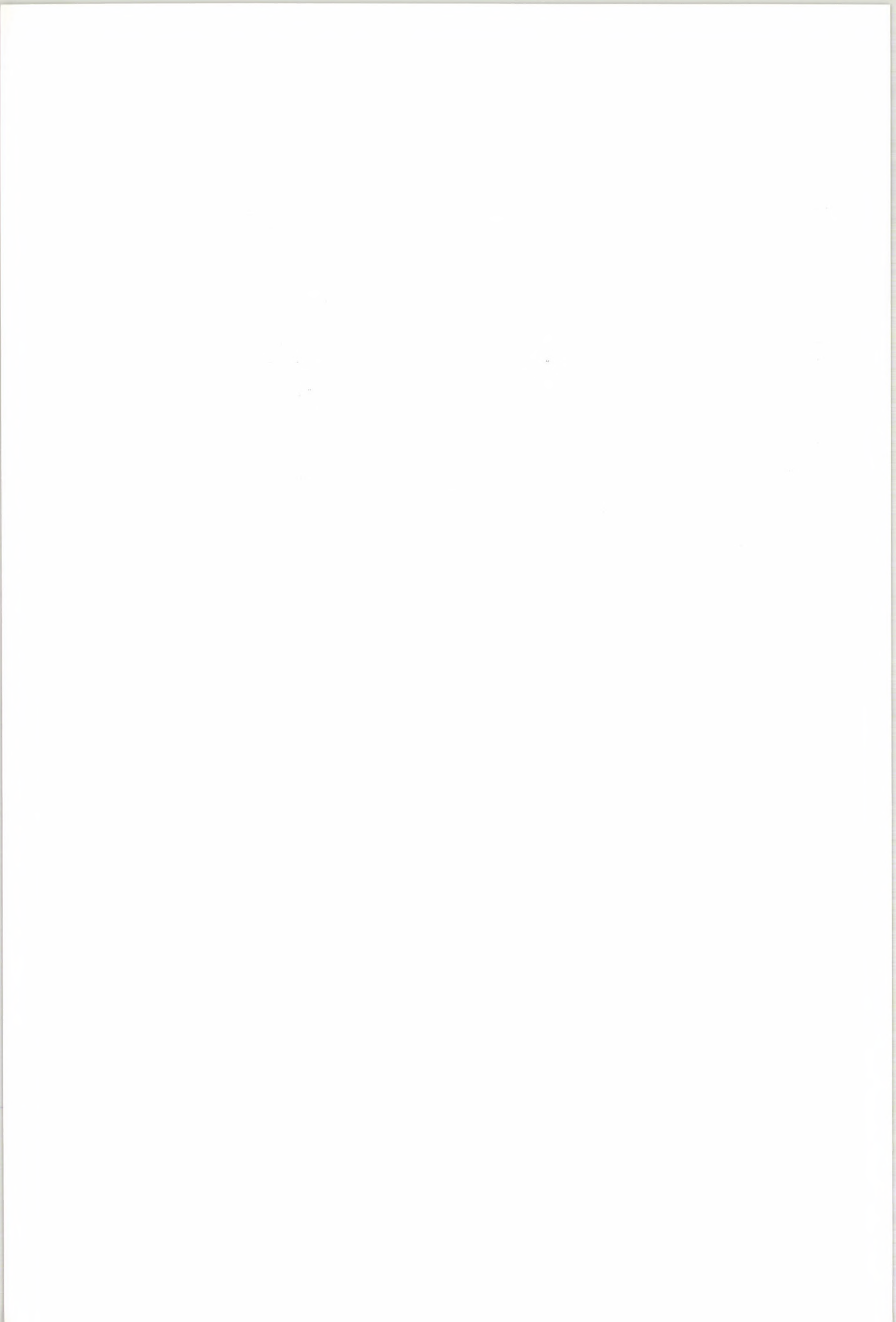

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"EARLY, NON-VIRION" ANTIGENS IN HERPES SIMPLEX VIRUS INFECTED TISSUE CULTURE CELLS

I. DETECTION OF "EARLY" ANTIGENS WITH COMPLEMENT FIXATION TEST

By

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Summary. It has been shown by complement fixation test that in BS-C-1, human embryonic fibroblast, and HeLa cells infected with multiplicity of 1—8 TCD_{50} per cell of Herpes simplex virus (HSV) there is a production of a virus specific, early antigen component. Its production is not inhibited by the presence of 10 μg/ml of cytosine arabinoside. The antigen is heat labile, ether labile, sensitive to repeated freezing and thawing, and is not sedimented by centrifugation at 100 000 g for 1 hour.

3 of 8 HSV immune rabbit sera reacted with the early component, but there was no correlation between the anti-early and anti-viral CF antibody titres. None of 6 human convalescent sera reacted with the early antigen in dilution of 1 : 4 or higher.

In recent years experimental cancers have been produced with certain DNA viruses [1—4], which under natural conditions exist in mice, monkeys and human beings, and produce either no recognizable disease, or only mild illness. These viruses multiply in certain normal tissue culture cells with destructive effect, and prior to the onset of viral DNA synthesis there appears in the cells a new antigenic component, identical with the specific antigen present in the tumour cells caused by the same virus.

On the basis of these findings increased attention has been focussed on the eventual role of human DNA viruses in human cancerogenesis. Until now experiments have failed to detect a specific antigen in human tumours. This might have been due to the small amount of antigens present in the tumour cells and/or to the methods being unsuitable for detecting such antigens.

As one of the new approaches it has been studied whether sera of humans with malignancies contain antibodies against the early antigens present in normal tissue culture cells infected with different human DNA viruses, thus trying to detect the viral aetiology of certain human tumours [5].

As a first step in this research we have detected the "early" antigen in the extract of Herpes simplex virus infected tissue culture cells, and have determined certain characteristics of this component.

Materials and methods

Tissue cultures used for the preparation of antigens. BS-C-1 stable line of Cercopithecus monkey kidney cells (kindly supplied by Dr. A. B. SABIN). The cultures were grown in 1 liter Roux bottles by plating about 10^7 trypsinized cells in 80 ml of medium No. 199 (0.125 per cent $NaHCO_3$), containing 0.1 per cent yeastolate and 20 per cent foetal calf serum. The medium was changed 2 and 5 days after plating.

HeLa cell line was grown in 1 liter Roux bottles. 3×10^6 trypsinized cells were plated in one bottle, in 80 ml of medium No. 199 (0.125 per cent $NaHCO_3$) containing 10 per cent bovine serum. The medium was changed on the 7th day after plating.

Human embryonic fibroblast tissue culture was prepared from 4—6 week old embryos with trypsinization as described previously [6]. Secondary tissue cultures were used for the preparation of antigens. 10^7 trypsinized cells were added to 1 liter Roux bottles in 80 ml of medium No. 199 (0.125 per cent $NaHCO_3$) with 10 per cent bovine serum. The medium was changed on the 2nd day for the same medium with 0.25 per cent $NaHCO_3$ content.

Virus strain used was HSV—"OS" in passages 133—137. This is a syncitium-forming strain isolated from the vesicle fluid of a patient with simple labial herpes and maintained by passages on human embryonic fibroblast tissue cultures. Its infective titre was $10^{6.5}$—$10^{6.75}$ TCD_{50} per ml.

Preparation of CF antigens. The tissue cultures were washed three times with pH 7.2 phosphate buffer solution when dense cell sheets had formed. Herpes simplex virus suspension was added in different amounts in different experiments, the multiplicity ranging from 0.2 to 8 TCD_{50} per cell. Inoculated tissue cultures were incubated at + 37°C for 1 hour, then the inoculum was poured off and the cell sheets were washed three times with Basal Medium (Eagle) (BME) (0.125 per cent $NaHCO_3$) without serum or phenol red, to remove unadsorbed viruses. 80 ml of BS-C-1 maintenance medium consisting of Medium No. 199 and 2 per cent calf serum (0.25 per cent $NaHCO_3$) was added per bottle. The early antigen was prepared after 5 1/2 hours of incubation, the "viral" antigen after 15 hours of incubation at +37°C.

The medium was poured off and discarded. The cell sheets were washed three times with BME (0.125 per cent $NaHCO_3$) without serum or phenol red. Cells were scraped off with "rubber policeman" into BME. The cell suspension was centrifuged at 2500 r. p. m. for 5 minutes. The supernatant was discarded, the sediment resuspended in BME, and centrifuged again. The procedure was repeated once. The final sediment was weighed, and a 10 per cent suspension was prepared in BME. Cells were disrupted by freezing and thawing four times at — 78°C in a REVCO ultra-low-temperature cabinet. Occasionally the 10 per cent suspension was centrifuged at 100 000 *g* for one hour, and the sediment was resuspended to original volume in BME. The upper and lower halves of the supernatant and the reconstituted sediment were saved separately.

CF test was carried out in precipitating tubes in a total amount of 0.5 ml. 1.5 units of complement were used. Before use, the dose of complement was determined in the presence of the components used in the test.

Cytosine arabinoside was added at a concentration of 10 μg per ml to the tissue culture medium which was added to the HSV inoculated tissue cultures as maintenance medium after the 1 hour adsorption period to detect the effect on the development of the "early" antigen in HSV infected cells. The compound was stored as 1 mg/ml stock solution in medium No. 199 in the dark at —78°C.

Determination of ether sensitivity. Twenty per cent of ethyl ether was added to the antigen, kept at +4°C overnight with repeated shaking. Next day it was centrifuged at 2500 r. p.m. for 10 minutes. The lower clear layer was removed, put into an Erlenmeyer flask covered with gauze, and evaporated at +37°C for 10 minutes. After shaking for 10 minutes at room temperature it was stored at — 78°C until use. The control antigen suspension was kept under the same conditions.

Heat stability test. Antigen was kept at 56°C, and other samples were boiled at 100°C in a water bath for 30 minutes.

Repeated freezing and thawing. An aliquot of antigen was frozen ten times at — 78°C in a REVCO cabinet and thawed under running tap water.

Results

Selection of Herpes simplex immune rabbit sera reacting with HSV-early antigen. SABIN has shown [5, 7] that some African green monkeys inoculated

with massive dose of SV-40 virus developed antibodies not only against the viral antigens but also against the "early", "tumour-like" antigens which are present in the SV-40 tumour cells and also in normal tissue culture cells early after inoculation. On the basis of these results we have attempted to find HSV-immune rabbit sera which would react with both "early" and "viral" HSV-antigens, and sera which would only react with viral components. The use of both kinds of immune sera allows the detection of "early" antigens in HSV infected cells.

For the selection of anti-"early-antigen" immune rabbit sera we have prepared our first early antigen 5 1/2 hours after inoculation of BS-C-1 tissue culture cells (see Methods). To eliminate the viral particles still present in the suspension, the antigens were centrifuged at 100 000 *g*/1 hr. and both the sediment (reconstituted to the original volume) and the supernatant were saved and tested separately. The viral antigens were prepared similarly, 15 hrs after inoculation.

Table I

Complement fixing antibody titres of HSV-immune rabbit sera against HSV/BS-C-1 "early" and "viral" antigens of 3—8—66

Serum	CF antibody titres against 10 per cent antigen suspensions					
	BS-C-1 control 100,000 g/1 hr		HSV/BS-C-1 "early" 100,000 g/1 hr		HSV/BS-C-1 "viral" 100,000 g/1 hr	
	Supernatant	Sediment	Supernatant 1 unit	Sediment 1 unit	Supernatant 1 unit	Sediment 1 unit
R No. 6	<4	<4	<4	<4	16(32)	16(32)
R No. 4	<4	<4	8(16)	4(8)	16(32)	16(32)
R No. 12	<4	<4	<4	<4	<4	4
R No. 22	≥4	≥4	(4)	≥4	≥4	≥4

According to the data of Table I, two of four HSV immune rabbit sera R No. 4, and R No. 6 were identical in anti-viral antibody titre. On the other hand R No. 6 did not react with the early extract of HSV infected cells, while R No. 4 reacted with this component at a lower titre than with the viral ones. The early supernatant had no infectivity when titrated on human embryonic tissue culture, while the early sediment showed an infectivity of $10^{1.5}$ TCD_{50} per 0.1 ml of 10 per cent homogenate.

R No. 22 serum reacted with the BS-C-1 control antigen, while R No. 12 serum reacted with the viral antigen at a very low titre and failed to react with the early component.

The effect of cytosine arabinoside on the formation of "early" antigen in HSV infected cells. To ascertain that the component present in the early extract of HSV infected cells is an early antigen which is formed before viral DNA has been synthetized, we have tested the R No. 6 and R No. 4 rabbit sera against antigens prepared from HSV infected tissue cultures which were incubated in the presence of 10 μg per ml of cytosine arabinoside.

According to the data of Table II, the presence of cytosine arabinoside did not inhibit the formation of the "early" antigen, thus proving its non-virion nature. The formation of viral components was inhibited even 15 hours after inoculation, as indicated by the lack of reactivity with the 15 hour antigen prepared in the presence of cytosine arabinoside of serum R No. 6.

Table II

Effect of 10 μg/ml of cytosine arabinoside on the formation of "early" and "viral" antigens in HSV-inoculated BS-C-1 continuous cell line

Serum	Complement-fixing antibody titres against 10 per cent antigens of 4/20/66				
	BS-C-1 control 100,000 g/l hr supernatant	HSV/BS-C-1 "0" time* 100,000 g/l hr supernatant	HSV/BS-C-1** "early" with CA+ 100,000 g/l hr supernatant 1 unit	HSV/BS-C-1*** "late" with CA+ 100,000 g/l hr supernatant 1 unit	HSV/BS C-1*** "viral" 100,000 g/l hr supernatant 1 unit
R No. 4	<4	(4)	8(16)	8(16)	16(32)
R No. 6	<4	<4	<4	<4	16(32)

*"0" time antigen was prepared at the end of the incubation period after inoculation with HSV, before cytosine arabinose had been added.
**5 1/2 hours after inoculation.
***15 hours after inoculation.
+Cytosine arabinoside.

Data of different batches of 10 per cent HSV-early antigen. Using sera R No. 4 and R No. 6, we have tested 8 different antigen preparations for the presence of "early" components in BS-C-1, human embryonic fibroblast and HeLa tissue cultures (Table III).

All the preparations but two contained the "early" antigen at a low concentration in the 100,000 *g* supernatants. Two, not ultracentrifuged preparations, labelled as 3—31—66 and 5—18—66, contained somewhat more of the antigen. One of these preparations, which did not contain the early component, was made in PBS instead of BME (labelled as 5—4—66). The other was prepared from a tissue culture inoculated with only 0.2 TCD_{50} per cell, an amount probably too low for the production of the early component in detectable amounts.

The reactivity of HSV immune rabbit and human sera with "early" and "viral" antigens. Among 8 HSV immune rabbit sera 3 reacted with the early

Table III

Data of 10 per cent "early" antigens prepared from HSV-infected tissue culture cells

Antigen	HSV strain used for inoculation	Inoculum TCD_{50}/cell	Tissue culture used for preparation	CF "early" antigen titre when tested with rabbit No 4 serum (2u. of antibodies) in		
				100,000 g/l hr supernatant	100,000 g/l hr sediment	not ultra-centrifuged
3— 8—66	HSV—OS	1	BS-C-l	und.** (2)	und. (2)	N. T.***
3—29—66	HSV—OS	8	Human embryonic fibroblast	und.	und.	N. T.
3—31—66	HSV—OS	7	BS-C-l	N. T.	N. T.	$\geqq$ und.
4—20—66	HSV—OS	6	BS-C-l	und.	und.	N. T.
5—4—66*	HSV—OS	1	BS-C-l	—	—	N. T.
5—18—66	HSV—OS	1	BS-C-l	N. T.	N. T.	2 (4)
5—27—66	HSV—OS	1	He La	und.	N. T.	N. T.
6— 3—66	HSV—OS	0.2	BS-C-l	—	—	N. T.

*Antigen suspended in PBS instead of BME
**undiluted
***not tested

antigen. None of 6 human convalescent sera reacted with the early component in dilutions 1 : 4 or higher in complement fixation test (Table IV).

Effect of heat, repeated freezing and thawing, ether and storage, on the HSV-early antigen. We have found that the early antigen is heat labile, ether sensitive, and it is destroyed by repeated freezing and thawing. It is not injured by 60 days of storage at —78°C. At least some part of it is soluble, as it is not sedimented by 100 000 *g* centrifugation for 1 hour (Table V).

Discussion

Our finding concerning the presence of "early", "non-virion" antigens in HSV infected normal tissue culture cells are in agreement with the results of SHEDDEN, WATSON and WILDY [8]. These authors have used the gel diffusion technique instead of CF, and have prepared their antigens 12 hours after inoculation with high multiplicity of virus (10 P. F. U. per cell).

The fact that cells exposed to cytosine arabinoside synthetize the early component confirms that the component is produced by the infected cell

Table IV

Anti-early and anti-viral CF antibody titre of different HSV-immune human and rabbit sera

	Serum	CF antibody titre against antigens: HSV/early 10 per cent 100 000 g/l hr supernatant 1 unit	CF antibody titre against antigens: HSV/viral 10 per cent 100 000 g/l hr sediment 1 unit
Human sera	K. K.	<4	8
	K. Sz.	<4	8
	B.	<4	32
	T. E.	<4	8
	S. B.	<4	8
	M.	<4	4
Rabbit sera	R No. 4	8(16)	16(32)
	R No. 6	<4	16(32)
	R No. 11	<4	⋝4
	R No. 10	<4	⋝4
	R No. 5	4	⋝4
	R No. 34	<4	8(16)
	R No. 37	<4	⋝4
	R No. 555	8(16)	16(32)

Table V

Effect of heat, repeated freezing and thawing, and ether, on the HSV/BS-C-l "early" 10 per cent antigen of 5—18—66

Serum used for test	Antibody units		CF antigenic units in preparations treated				Untreated control
	Anti-"early"	Anti-viral	56°C 30 min.	100°C 20 min.	Frozen and thawed ten times	Ether	
Rabbit No. 555	2	4	—	—	(und.)*	—	4
Rabbit No. 6	0	4	—	—	—	—	—

*undiluted

before viral DNA has been synthetized. Rapp et al. [9] arrived at the same result with the production of "tumour-like" antigen in SV-40 infected tissue culture cells.

The properties of our "early" antigen are very similar to those of SV-40 tumour and tumour-like antigens [10]. It is heat labile, ether sensitive, sensitive to repeated freezing and thawing, and it remains at least partly in the

supernatant after centrifugation at 100 000 *g* for 1 hour. In agreement with the results of SHEDDEN et al. [8] it consists of several distinct components, as not only the 100 000 *g* supernatant, but also the reconstituted sediment reacted with our indicator serum R No. 4 (Table I).

We failed to find a correlation between the antiviral and anti-early antibody titre of the rabbit sera, though the anti-early titre was always lower than the former one.

The fact that some HSV immune rabbit sera react with the early antigen is in agreement with SABIN's finding concerning the sera of some African green monkeys inoculated with a massive dose of SV-40 virus [5]. These phenomena call for a study with different methods of human HSV-convalescent sera for the presence of anti-early antibodies, before sera of patients with malignancies are tested against the "early" antigens produced by different kinds of human DNA virus.

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"EARLY, NON-VIRION" ANTIGENS IN HERPES SIMPLEX VIRUS INFECTED TISSUE CULTURE CELLS

II. INTRACELLULAR LOCALIZATION OF "EARLY" ANTIGEN WITH INDIRECT IMMUNOFLUORESCENCE TECHNIQUE

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Summary. Production of a virus specific "early" antigen component localized in the nucleus, especially at its periphery, has been shown immunofluorescence in BS-C-1 and chicken embryonic fibroblast cells, 5 1/2 and 8 hours after infection with Herpes simplex virus (multiplicity of 4.5 TCD_{50}). Production of the antigenic component was not inhibited by 10μg per ml of cytosine arabinoside.

Virus-specific antigens in tumour cells and in normal ones subjected to lytic infection by SV-40, adeno-12 and polyoma viruses may be detected not only by the complement fixation (CF) reaction but also by the fluorescent antibody (FA) technique [1—7].

On the basis of previous results [8], we have attempted to localize the "early" antigen present in tissue culture cells infected with Herpes simplex virus (HSV), using HSV-immune rabbit sera and the indirect immunfluorescence technique.

Materials and methods

Tissue cultures. (1) BS-C-1 stable line of Cercopithecus monkey kidney cells was as described previously [8]. 7.5×10^5 cells were added per coverslip-tube in 2 ml of Medium BS-C-1 growth. (2) Chicken embryonic fibroblast tissue culture was prepared from 9—10 days old chicken embryos with trypsinization according to the method used for preparation of human embryonic tissue cultures as described previously [9]. 10^6 cells were added to 1 cover-slip-tube in 2 ml of Medium No 199 (0.125 per cent $NaHCO_3$) with 10 per cent bovine serum.

Slightly confluent growth was formed 2 days after plating, in both tissue cultures inoculated with HSV.

Virus strain was the same as used previously [8].

Inoculation. Tissue cultures were washed 3 times with pH 7.2 phosphate buffer solution. HSV suspension was added to get a multiplicity of 4.5 TCD_{50} per cell. Inoculated tissue cultures were incubated at 37°C for 30 minutes, then the inoculum was poured off to remove unadsorbed viruses and the cell sheets were washed 3 times with Basal Medium Eagle (BME) (0.125 per cent $NaHCO_3$) without serum or phenol red. 2.5 ml of BS-C-1 maintenance medium (Medium No 199 with 2 per cent bovine serum, and 0.25 per cent $NaHCO_3$) containing 10 μg per ml of cytosine arabinoside was added per bottle. Fixation and staining of the cover slips was done 3, 5 1/2, 6, 8, 10, 15, 21 and 24 hours after inoculation.

Fixation and staining of cover slips. Cover slips were washed twice in PBS pH 7.4 and then once in distilled water, subsequently dried in air for 30 minutes, fixed in acetone for 3

minutes, again dried in air for 30 minutes and stored at +4°C in rubber-stoppered centrifuge tubes.

HSV-rabbit immune sera No. 4 and No. 6 were the same as used previously for the CF test [8]. R No. 4 had an anti-early antibody titre of 1 : 8, and R No. 6 a titre of less than 1 : 4. Both sera had an antiviral CF antibody titre of 1 : 16 [32].

Fluorescein-conjugated anti-rabbit goat globulin was supplied by the Serum and Vaccine Institute, Praha, Czechoslovakia.

Immune rabbit sera and conjugated anti-rabbit globulin were adsorbed with 10^7 tissue culture cells. The cell suspension was added to the serum, frozen and thawed twice at −76°C, left 1 hour at room temperature and overnight at +4°C under repeated shaking, then centrifuged at 10,000 r. p. m. for 1 hour, and stored at +4°C until use.

Staining. Cover slips were covered with immune sera in a moist chamber for 30 minutes, washed in PBS pH 7.4 for 1 hour, changing the PBS 3 times, then covered with fluorescein-conjugated anti-rabbit globulin for 30 minutes, washed with PBS pH 7.4 for 1 hour changing the PBS 3 times and mounted in 9 parts glycerol and 1 part of PBS pH 7.4.

Results

Selection of HSV-immune rabbit sera for the detection of "early" antigens in tissue culture cells. Of 8 HSV immune rabbit sera 3 reacted with both "early" and "viral" antigens, while 5 did not react with the "early" component at a dilution of 1 : 4 or higher in CF test. The use of two HSV-immune rabbit sera, — one reacting with both kinds of antigen, and one only with the viral one — allowed the detection of "early" antigen [8]. The reactivity to "early" and "viral" antigens of the HSV-immune rabbit sera selected for the immunofluorescent test is shown in Table I.

Table I

Anti-"early" and anti-"viral" CF antibody titre of rabbit sera R No. 4 and R No. 6 used for the detection by the immunofluorescent technique of "early" antigens in cytosine arabinoside treated tissue culture cells

Serum	CF antibody titre against antigen	
	HSV/Early 1 u.	HSV/Viral 1 u.
R No. 4	8(16)*	16(32)
R No. 6	4	16(32)

* + + + + fixation in dilution of 1 : 8 and + + fixation in dilution of 1 : 16 of the serum.

Development of "early" antigen in HSV-infected BS-C-1 and chicken embryonic fibroblast tissue culture cells detected by indirect immunofluorescence. As described in "Materials and methods", we have added 10 μg/ml of cytosine arabinoside to the medium of the HSV inoculated coverslip cultures just after the adsorption period, to inhibit the formation of viral antigens and infective

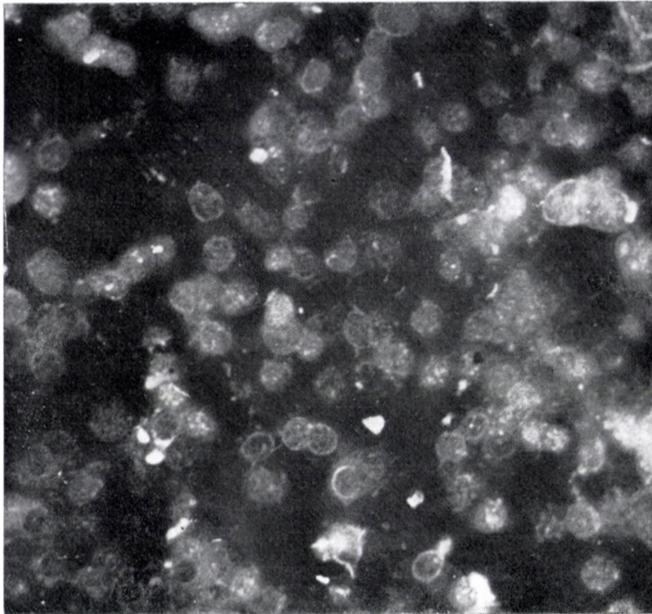

Fig. 1. Distribution of HSV- "early" antigens in BS-C-1 tissue culture cells 15 hours after inoculation. The antigen was synthetized in the presence of 10 μg/ml of cytosine arabinoside and stained with R No. 4. HSV-immune rabbit serum. Magnification, × 160

viral particles. Table II and Fig. 1 show the development of "early" antigen in HSV-infected BS-C-1 tissue culture cells, as tested with R No. 4 serum, as compared to the appearance of viral components in untreated controls determined with R No. 6 serum.

Table II

Synthesis of "early" antigen in Herpes simplex virus infected BS-C-1 continuous tissue culture cells in the presence of 10 μg/ml of cytosine arabinoside, and development of "viral" antigens in untreated controls

Hours after inoculation	Inoculum TCD_{50}/cell	Immunofluorescence* of		
		cytosine arabinoside treated cells stained with serum		untreated cells stained with serum R No. 6
		R No. 4	R No. 6	
3	4.5	0	0	0
5 1/2	4.5	10	0	5
15	4.5	100	0	50
21	4.5	100	5	100

*Numbers represent approximate percentage of cells containing respective antigens.

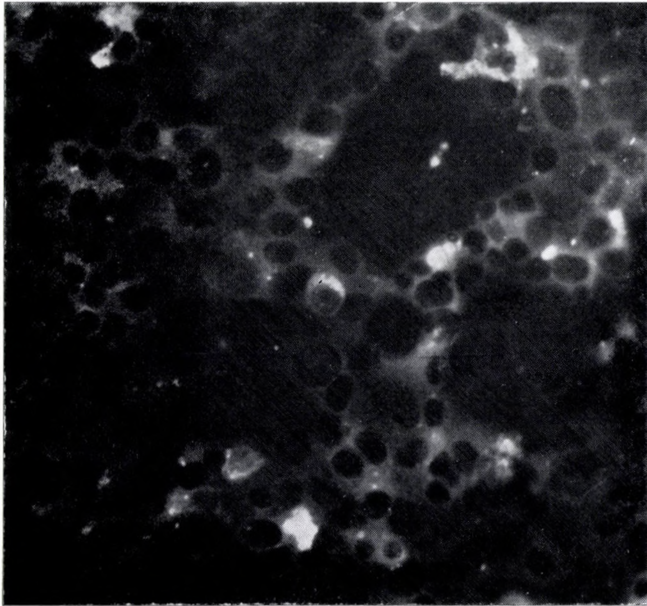

Fig. 2. Same culture as in Fig. 1, allowed to react with viral-antigen reactive R No. 6 HSV immune rabbit serum, followed by fluorescein labelled goat anti-rabbit serum. Magnification, ×160

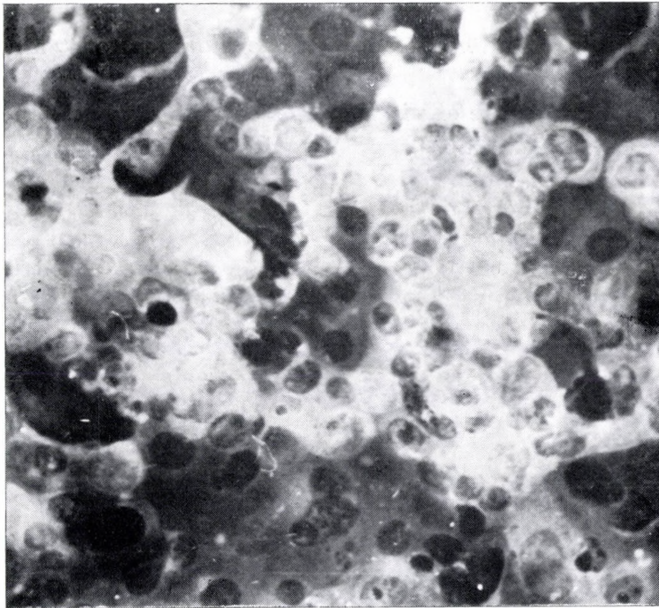

Fig. 3. Distribution of HSV- "viral" antigens in BS-C-1 tissue culture cells 15 hours after inoculation, incubated without cytosine arabinoside, stained with R No. 6 HSV-immune rabbit serum. Magnification, ×180

The "early" antigen was first detectable 5 1/2 hours after inoculation in about 10 per cent of the BS-C-1 cells. The antigen was concentrated at the periphery of the nucleus. At 15 hours after inoculation all the cells contained it.

The 15 hour preparation stained with R No. 6 serum showed no fluorescence (Fig 2).

21 hours after infection about 5 per cent of the nuclei displayed a slight granular fluorescence when stained with R No. 6 serum.

In preparations propagated without cytosine arabinoside, the first fluorescing intranuclear granules appeared 5 1/2 hours after inoculation in about 5 per cent of the cells. 15 hours after inoculation about 80 per cent of the cells showed inclusion-like intranuclear and homogeneous cytoplasmic fluorescence (Fig. 3).

In chicken embryonic fibroblast cells, in accordance with the slower multiplication of the HSV in these cells as compared to growth in BS-C-1 cells, the "early" antigen appeared 8 hours after inoculation in about 5 per cent of the nuclei of the cells treated with cytosine arabinoside. The ratio of positive nuclei increased to 80 per cent by 10 hours, and to 100 per cent by 24 hours.

In preparations fixed 10, 15 and 24 hours after inoculation, 5, 10, 15 per cent, respectively, of the nuclei showed fluorescence when stained with R No. 6 serum.

The first viral antigens in untreated control cells were visible 10 hours after infection (Table III).

Uninoculated tissue cultures stained with the immune sera, and inoculated cultures stained with normal rabbit serum showed no fluorescence, thus proving the specificity of the observed reactions.

Table III

Synthesis of "early" antigen in Herpes simplex virus infected chicken embryonic fibroblast tissue culture cells in the presence of 10 μg/ml of cytosine arabinoside, and development of "viral" antigens in untreated controls

Hours after inoculation	Inoculum TCD_{50}/cell	Immunofluorescence* of: cytosine arabinoside treated cells stained with serum R No. 4	Immunofluorescence* of: cytosine arabinoside treated cells stained with serum R No. 6	Immunofluorescence* of: untreated cells stained with serum R No. 6
6	4.5	0	0	0
8	4.5	5	0	0
10	4.5	80	5	5
15	4.5	90	10	100
24	4.5	100	25	100

*Numbers represent approximate percentage of cells containing respective antigens.

Discussion

As it has been shown previously [8] the demonstration of "early" antigens in lytic infections caused by human DNA-viruses, may help to clear the role of these viruses in human carcinogenesis. We have succeeded in detecting HSV- "early" antigens in the extracts of virus infected normal tissue culture cells by the complement fixation reaction, in this study we have tried to localize it in the cells by the indirect immunofluorescence technique.

RAPP's finding [11, 12, 13,] that cytosine arabinoside does not inhibit the formation of the "early" antigen of SV-40 virus, but inhibits the production of viral antigenic components in cells infected with SV-40, HSV and Herpes zoster viruses, has made us to use HSV-immune rabbit serum for detection of the antigen reacting with both viral and "early" antigens.

Localization of the "tumour" antigens is different. In the case of SV-40 tumour cells and normal tissue culture cells infected with SV-40 virus it is distributed diffusely throughout the nucleus [4, 12, 14] while the adeno-12 tumour antigen is to be found chiefly in the cytoplasm [7].

Our finding is partly in agreement with that of SHEDDEN, WATSON and WILDY [15]. We found the antigens to concentrate mostly near the nuclear membrane and less throughout the other parts of the nucleus. The mentioned authors found fluorescent granules in the cytoplasm, in connection with the perimembranal mass. They were using specific immune sera prepared against the "early" antigen, while we made our tests with HSV immune sera derived from rabbits immunized by corneal scarification.

It is hard to explain why the viral-antigen-reactive R No. 6 serum showed fluorescence in some of the HSV-infected cells only, at 10, 21 and 24 hours after inoculation in spite of the presence of cytosine arabinoside. This was probably due to the low amount of viral antigen synthetized even in the presence of cytosine arabinoside [13, 14].

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ТОМ XIII

РЕЗЮМЕ

DIE STRAHLENEMPFINDLICHKEIT VON ESCHERICHIA COLI B-KULTUREN II. DIE WIRKUNG DER BEHANDLUNG VOR, WÄHREND UND NACH DER BESTRAHLUNG AUF DIE STRAHLENEMPFINDLICHKEIT DER ZELLEN

F. Hernádi, Zs. Nagy, P. Kovács, O. Mucsi

ЧУВСТВИТЕЛЬНОСТЬ К ИРРАДИАЦИИ КУЛЬТУР ESCHERICHIA COLI B. II. ВОЗДЕЙСТВИЕ ОБРАБОТКИ НА ЧУВСТВИТЕЛЬНОСТЬ КЛЕТОК К ИРРАДИАЦИИ ДО, ВО ВРЕМЯ И ПОСЛЕ ОБЛУЧЕНИЯ

Ф. ХЕРНАДИ, Ж. НАДЬ, П. КОВАЧ, О. МУЧИ

Исследовалась чувствительность к Rtg. и ^{60}Co лучам культур *E. coli B* в условиях культивирования до и после облучения, а также в зависимости от температуры и аноксии во время облучения.

Перед облучением чувствительность культур *E. coli*, выращенных на минимальной питательной среде, была выше, чем чувствительность 14-и часовых культур, полученных в «цельной» питательной среде (в бульоне).

Переживание клеточных суспензий после облучения усиливалось выращиванием на минимальной среде. При этом благоприятный эффект умеривался при объединении синтетической питательной среды с органическими составными частями (дрожжи, пептон, костный мозг). В то же время более неблагоприятное воздействие «цельной» питательной среды, развитое в ответ на переживание, можно было улучшить ингибиторами обмена веществ (аноксия, предварительная инкубация с хлорфениколом).

Повышение температуры во время облучения при работе с низкой дозой (260 r/min) обусловливало усиление чувствительности к иррадиации, тогда как этого не удалось наблюдать при работе с высокой дозой (3200 r/min).

Аноксия во время иррадиации, вызванная атмосфером азота, уменьшила чувствительность клеточной суспензии к иррадиации приблизительно на 2,4 DRF.

SENSITIVITY OF E. COLI B TO IRRADIATION IV. DEPENDENCE OF THE PROTECTIVE EFFECT OF CYSTEINE-CYSTEAMINE-TYPE COMPOUNDS ON PREIRRADIATION OXYGENATION AND ON PRE- AND POSTIRRADIATION CONDITIONS OF CULTURING

F. Hernádi, Zs. Nagy, P. Kovács, T. Vályi-Nagy

ЧУВСТВИТЕЛЬНОСТЬ К ИРРАДИАЦИИ КУЛЬТУР ESCHERICHIA COLI B IV. ЗАВИСИМОСТЬ ЛУЧЕЗАЩИТНОГО ЭФФЕКТА СОЕДИНЕНИЙ ТИПА ЦИСТЕИН-ЦИСТЕАМИН ОТ ОБРАБОТКИ КИСЛОРОДОМ ДО ОБЛУЧЕНИЯ, А ТАКЖЕ ОТ ПРЕ- И ПОСТИРРАДИАЦИОННЫХ УСЛОВИЙ КУЛЬТИВИРОВАНИЯ

Ф. ХЕРНАДИ, Ж. НАДЬ, П. КОВАЧ, Т. ВАЛИ-НАДЬ

Изучалось взаимодействие факторов, изменяющих Rtg-чувствительность клеточной суспензии *E. coli* B.

Аноксия снижала или прекращала (по сравнению с контролем с аноксией) лучезащитный эффект группы цистеин-цистеамин, если оставлялись «период реакции» и «поздний период».

В случае применения «периода оптимальной реакции и позднего периода» оптимальные пре- и пострирадиационные условия инкубирования не усиливали защитный эффект упомянутых выше соединений.

Из наших экспериментов вытекает, что лучезащитное действие соединений группы цистеин-цистеамин во время облучения происходит на физико-химическом уровне, а до и после облучения — на метаболическом.

SENSITIVITY OF E. COLI B TO IRRADIATION VI. EFFECT OF CYSTEINE ON DNA BREAKDOWN BY IONIZING IRRADIATION

Zs. Nagy, F. Hernádi, P. Kovács

ЧУВСТВИТЕЛЬНОСТЬ К ИРРАДИАЦИИ КУЛЬТУР E. COLI B VI. ДЕЙСТВИЕ ЦИСТЕИНА НА РАСПАД ДНК, ИНДУЦИРОВАННЫЙ ИОНИЗИРУЮЩИМ ИЗЛУЧЕНИЕМ

Ж. НАДЬ, Ф. ХЕРНАДИ, П. КОВАЧ

Изучалось действие 0,1 М — 0,01 М цистеина на индуцированный излучением распад ДНК в культуре *E. coli B* и на формирование числа переживающих клеток.

0,1 *M* — 0,01 *M* цистеин подавлял распад ДНК, в то время как в концентрации 0,001 *M* он не оказывал эффекта.

Прямую связь между содержанием ДНК и переживанием клеток выявить не удалось.

BREAKDOWN OF AMINO ACIDS BY ENTEROBACTERIACEAE V. BREAKDOWN OF GLYCINE IN SIMPLE MEDIUM

B. Serény

РАСЩЕПЛЕНИЕ АМИНОКИСЛОТ ЭНТЕРОБАКТЕРИЯМИ V. РАСЩЕПЛЕНИЕ В ПРОСТОМ РАСТВОРЕ ГЛИЦИНА

Б. ШЕРЕНЬ

Изложенная ранее автором реакция глицин-распада может быть проведена в простом растворе, содержащем кроме глицина только фосфат. В этом растворе кишечные бактерии являются глицин-положительными в большем проценте, чем в комплексной среде.

Процесс представляется надежным для дифференцировки бактерий кишечного тракта, так как в макротесте подщелачивания негативна только часть штаммов ***S. paratyphi A***, ***Shigella*** и ***Proteus—Providencia***, а в ninhydrin-пробе — ***Shigellae.*** В микротесте подщелачивания штаммы B ***S. paratyphi*** A, ***Shigella***, ***Morganella*** и ***Providencia*** не дают раннего положительного результата.

STABILITY AND PURIFICATION OF ANTIPHOSPHORYLASE

I. Jókay, M. Szabolcs

СТАБИЛИЗАЦИЯ И ОЧИСТКА АНТИФОСФОРИЛАЗЫ

И. ЙОКАИ, М. САБОЛЧ

Изучалась стабильность антител, полученных в петухе против фосфорилазы б при разных (кислых и щелочных) pH и температурах. На основе полученных данных разработана очистка антифосфорилазы из специфического преципитата. После растворения преципитата в глицин-сахарозном буфере с pH 11 и температурной обработки при 56° С можно получить обратно 60—75% антител.

REDUCTION OF HEAT AND RADIATION RESISTANCE OF BACILLUS CEREUS SPORES BY INITIATING GERMINATION

J. Farkas, I. Kiss, E. Andrássy

СНИЖЕНИЕ ТЕМПЕРАТУРНОЙ И РАДИАЦИОННОЙ РЕЗИСТЕНТНОСТИ СПОР BACILLUS CEREUS ПОСРЕДСТВОМ ПРОРАСТАНИЯ

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Активированные температурой при 60° С в течение 30 минут споры выделенного из зеленого горошка штамма *B. cereus* проращивались — при 30° С в течение 50 минут — в 90%-ной пропорции в водном растворе, содержащем только 0,4 мг/мл аденозина и 0,4 мг/мл L-аланина, или в фосфатном буфере. Активирующий эффект 60° С-ной температуры появлялся спустя 4 минуты от начала обработки температурой и возрастал пропорционально с увеличением времени воздействия.

Стимулированные к прорастанию споры вместе с понижением их светопреломляющей способности теряли не только температурную, но и радиационную резистентность.

Добавление аденозина и L-аланина повышало скорость и пропорцию прорастания спор, привитых в экстракт горошка или в смесь зернистого зеленого горошка и сахарно-солевого раствора. Действие добавленных веществ, стимулирующих прорастание, проявлялось уже через несколько минут инкубации при 30° С в понижении температурной и радиационной резистентности популяции, что согласовывалось с результатами исследования фазово-констрастным микроскопом распределения состояния клеток.

При комбинированном применении температурной активации и добавления веществ, стимулирующих прорастание спор, даже в случае значительной, 10^8—10^9/мм, клеточной плотности можно было вызвать прорастание и этим повысить их чувствительность к последующим стерилизующим воздействиям, даже в той среде, в которой споры образовывались.

INTESTINAL MICROFLORA OF THE LARVAE OF ST. MARK'S FLY

I. A COMPARATIVE STUDY OF STREPTOMYCES STRAINS BELONGING TO THE GRISEUS GROUP ISOLATED FROM THE INTESTINAL CANAL

I. Szabó, M. Marton, I. Buti, G. Pártai

МИКРОФЛОРА ПИЩЕВАРИТЕЛЬНОГО ТРАКТА ЛИЧИНКИ МАРТОВСКОЙ БАРХАТНОЙ МУХИ

I. ШТАММЫ, ИЗОЛИРОВАННЫЕ ИЗ ПИЩЕВАРИТЕЛЬНОГО ТРАКТА И ОТНОСЯЩИЕСЯ К ГРУППЕ STREPTOMYCES GRISEUS

И. САБО, М. МАРТОН, И. БУТИ, Г. ПАРТАИ

Штаммы, выделенные из кишечного тракта и экскрементов трех личиночных популяций мартовской бархатной мухи *(Bibio marci* L.*)* и относящиеся к группе *Streptomyces griseus*, были идентифицированы как *Act. vulgaris Nikitina et al.* 1960 и *Act. citreofluorescens Koreniako et al.* 1960. Те «griseus-штаммы», которые были выделены из почвенного слоя АН из-под популяций *Bibio*, оказались тождественными с видами *Act. levoris Koreniako et al.* 1960 и частично *Str. michiganensis Corbaz et al.* 1957. В связи с этой работой был «сконструирован» ключ для определения видов и разновидностей, принадлежащих к группе *Streptomyces griseus.*

INCIDENCE OF RHODOTORULA SPECIES IN URBAN AIR

Gy. Vörös-Felkai

ВСТРЕЧАЕМОСТЬ ВИДОВ RHODOTORULA В ГОРОДСКОМ ВОЗДУХЕ

ДЬ. ВЁРЁШ-ФЕЛКАИ

В рамках исследовательской темы по загрязнению городского воздуха была изучена дрожжевая флора воздуха. Исследования, продолжающиеся и в настоящее время, показывают, что чаще всего из городского воздуха можно выделить виды *Rhodotorula* и *Cryptococcus*.

Во встречаемости выделенных 100 штаммов *Rhodotorula*, представляющих 12 видов, сезонного различия не наблюдалось. Среди выделенных штаммов 34% принадлежало к *Rhodotorula glutinis*, 26% — *Rhodotorula mucilaginosa*, 16% — *Rhodotorula rubra* и 11% — *Rhodotorula minuta*.

Эти данные обращают внимание на то, что в случае поиска источника заражения человеческими микозами нельзя оставлять без внимания и микрофлору воздуха.

ORGANIC AND AMINO ACID ASSIMILATION BY YEASTS AS STUDIED BY THE REPLICA PLATING TECHNIQUE

Gy. Vörös-Felkai, E. K. Novák

ОРИЕНТИРОВОЧНОЕ ИССЛЕДОВАНИЕ АССИМИЛЯЦИИ ОРГАНИЧЕСКИХ КИСЛОТ И АМИНОКИСЛОТ ДРОЖЖЕВЫМИ ГРИБАМИ МЕТОДОМ «REPLICA PLATING»

ДЬ. ВЁРЁШ-ФЕЛКАИ, Е. К. НОВАК

Методом «replica plating» у каждого штамма из 25 видов дрожжевых грибов были исследованы ассимиляция и утилизация 11 аминокислот как угольных и нитрогенных источников в одно и то же время. Результаты оценивались с таксономической и физиологической точек зрения. Было установлено, что метод «replica plating» вследствие малой потребности в нитрогене, проявляющейся у большинства видов, непригоден для определения нитрогенного источника.

INTRAVASCULAR PRECIPITATE FORMATION DURING ANAPHYLACTIC SHOCK IN THE GUINEA PIG

T. Szilágyi, L. Miltényi, G. Lévai, K. Benkő

ОБРАЗОВАНИЕ ИНТРАВАСКУЛЯРНОГО ПРЕЦИПИТАТА В МОРСКОЙ СВИНКЕ В ТЕЧЕНИЕ АНАФИЛАКТИЧЕСКОГО ШОКА

Т. СИЛАДЬИ, Л. МИЛТЕНЬИ, Г. ЛЕВАИ, К. БЕНКЁ

На морских свинках, активно и пассивно сенсибилизированных к ферритину, а в дальнейшем гипериммунизированных им, вызывали анафилактический шок путём введения антигена в v. jugularis. С помощью исследований, проведенных световым и электронным микроскопом, было установлено, что в легочных капиллярах возникает преципитат, который соответствует ферритин-антиферритин комплексу. У животных, подвергнутых гипотермии, шок не проявлялся, и образование преципитата происходило в пониженной степени. Хотя анафилактический шок полностью предотвращался хлорпромазином, всё-таки и в этом случае в легочных капиллярах животного образование преципитата, характерного ферритин-антиферритин комплексу, было значительное. В мелких легочных сосудах морских свинок с большим титром антител интраваскулярный преципитат появлялась в меньшем количестве.

На основе наших экспериментов установили, что при анафилактическом шоке морской свинки, как результат реакции антиген-антитело, возникает интраваскулярный преципитат, но его патогенетическая роль незначительна.

LIPID COMPOSITION OF TREPONEMAL STRAINS

L. Váczi, K. Király, A. Réthy

ЛИПИДНЫЙ СОСТАВ ТРЕПОНЕМНЫХ ШТАММОВ

Л. ВАЦИ, К. КИРАЙ, А. РЕТИ

Посредством газовой и тонкослоевой хроматографии был проанализирован состав жирных кислот и фосфолипидов патогенного *T. pallidium* (штамм Будапешт) и различных культивируемых штаммов трепонем *T. reiteri*, *Kazani 5*, *minutum* и *refrigentis)*. Спектры жирных кислот были простыми и почти одинаковыми у всех штаммов. Они содержали, главным образом, пальмитиновую и ненасыщенные (олеивую и оленииновую) жирные кислоты, последние в относительно высокой пропорции. Жирные кислоты, содержащие циклопропан-кольцо, не были обнаружены. Фосфолипидный состав был довольно сложный. В отношении их ненасыщенной жирной кислоты трепанемы и стрептококки оказались подобными. Данные указывают на повышенную проницаемость их клеточной мембраны и в результате этого — более высокую уязвимость при воздействии окружающей среды.

Трепонемы в сравнении с другими бактериями относительно богаты липидами. Наши знания в отношении их химического состава, структуральной и биологической роли недостаточны. В настоящей статье сравнивается жирно-кислотный и фосфолипидный состав патогенных и различных культивируемых трепонем.

STUDIES ON THE INITIAL PHASES OF POLIOVIRUS REPRODUCTION CYCLE

A. Koch, E. György

ИЗУЧЕНИЕ НАЧАЛЬНЫХ СТАДИЙ РЕПРОДУКЦИИ ПОЛИОВИРУСА

А. КОХ, Э. ДЁРДЬ

Были использованы полиовирус 1 типа (Штамм Mahoney) и стабильная клеточная линия почки обезьяны (РМК III/1). Эта система давала полный выход вируса в среде Паркера № 199 независимо от того, добавлялся телячий альбумин или нет. Для получения полного выхода вируса в буферном солевом растворе Хенкса наличие телячьего альбумина было необходимо, и его отсутствие исчислялось 90% (или даже больше) редукции количества продуцируемого вируса.

Присутствие телячьего альбумина в буферном солевом растворе Хенкса требовалось только в течение первых 30 минут цикла, в то время как его добавление между 30-ой и 60-ой минутами обуславливает редукцию выхода вируса приблизительно в 50—80%. Телячий альбумин, добавленный на втором часу цикла или позже, уже не делал выход вируса выше, чем это наблюдалось в случае одного только буферного солевого раствора Хенкса.

Отсутствие или запаздывающее прибавление (2 часа или позже) телячьего альбумина вызывало задержку начала цикла в буферном солевом растворе Хенкса на 30—40 мин.

Репликационное соотношение было во всех случаях практически одинаково, время удвоения равнялось приблизительно 10 минутам.

Телячий альбумин представляется фактором, вовлеченным в начальные механизмы вирусной инфекции (внедрение или эклипс). Его вероятная роль в «виртуальном эклипсе» адсорбированных вирионов дискутируется.

Чтобы тесно приблизиться к возможному механизму действия телячьего альбумина, было предпринято изучение эффекта цистеина, глютатиона и К-линолеата на начальную фазу вирусного цикла.

THE SIGNIFICANCE OF SEROLOGICAL TESTS IN CONTROLLING THE SUCCESS OF SMALLPOX REVACCINATION

G. Nyerges, I. Hollós, G. Barsy

ЗНАЧЕНИЕ СЕРОЛОГИЧЕСКИХ ИССЛЕДОВАНИЙ В ОЦЕНКЕ ЭФФЕКТИВНОСТИ РЕВАКЦИНАЦИИ ПРОТИВ ОСПЫ

Г. НЬЕРГЕШ, И. ХОЛЛОШ, Г. БАРШИ

Была проведена ревакцинация оспенной вакциной взрослых людей, которые в прошлом получили прививку в период новорожденности и в 6—10-летнем возрасте. С помощью статистических методов искали взаимосвязь между следующими 4 факторами: (1) результатом прививок, (2) возрастом привитых, (3) титром нейтрализующих и ГА подавляющих антител до ревакцинации, (4) повышением титра нейтрализующих и ГА подавляющих антител после ревакцинации.

Между титром нейтрализующих антител до ревакцинации и результатом прививки обнаружить связь не удалось. Не было также статистической взаимосвязи и между титром РПГА до ревакцинации и результатом прививки. Единственным фактором, стоящим в оцениваемой связи с результатом прививки, является возраст привитых, то есть период, прошедший со времени последней оспенной прививки в детстве.

Перед успешной (сопровождающейся образованием пустул) ревакцинацией корреляция между титрами нейтрализующих и ГА подавляющих антител отсутствовала. Перед неэффективными (не сопровождающимися образованием пустул) прививками была показана позитивная взаимосвязь между двумя видами антител.

После эффективной ревакцинации титры нейтрализующих антител значительно повысились, степень повышения не зависела от титра до ревакцинации. После эффективной прививки между титрами ГА подавляющих и нейтрализующих антител выявилась отчётливая связь. Титры в нейтрализации 1:128 и в РПГА 1:32 с большой вероятностью указывают на эффективную ревакцинацию. После безуспешной ревакцинации титры нейтрализующих антител значительно возрасли, но степень повышения была меньше, чем это наблюдалось в случае эффективной прививки. В противоположность эффективной прививке повышение титра нейтрализующих антител, следующее за безуспешной прививкой, находилось в резкой негативной корреляции с титрами нейтрализующих антител до ревакцинации. После неэффективной ревакцинации исчезла выявляемая до прививки связь между титрами ГА подавляющих и нейтрализующих антител сыворотки. Причина исчезновения взаимосвязи заключается в том, что после безуспешной прививки титры ГА подавляющих антител или совсем не повышались , или в порядке исключения повышались в незначительной степени. Из последнего факта следует, что изменение ГА подавляющих титров более достоверно отражает результат прививки, чем титры нейтрализующих антител.

STUDY ON THE EFFECT OF FLAVONOIDS AND RELATED SUBSTANCES

I. THE EFFECT OF QUERCETIN ON DIFFERENT VIRUSES

R. Pusztai, I. Béládi, M. Bakai, I. Mucsi, E. Kukán

ИЗУЧЕНИЕ ДЕЙСТВИЯ ФЛАВОНОИДОВ И РОДСТВЕННЫХ ВЕЩЕСТВ

I. ДЕЙСТВИЕ КВЕРЦЕТИНА НА РАЗЛИЧНЫЕ ВИРУСЫ

Р. ПУСТАИ, И. БЕЛАДИ, М. БАКАИ, И. МУЧИ, Е. КУКАН

Изучалось воздействие кверцетина на различные вирусы. ***Herpesvirus hominis***, ***Herpesvirus suis***, вирус парагриппа 3 типа и вирус Sindbis оказались чувствительными, полиовирус 1 типа — умеренно чувствительным, а полиовирус 2 и 3 типов и аденовирусы 3 и 4 типов — резистентными к воздействию кверцетина. Кверцетин проявил себя как вируцидное средство, так как оказывал действие только на внеклеточный вирус. Было найдено, что действие морина на ***Herpesvirus suis*** подобно таковому кверцетина, а рутин, можно сказать, оказался неэффективным.

AGE-INCIDENCE OF HAEMAGGLUTINATION-INHIBITING ANTIBODIES TO REOVIRUS TYPES 1, 2 AND 3

M. Tóth, A. Honty

ЧАСТОТА ВСТРЕЧАЕМОСТИ ГА ПОДАВЛЯЮЩИХ АНТИТЕЛ К РЕОВИРУСАМ 1,2 И 3 ТИПОВ В РАЗНЫХ ВОЗРАСТНЫХ ГРУППАХ

М. ТОТ, А. ХОНТИ

С целью выяснения частоты встречаемости реовирусов в Венгрии были исследованы 1982 сывороточных пробы от людей различного возраста в РПГА против всех трех серотипов. Встречаемость антител к 1 и 2 типам оказалась почти одинаковой — 73% и 74%, в 81% случаев в сыворотке содержались ГА подавляющие антитела к 3 типу. Пропорция серопозитивных лиц в отношении 1 и 2 типов постепенно увеличивалась до 40-летнего возраста, в то время как в случае 3 типа наиболее высокие пропорции наблюдались у 3—5-летних детей.

В 273 случаях острых заболеваний верхних дыхательных путей и 114 случаях гепатита было предпринято исследование частоты повышения титров антител к трем вирусным типам. Между двумя группами больных существенной разницы с этой точки зрения обнаружено не было.

Для определения присутствия антител в сыворотках РПГА и нейтрализационная проба оказались одинаково приемлемыми. Была обнаружена заслуживающая внимания связь между ГА подавляющими и нейтрализующими титрами антител сывороток.

SIMULTANEOUS DETERMINATION OF ERYTHRO- AND LEUKOCYTOTROPINES IN VIVO

Gy. Vajda, J. Tóth, V. Tax

ОДНОВРЕМЕННОЕ ВЫЯВЛЕНИЕ ЭРИТРОЦИТО- И ЛЕЙКОЦИТОТРОПИНОВ IN VIVO

ДЬ. ВАЙДА, Й. ТОТ, В. ТАКС

Авторы выработали новый метод одновременного определения эритроцито-лейкоцито-тропинов *in vivo*. Сущность процесса состоит в том, что к 0,5 мл разведений исследуемой сыворотки прибавляют 0,25 мл 10%-ной суспензии трижды отмытых эритроцитов идентичной группы и 0,25 мл суспензии лейкоцитов (20 000/мм³). Каждой смесью прививают трёх мышей внутриперитонеально, затем в мазках, приготовленных из брюшнополостного эксудата, определяют %-ное распределение фагоцитоза эритроцитов и лейкоцитов. Положительным считался фагоцитоз выше 3%. Из обследованных 25 иммунгематологических диагнозов эритрофагоцитоз встречался в 14 случаях, лейкоцитофагоцитоз — в 9 и одновременно эритролейкоцито-фагоцитоз — в 9. В то же время в опытах, проведенных *in vitro*, эритроцитные антитела в 12, лейкоцитные антитела в 5, эритроцитные и лейкоцитные антитела вместе в 4 случаях были положительными. 20 контрольных исследований дали отрицательный результат. Процесс одинаково успешно применим для выявления гетероцитотропинов, комплетных и инкомплетных аутоантител. Реакция цитотропина может быть проведена по прямому и непрямому методу.

EFFECT ON INFLUENZA VIRUS OF A MODIFIED FRANCIS INHIBITOR AND ITS ACETONE-SOLUBLE FRACTION

I. STUDIES IN ROLLER DRUM, AND IN DE-EMBRYONATED EGGS

I. Hollós

ВЛИЯНИЕ ВИДОИЗМЕНЕННОГО ФРАНСИС-ИНГИБИТОРА И ЕГО АЦЕТОН-РАСТВОРИМОЙ ФРАКЦИИ НА ВИРУС ГРИППА

I. ИССЛЕДОВАНИЯ МЕТОДОМ ВРАЩАЮЩЕГОСЯ БАРАБАНА И В ДЕЭМБРИОНИРОВАННОМ ЯЙЦЕ

И. ХОЛЛОШ

Автор провел сравнение свойств Франсис-ингибитора *(K_0)*, видоизмененного Франсис-ингибитора *(8m)* и оставшихся в растворе фракций *(K_0ac и 8ac)* после ацетонового фракционирования.

2%-ный раствор *8 ac* оказывал **in vitro** значительное вируцидное действие, в то время как остальные три вещества были лишены этого влияния.

Методом вращающегося барабана было определено воздействие различных концентраций отдельных веществ на размножение вируса гриппа. О последнем автор судил на основе гемагглютинационного титра. При статистической оценке результатов автор установил, что в случае использования средних концентраций вещества между дозой и действием имеется прямопропорциональная зависимость.

Среди испытуемых веществ наиболее активным оказался *8ac*. Для 50%-ного подавления вирусного размножения в отношении одной и той же дозы вируса требовалось в 39 раз меньшее количество *8ac* по сравнению с K_0. Последовательность отдельных веществ по их эффективности против одного и того же вирусного штамма была следующей: *8ac, 8m, K_0ac, K_0*. Сила воздействия разных вирусных штаммов была различной.

Вирусподавляющее действие идентичного количества *8ac* было тем сильнее, чем раньше после заражения проводилось его прибавление к системе и чем меньше была доза вируса при прививке.

8ac на образование гемагглютинина вируса вакцины подавляющего эффекта не оказывал.

STUDIES ON THE INFLUENCE OF ENDOGENOUS REGULATORY FACTORS ON THE GROWTH OF HERPES SIMPLEX VIRUS

Gy. Hadházy, F. Lehel, L. Gergely

ИЗУЧЕНИЕ ВЛИЯНИЯ ФАКТОРОВ ЭНДОГЕННОЙ РЕГУЛЯЦИИ НА РАЗМНОЖЕНИЕ ВИРУСА HERPES SIMPLEX

ДЬ. ХАДХАЗИ, Ф. ЛЕХЕЛ, Л. ГЕРГЕЙ

Несколько биогенных аминов и гормонов (гистамин, адреналин, серотонин, гидрокортизон, АКТГ) усиливали подавляющий эффект гепарина на размножение вируса ***Herpes simplex*** во вторичной культуре фибробластов эмбриона человека и в клеточной культуре Hela. Гепарин оказывал подавляющее действие на вирус герпеса и при подкожном заражении кроликов. Подавление проявлялось в уменьшении величины поражения кожи и в снижении степени местного размножения вируса.

TYPE DISTRIBUTION OF STREPTOCOCCUS PYOGENES STRAINS IN THE YEARS 1964—1965. ACTIVITIES WITHIN AN INTERNATIONAL SURVEY OF THE DEPARTMENT OF BACTERIOLOGY, NATIONAL INSTITUTE OF PUBLIC HEALTH, BUDAPEST

J. Szita, G. Hegyessy

РАСПРЕДЕЛЕНИЕ ПО ТИПАМ ШТАММОВ STREPTOCOCCUS PYOGENES ЗА 1964—65 ГОДЫ. ДЕЯТЕЛЬНОСТЬ ОТДЕЛА БАКТЕРИОЛОГИИ ГОСУДАРСТВЕННОГО ИНСТИТУТА ЗДРАВООХРАНЕНИЯ В РАМКАХ МЕЖДУНАРОДНОГО СОТРУДНИЧЕСТВА

Й. СИТА, ДЬ. ХЕДЬЕШШИ

В рамках сотрудничества, организованного Международным Комитетом по стрептококкам и пневмококкам, с территорий, относящихся к 11 учреждениям страны, с 1 июня 1964 года по июнь 1965 года было собрано 395 штаммов *Str. pyogenes*. Штаммы относились к 26 различным типам. Всего 0,8% штаммов невозможно было определить ни агглютинацией, ни преципитацией. Доминирующие штаммы (встречающиеся с 5%-ной или выше частатой) представляли собой: 5,11,12....., 3,13, B_{3264} — комплексы и 3,12,1 и 6 — типы. Полученные результаты подкрепляют уже известное в литературе положение, что между заболеванием и вызывающим его типом *Streptococcus pyogenes* (за исключением нефрита) достоверной связи нет. С целью контроля 5% исследованных авторами штаммов были отосланы в Прагу. Те штаммы, в отношении которых при определении типа в двух лабораториях возникли расхождения, были отправлены для дальнейшего контроля в Генову. Все 395 штаммов оказались чувствительными к пенициллину, а 5,3% из них — резистентными к тетрациклину.

CHROMATOGRAPHY OF POLIOVIRUS STRAINS ISOLATED IN HUNGARY PRIOR TO AND AFTER THE INTRODUCTION OF LIVE POLIOVIRUS VACCINE

E. Szöllősy, Gy. Lengyel, É. Ágoston

ХРОМАТОГРАФИЧЕСКАЯ ХАРАКТЕРИСТИКА ШТАММОВ ПОЛИОВИРУСА, ВЫДЕЛЕННЫХ В ВЕНГРИИ ДО И ПОСЛЕ ВВЕДЕНИЯ МАССОВЫХ ВАКЦИНАЦИЙ ЖВС

Е. СЁЛЛЁШИ, ДЬ. ЛЕНДЬЕЛ, Е. АГОШТОН

Авторы характеризовали 16 штаммов полиовируса 3 типа на основе их адсорбционной способности на Al $(OH)_3$-геле и ДЕАЕ-целлулозе. Исследованные штаммы происходили частью из периода времени перед введением вакцинации живой вакциной против полиомиелита, частью—после введения этого мероприятия. Хроматографическая характеристика первых отличалась от таковой последних, так как их максимумы элюции на ДЕАЕ целлулозной колонке были выше (0,05 — 0,25 *M* NaCl), чем у штаммов, выделенных после введения вакцинации, максимумы элюции которых подобно аттенуированному стандартному штамму (Leon 12 a_1 в) находились в пределах 0,035—0,05 M. В Al $(OH)_3$ — элюционных опытах в обеих группах были получены идентичные результаты. Хроматографическое свойство штаммов авторы сравнили с их rct/40 маркерами.

BREAKDOWN OF AMINO ACIDS BY ENTEROBACTERIACEAE

VI. BETA-ALANINE MACRO- AND MICROTEST

B. Serény

РАСЩЕПЛЕНИЕ АМИНОКИСЛОТ У ЭНТЕРОБАКТЕРИЙ

VI. β-АЛАНИНОВЫЙ МАКРО- И МИКРОТЕСТ

Б. ШЕРЕНЬ

Среди бактерий, относящихся в семейство *Enterobacteriaceae*, реакцию подщелачивания в растворе β-аланина даёт часть культур ***Serratia***, ***Enterobacter***, ***Arizona*** и ***Klebsiella***. Реакция может быть использована для дифференцировки биотипов внутри их родов. Основой подщелачивания, по всей вероятности, является эндогенное окисление, а не распад β-аланина.

TYPING OF BRUCELLA WITH HUNGARIAN PHAGE STRAINS

A. A. El Naasan

ТИПИРОВАНИЕ БРУЦЕЛЛ ВЕНГЕРСКИМИ ФАГОВЫМИ ШТАММАМИ

А. А. ЕЛ НААСАН

В процессе экспериментов по типированию, проведенных с суспензиями в концентрациях **RTD (routine-test dilution)** и 10 000×**RTD** на 117 штаммах ***Br. abortus***, 44 — ***Br. suis***, 26 — ***Br. melitensis*** 6 фаговыми штаммами, выделенными в Венгрии из естественного материала, и 4 — полученными в Венгрии из культур бруцелл, на действие обеих концентраций растворилось 94,8% штамма ***Br. abortus***, 11,4% — ***Br. suis***, 19,3% — ***Br. melitensis***; и только на действие концентрации 10 000 × **RTD** растворились дальнейшие 2,6% ***abortus*** штаммов и 61,4% — ***suis*** штаммов. 2,6% ***abortus*** штаммов, 27,2% ***suis*** штаммов и 80,7% ***melitensis*** штаммов противостояли концентрированным фаговым суспензиям. Фаги, выделенные в Венгрии, располагают одинаковой с заграничными фагами способностью растворять. В Венгрии удалось выделить только *abortus*-фаг. На том основании, что виды животных, от которых происходят штаммы, известны, для дифференцировки 3 бруцеллавариантов был разработан соответствующий этой цели «ключ». Из-за промежуточных штаммов с точки зрения их фаговой чувствительности в отдельных случаях нельзя пренебрегать и конвенциональными методами типирования.

LYSOGENIC PROPERTIES OF VARIOUS STAPHYLOCOCCUS AUREUS PHAGE-TYPES

J. Lantos

РАЗЛИЧНЫЕ ПО ФАГОВЫМ ТИПАМ ШТАММЫ STAPHYLOCOCCUS AUREUS

Й. ЛАНТОШ

На основе многосторонних исследований была показана тесная связь между мультирезистентным к антибиотикам штаммом ***Staphylococcus aureus*** I группы по фаговым типам, выделенным во время эпидемии, протекавшей в одном из отделений больницы, и штаммом по фаговым типам «52 B», встречавшемся в том же отделении. Лизогенизацией, проведенной соответствующими фагами, штаммы удалось преобразовать по типу фага в другой тип.

В связи с эпидемиологическими исследованиями даже в случае выделения различных по типам фагов стафилококковых штаммов надо считаться с той возможностью, что штаммы происходят из одного и того же источника.

ACRIDINE ORANGE FLUORESCENCE OF TISSUE CULTURES INFECTED WITH AUJESZKY'S DISEASE VIRUS

L. Bodon, E. Gréczi

АКРИДИН-ОРАНЖЕВАЯ ФЛУОРЕСЦЕНЦИЯ ТКАНЕВЫХ КУЛЬТУР, ЗАРАЖЕННЫХ ВИРУСОМ БОЛЕЗНИ АУЕСКОГО

Л. БОДОН, Е. ГРЕЦИ

Были исследованы два штамма вируса болезни Ауеского (дикий и вакцинный) в двух различных клеточных культурах (культура яичка теленка и культура почки эмбриона свиньи). Оба штамма спустя 24—28 часов после заражения вызывали в ядрах обоих видов клеток хроматиновую грануляцию, характерную для ДНК-вирусов. Дикий штамм вируса болезни Ауеского образовывал в клеточных культурах огромные синцитии, которые иногда содержали до 50—100 клеточных ядер, в то время как вакцинные штаммы обычно вызывали округливание клеток, а если и возникали синцитии, то они были очень маленькими и содержали в себе всего 2—5 ядер.

FURTHER STUDIES ON HAEMAGGLUTINATING AGENTS OBTAINED FROM ACUTE HEPATITIS SERA

S. Nagylucskay, J. Angyal

ДАЛЬНЕЙШЕЕ ИЗУЧЕНИЕ ГЕМАГГЛЮТИНИРУЮЩИХ АГЕНТОВ, ВЫДЕЛЕННЫХ ИЗ СЫВОРОТОК, ПОЛУЧЕННЫХ В ОСТРОЙ СТАДИИ ГЕПАТИТА

Ш. НАДЬЛУЧКАИ, Й. АНДЬАЛ

В сообщении излагается продолжение предшествующих исследований, согласно которому в аллантоисном мешке оплодотворенных яиц, привитых сыворотками, взятыми в период острого гепатита, размножается гемагглютинирующий агент, гемагглютинирующая способность которого подобна таковой агента, выявляемого из гепатитных сывороток. Агент без снижения титра может сохраняться путём многочисленных пассажей в оплодотворенных яйцах. Большинство гепатитных реконвалесцентных сывороток подавляет гемагглютинирующий агент, присутствующий как в аллантоисной жидкости, так и в гепатитной сыворотке острого периода. Пробы нейтрализации в яйце, проведенные в предварительных экспериментах, дали тот же результат, что и РПГА. Агент оказался очень резистентным к температуре. При 100° С в течение 20 минут не инактивировались ни его гемагглютинирующая способность, ни его способность размножаться в яйце.

SIMULTANEOUS INHIBITORY ACTION ON VIRUS MULTIPLICATION OF INTERFERON AND SOME NATURAL MUCOPOLYSACCHARIDES (HEPARIN, HYALURONIC ACID)

Gy. Hadházy, É. Horváth, L. Gergely

СОВМЕСТНОЕ ИНГИБИРУЮЩЕЕ ДЕЙСТВИЕ ИНТЕРФЕРОНА И НЕКОТОРЫХ ЕСТЕСТВЕННЫХ МУКОПОЛИСАХАРИДОВ (ГЕПАРИН, ГИАЛУРОНОВАЯ КИСЛОТА) НА РАЗМНОЖЕНИЕ ВИРУСА

ДЬ. ХАДХАЗИ, Е. ХОРВАТ, Л. ГЕРГЕЙ

Концентрации гепарина 10, 100 и 100 μg/мл и гиалуроновой кислоты 1, 10 и 100 μg/мл не влияли на размножение в кусочках хориоаллантоисной мембраны штаммов вируса гриппа типа А (PR8) и парагриппа 1 типа (Сендай). Присутствие этих веществ не препятствовало подавляющему действию интерферона на размножение штамма вируса

Сендай. Гепарин подавлял размножение штамма ***Herpes simplex*** в культуре фибробластов куриного эмбриона, гиалуроновая кислота же подобного действия не оказывала. Если эти два вещества прибавлены вместе с интерфероном, то действие первого из них суммируется с таковым интерферона, а второе действие интерферона не изменяет.

EFFECT OF HEPARIN ON HERPES SIMPLEX VIRUS INFECTION IN THE RABBIT

F. Lehel, Gy. Hadházy

ВЛИЯНИЕ ГЕПАРИНА НА ЗАРАЖЕНИЕ КРОЛИКОВ ВИРУСОМ HERPES SIMPLEX

Ф. ЛЕХЕЛ, ДЬ. ХАДХАЗИ

Авторы изучали действие экзогенного гепарина на размножение штаммов HSV, происходящем в коже кролика. Было установлено, что гепарин, примененный за 1 и 18 часов до заражения, в зависимости от концентрации подавляет размножение многочисленных штаммов HSV, а также развитие поражения кожи. Гепарин в существующих экспериментальных условиях не подавлял размножение одного штамма HSV, располагающего выраженной нейровирулентцией. Обсуждается возможная защитная роль гепарина против вирусной инфекции, далее — вопрос предполагаемой связи между нейровируленцией и чувствительностью к гепарину.

OLIGOSACCHARIDE UTILIZATION BY SAPROPHYTIC CLAVICEPS STRAINS

T. Perény, É. Udvardy-Nagy, G. Wack, E. K. Novák

УТИЛИЗАЦИЯ ОЛИГОСАХАРИДОВ САПРОФИТНЫМИ ШТАММАМИ CLAVICEPS

Т. ПЕРЕНЬИ, Е. УДВАРДИ-НАДЬ, Г. ВАК, Е. К. НОВАК

Изучали утилизацию олигосахаридов алкалоид-позитивными и -негативными штаммами *Claviceps*, выделенными из ржи и трав. В отношение 3 штаммов было доказано наличие β-фруктофуранозидазы и у одного штамма — другого характера эндосахарозы. Авторы установили, что первый энзим и в случае штаммов *Claviceps* располагает трансфер-активностью. У штаммов, свежевыделенных из склероциум, наблюдали высокую активность энзима трехалазы. Расщепление мальтозы в зависимости от приема и индукции можно было показать только в отдельных случаях; ни один из исследуемых штаммов не утилизировал лактозу, мелибиозу.

ISOLATION OF ANTIBODIES BY GEL-FILTRATION

M. Kávai, S. Jusupova, B. Csaba

ВЫДЕЛЕНИЕ АНТИТЕЛ ГЕЛЬ-ФИЛЬТРАЦИЕЙ

М. КАВАИ, С. ЮСУПОВА, Б. ЧАБА

Разработали метод для получения кроличих чистых антител против овальбумина. Количественно преципитированный овальбумин-антиовальбумин комплекс диссоциировался в N уксусной кислоте, затем фильтровался на геле Сефадекс G—200. Выделенные таким способом антитела на основе ультрацентрифужного измерения и иммунэлектрофоретического исследования представляют собой гаммаглобулин с седиментацией 6,4 S. Способность к преципитации с овальбумином сохранялась почти на 100%. При анафилактическом шоке в 4 раза эффективнее, чем иммунная сыворотка. Этим методом смогли выделить в чистом виде 60—80% антител иммунной сыворотки.

ALLERGIC-TYPE SKIN REACTIONS IN ANIMALS WITH ALLOXAN DIABETES

T. Szilágyi, A. Kiss, M. Kávai

КОЖНЫЕ РЕАКЦИИ АЛЛЕРГИЧЕСКОГО ХАРАКТЕРА У ЖИВОТНЫХ С АЛЛОКСАН-ДИАБЕТОМ

Т. СИЛАДЬИ, А. КИШШ, М. КАВАИ

На кроликах и мышах с аллоксан-диабетом вызывали пассивную реакцию **Arthus** и антиген-антитело комплекс, а также пассивную кожную анафилаксию. Установили, что диабет подавляет или понижает развитие этих кожных реакций аллергического характера. Гипергликемия, достигнутая дачей глюкозы, также оказывала подавляющий эффект. Вероятно, что за снижение интенсивности кожных реакций в первую очередь ответственны подавляемые высоким уровнем сахара в крови освобождение гистамина и образование грануляционных тканей.

ENHANCING EFFECT OF HUMAN ERYTHROCYTE EXTRACTS ON THE SUSCEPTIBILITY OF MONKEY KIDNEY CELLS TO CERTAIN ENTEROVIRUSES

M. Simon, I. Dömök

ПОВЫШЕНИЕ ЧУВСТВИТЕЛЬНОСТИ КЛЕТОК ПОЧЕК ОБЕЗЬЯН К НЕКОТОРЫМ ЭНТЕРОВИРУСАМ ДОБАВЛЕНИЕМ ЭКСТРАКТА ЧЕЛОВЕЧЕСКИХ ЭРИТРОЦИТОВ

М. ШИМОН, И. ДЁМЁК

В предшествующем эксперименте авторы показали, что некоторые гемагглютинирующие ЕСНО вирусы лучше размножались в таких первичных клеточных культурах почек обезьян, которые обрабатывались экстрактом человеческих эритроцитов. Значение этого явления для практики было изучено в опытах по выделению вируса, проведенных параллельно в нормальных культурах почек обезьян и в культурах, обработанных экстрактом человеческих эритроцитов. Из стула 175 здоровых новорожденных всего удалось выделить 53 штамма вирусов, из которых 32 могли быть выделены в обоих видах тканевых культур, 1 — только в нормальной и 20 — только в тканевой культуре, обработанной экстрактом человеческих эритроцитов. Среди последних 13 штаммов относились к гемагглютинирующим энтеровирусным типам.

На основе своих результатов авторы предлагают использовать для рутинных диагностических целей тканевые культуры, обработанные экстрактом человеческих эритроцитов.

STUDIES ON THE INITIAL PHASES OF POLIOVIRUS REPRODUCTION CYCLE

II. COMPARATIVE STUDIES ON HELA AND PERMANENT MONKEY KIDNEY CELLS

E. György, B. Lomniczi, A. Koch

ИЗУЧЕНИЕ НАЧАЛЬНЫХ СТАДИЙ ЦИКЛА РЕПРОДУКЦИИ ПОЛИОВИРУСА

II. СРАВНИТЕЛЬНЫЕ ОПЫТЫ НА КЛЕТКАХ HELA И СТАБИЛЬНОЙ КУЛЬТУРЕ ПОЧКИ ОБЕЗЬЯНЫ

Э. ДЬЁРДЬ, Б. ЛОМНИЦИ, А. КОХ

Клетки **HeLa** и стабильные клетки почки обезьяны РМК III/1 перед заражением их полиовирусом 1 типа (**Mahoney**) в течение различного времени обрабатывались версеном и трипсином. Было проведено сравнение цикла репродукции вируса в клетках, поддерживаемых в питательном растворе, содержащем телячий альбумин (HBS + BA), и в питательной среде без последнего (HBS).

В клетках РМК III/1 конечный выход вируса был в 10 раз выше в случае питательной среды HBS + BA, чем в среде HBS. Эта разница не изменялась в зависимости от того, версеном или трипсином обрабатывались предварительно клетки, но абсолютное количество вируса умеренно понижалось в связи с продолжительностью обработки трипсином. Наиболее высокие титры были получены при обработке клеток версеном.

При продолжительной (30, 60 минут) обработке клеток HeLa версеном или трипсином выход вируса был одинаковым в случае использования двух видов питательной среды. Однако после 5—20 минутной обработки трипсином выход вируса в отношении клеток, суспендированных в HBS + BA, оказался в 10 раз выше.

Авторы предполагают, что клетки HeLa располагают тремя видами рецепторов, а клетки РМК III/1 — двумя.

STUDIES ON THE INITIAL PHASES OF POLIOVIRUS REPRODUCTION CYCLE

III. ACTION OF FATTY ACIDS AND TWEEN 80

A. Koch, B. Lomniczi, E. György

ИЗУЧЕНИЕ НАЧАЛЬНЫХ СТАДИЙ ЦИКЛА РЕПРОДУКЦИИ ПОЛИОВИРУСА

III. ВЛИЯНИЕ ЖИРНЫХ КИСЛОТ И TWEEN 80

А. КОХ, Б. ЛОМНИЦИ, Э. ДЬЁРДЬ

После заражения суспендированных клеток перевиваемой культуры (РМК III/1) почки обезьяны полиовирусом 1 типа (Mahoney) в растворе Хэнкса (HBS) выход вируса оказывается низким. Если к раствору Хэнкса прибавляли 0,2% телячьего альбумина (БА), выход вируса увеличивался в 10 раз. Было предположено, что за действие телячьего альбумина ответственны жирные кислоты, присутствующие в последнем как загрязнение, поэтому изучалось размножение вируса в присутствии различных жирных кислот и Tween-а 80.

Линолевая и олеиновая кислоты в конечной концентрации 10^{-4} *M* полностью подавляли размножение вируса. Арахиновая и стеариновая кислоты, взятые в той же концентрации, стимулировали размножение вируса в степени, равной половине эффекта БА. Пальмитиновая кислота оказывала меньший эффект. При сопоставлении с действием БА жирные кислоты в концентрации 10^{-5} *M* обладали различной активностью: арахиновая — 90%, линолевая и олеиновая — 40%, стеариновая и пальмитиновая — 32% и 22%. При концентрации 10^{-7} *M* эти соотношения были следующие: арахиновая кислота — 50%, олеиновая — 30%; стеариновая и пальмитиновая кислоты в такой концентрации оказались неэффективными.

Tween 80 в концентрации 150 μg/мл подавлял размножение вируса; его концентрация 1,5 μg/мл не влияла, а 15 μg/мл подобно БА стимулировала размножение вируса. Активность жирных кислот, взятых в концентрации 10^{-4} — 10^{-7} *M*, зависела от длины углеродной цепочки и концентрации. Активность была тем выше, чем длиннее была углеродная цепочка соединения и чем выше концентрация.

Для того, чтобы развилась полная активность, жирным кислотам и Tween-у 80 надо присутствовать в первые часы цикла вирусного размножения. Прибавленные позже жирные кислоты не оказывают никакого эффекта на размножение вируса.

Авторы предполагают, что эти вещества влияют на пенетрацию вирионов, связанных с клетками. В этой связи излагается гипотеза, относящаяся к ранним стадиям взаимодействия вирус-клетка.

PATHOGENESIS OF THE WASTING SYNDROME FOLLOWING NEONATAL THYMECTOMY

I. Szeri, Zs. Bános, P. Anderlik, M. Balázs, P. Földes

ДАННЫЕ К ПАТОГЕНЕЗУ «WASTING SYNDROME», СЛЕДУЮЩЕГО ЗА УДАЛЕНИЕМ У НОВОРОЖДЕННЫХ ВИЛОЧКОВОЙ ЖЕЛЕЗЫ

И. СЕРИ, Ж. БАНОШ, П. АНДЕРЛИК, М. БАЛАЖ, П. ФЁЛДЕШ

(1) Интрацеребральное заражение мышей вирусом лимфоцитарного хориоменингита (LCM), происходящее после прекращения вскармливания молоком, способствует развитию **«wasting syndrome»** (одинаково в отношении количества и времени), возникающего как результат удаления вилочковой железы в период новорожденности.

(2) Мыши с удаленной в период новорожденности вилочковой железой и переживающие интрацеребральное заражение LCM, хотя и являются вирусоносителями, с иммунологической точки зрения не „ведут себя“ одинаково. В равной пропорции (50—50%) можно найти иммунных и не дающих иммунного ответа, толерантных животных. Большая часть животных, однако, независимо от их иммунного состояния погибает в течение 80 дней после прививки с **«wasting syndrome»**-ом.

(3) На основе своего опыта и литературных данных, касающихся этого вопроса, авторы считают, что организм, находящийся в иммунной депрессии, на различные антигенные раздрожители может реагировать только неспецифическим способом. Этот неспецифический ответ — возникновение самого **«wasting syndrome»**-а.

(4) Поднимается возможность аналогии между возникновением атрофий, развивающихся у человека на основе физиологической иммунной депрессии в период новорожденности и в старческом возрасте, и патогенезом **«wasting syndrome»**-а.

HAS THE NERVOUS SYSTEM A ROLE IN THE ENDOTOXIN-INDUCED ALTERATION OF CELL METABOLISM?

K. S. Vukán, P. Kertai

ИГРАЕТ ЛИ НЕРВНАЯ СИСТЕМА РОЛЬ В ИЗМЕНЕНИИ КЛЕТОЧНОГО МЕТАБОЛИЗМА, ВЫЗВАННОГО ЭНДОТОКСИНОМ?

К. Ш. ВУКАН, П. КЕРТАИ

Авторы не могли установить разницы между обменом веществ иннервированных и денервированных клеток после параэнтерального введения эндотоксина.

DATA ON THE CYTOTOXICITY OF ANTI-TUMOUR SERA

G. Elek, L. Vekerdi

ДАННЫЕ О ЦИТОТОКСИЧЕСКОМ ДЕЙСТВИИ ПРОТИВООПУХОЛЕВЫХ ИММУННЫХ СЫВОРОТОК

Г. ЭЛЕК, Л. ВЕКЕРДИ

Пользовались мышиной асцитической опухолью NK/Ly для получения крысиной иммунной сыворотки. Сыворотка содержала инкомплектные антитела и цитотоксический эффект оказывала только в присутствии комплемента. Подобную, но с меньшим цитотоксическим действием сыворотку удалось получить при иммунизации крыс суспензией из селезенки здоровых мышей. Из последней сыворотки цитотоксические антитела могли истощаться клетками селезенки здоровых мышей. Противоопухолевая сыворотка, истощенная клетками мышиной селезенки, однако, оставалась эффективной при определенных количественных условиях.

EFFECT ON INFLUENZA VIRUS OF A MODIFIED FRANCIS INHIBITOR AND ITS ACETONE-SOLUBLE FRACTION

II. STUDIES ON THE MODE OF ACTION IN DE-EMBRYONATED EGGS

I. Hollós

ВЛИЯНИЕ ВИДОИЗМЕНЕННОГО ФРЭНСИС-ИНГИБИТОРА И ЕГО АЦЕТОН-РАСТВОРИМОЙ ФРАКЦИИ НА РАЗМНОЖЕНИЕ ВИРУСА ГРИППА

II. ИЗУЧЕНИЕ МЕХАНИЗМА ДЕЙСТВИЯ В ДЕЭМБРИОНИРОВАННОМ ЯЙЦЕ

И. ХОЛЛОШ

Автор изучал влияние Фрэнсис-ингибитора (K_0), его диэтил-р-фенилен-диамин-диазониум-хлоридного деривата (*8m*), а также их фракции (K_0ac *и* *8ac*), остающиеся в растворе во время ацетонового фракционирования, с точки зрения их подавляющего действия на размножение вируса гриппа А—1, штамм Будапешт 4/49, по методике деэмбрионированного яйца.

20 мг/мл K_0ac и 5 мг/мл *8ac* полностью подавляли размножение вируса в хорио-аллантоисной мембране, если их прибавляли к питательной среде спустя 30 минут после заражения. 20 мг/мл K_0 и *8m* в аналогичных условиях оказались неэффективными.

20 мг/мл *8ac*, примененные спустя 1 час после заражения, останавливали размножение вируса. Такой же эффект наблюдался и через 5 часов: значительно понижается выход вируса в случае происходящего цикла, а дальнейшие циклы останавливаются. Прибавление через 10 часов сопровождается остановкой последующих циклов.

Автор установил, что период времени, при котором имеется возможность подавления, зависит и от дозы *8 ac:* процесс, чувствительный к дозе 5 мг/мл, находится в промежутке между 30 минутами и 2 часами эклипса. Процесс, который может быть подавлен 7,5 мг/мл или 10 мг/мл *8 ac*, начинается между 30 минутами и 2 часами эклипса и продолжается до его конца. Как видно, некоторые составные части вируса не стабилизируются до 6 часов и остаются чувствительными к действию *8 ac*. Темп чувствительного к подавлению синтеза идет параллельно с формированием инфективного вируса. После удаления *8ac* синтез вируса восстанавливается и, спустя короткую lag фазу, продолжается в таком же темпе, как и темп размножения вируса в контроле.

TICK-BORNE ENCEPHALITIS: A COMPARATIVE SEROLOGICAL SURVEY IN HUNGARY

E. Molnár, T. Kubászova

СРАВНИТЕЛЬНЫЕ СЕРОЛОГИЧЕСКИЕ ИССЛЕДОВАНИЯ В ОТНОШЕНИИ КЛЕЩЕВОГО ЭНЦЕФАЛИТА В ВЕНГРИИ

Э. МОЛЬНАР, Т. КУБАСОВА

В связи с острыми заболеваниями нервной системы, имевшими место в Венгрии в 1963—65 гг., были исследованы сыворотки крови от 1007 лиц в пробе нейтрализации вируса и в РПГА. Вируснейтрализующие антитела к вирусу клещевого энцефалита, выделенному в Венгрии (штамм $КЭМ_1$), были найдены в 23% случаев энцефалита и в 10% случаев асептического менингита. В РПГА положительный результат был получен в большем проценте; кроме того, нейтрализационные титры сывороток отличались от титров РПГА. На этом основании авторы предполагают, что в Венгрии кроме вируса клещевого энцефалита существует и другой арбовирус, относящийся согласно классификации по Казалс в группу Б.

SEROLOGICAL PROPERTIES OF PSEUDOMONAS AERUGINOSA

I. GROUP-SPECIFIC SOMATIC ANTIGENS

B. Lányi

СЕРОЛОГИЧЕСКИЕ СВОЙСТВА PSEUDOMONAS AERUGINOSA

I. ГРУППОВЫЕ СОМАТИЧЕСКИЕ АНТИГЕНЫ

Б. ЛАНЬИ

Соматические антитела к штаммам *Pseudomonas aeruginosa* наиболее выраженно агглютинируют живые и прогретые в течение 2 ½ часов при 100° С и в течение 1 часа при 130° С бактерии. Слабое температурное воздействие (60—75° С), а также обработка алкоголем, насыщенным NaCl и формалином прекращают агглютинабильность соматических антигенов. Агглютинабильность суспензий, прогретых при 100—130° С, усиливается пропорционально времени и степени температурного воздействия. Обработанные кислотами бактерии способны агглютинироваться подобно клеткам, прогретым при более высокой температуре. Соматические антигены свою иммуногенность и способность истощать антитела сохраняют после любой из упомянутых выше обработок.

На основе исследования 2197 штаммов *Pseudomonas aeruginosa* автор составил 13 антигенных схем, содержащих О антигенную группу. Штаммы, относящиеся в группы О3, О4, О5 и О10, на основе более важных соматических антигенных составов были подразделены далее на 15 подгрупп. Кроме групповых антигенов указывает на общий соматический фактор, которым располагает большинство штаммов *Pseudomonas aeruginosa*. На основе агглютинационных исследований термолабильные соматические антигены выявить не удалось.

Агглютинация, проведенная с прогретым антигеном в пробирке, и агглютинация, выполненная на предметных стеклах с живым антигеном, с точки зрения О антигена оказались равноценными. Автор подробно знакомит с типовыми штаммами и методиками, требующимися для производства, контроля и истощения используемых сывороток. Знакомит с распределением по серогруппам и подгруппам 2197 исследованных штаммов, происходящих из разного материала.

INCIDENCE OF PSEUDOMONAS AERUGINOSA SEROGROUPS IN WATER AND HUMAN FAECES

B. Lányi, M. Gregács, M. M. Ádám

ВСТРЕЧАЕМОСТЬ СЕРОГРУПП PSEUDOMONAS AERUGINOSA В ВОДЕ И ЧЕЛОВЕЧЕСКИХ ФЕКАЛИЯХ

Б. ЛАНЬИ, М. ГРЕГАЧ, М. М. АДАМ

Из 9219 проб питьевой воды, 1065 — речной, 419 — сточной воды, а также из стула 7650 здоровых взрослых, 650 новорожденных, содержавшихся в больнице, был изолирован 521 штамм *Pseudomonas aeruginosa*.

Было выявлено, что частота встречаемости *Pseudomonas aeruginosa* в колодезной воде составляла 1,6%, в воде из источника — 4,1%, в водопроводной воде — 0,6% и в речной воде — 1,8%. На той же самой территории бактерии были обнаружены в стуле взрослых в 1,5%, в стуле новорожденных — в 32,% в сточной воде — в 10%.

Распределение штаммов, выделенных из воды и из стула взрослых, по серологическим группам было заметно сходно. Чаще всего встречались члены 1, 3, 4, 5 и 6 серогрупп. По мнению авторов, присутствие в воде *Pseudomonas aeruginosa* может иметь фекальное происхождение.

INDUCTION AND MULTIPLICATION OF λ-PHAGE

I. THE EFFECT OF CHLOROMYCETIN

I. Gadó, G. Savchenko

ИНДУКЦИЯ И РАЗМНОЖЕНИЕ λ-ФАГА

I. ДЕЙСТВИЕ ХЛОРАМФЕНИКОЛА

И. ГАДО, Г. САВЧЕНКО

На действие митомицина в клетках, находящихся в состоянии «**Shift down**», общее число зараженных центров тотчас повышается. Повышение не подавляется хлорамфениколом, а трипафлавином — да. В присутствии хлорамфеникола первичная активность оборванного профага остается в течение продолжительного времени. Без «**Shift down**» в присутствии хлорамфеникола оборванный профаг инактивируется.

SOME PHYSICAL CHARACTERISTICS OF INFLUENZA VIRUS DETERMINED BY ELECTRONMICROSCOPY

I. Hollós, Á. Barna

ОПРЕДЕЛЕНИЕ ЭЛЕКТРОННЫМ МИКРОСКОПОМ НЕКОТОРЫХ СВОЙСТВ ВИРУСА ГРИППА

И. ХОЛЛОШ, А. БАРНА

После очистки и концентрации комплетные и некомплетные вирионы штамма вируса гриппа A—1, обозначенного как **Paris PL** 1/49, были смешаны с частицами полистирен латекс-а «**Dow**». Нефиксированные и с помощью OsO_4 фиксированные препараты были изучены частью без теневого напыления, частью с палладиумовым напылением. Соответствующие измерения проводились на снимках, изготовленных среди постоянных условий.

На основе измерения, проведенного на латекс-частицах, было определено значение S, характерное для электронного микроскопа. Определили градацию фотонегатива (γ) относительно используемой электронной экспозиции с 45 KV-ным ускорением.

Пристальным разглядыванием («**scanning**» метод) вирионов получили кривую плотности и компаратором измерили их диаметр и высоту.

Было установлено, что в течение подготовки и исследования происходит в большой степени уплощение вирионов. Сделали попытку определить истинные размеры вирионов. На основе измерений плотности высчитали сухую массу вирионов и сделали вывод относительно количества принятого OsO_4. Была сделана попытка и в вычислении истинной (влажной) массы и плотности.

REGULATION OF VALINE AND ISOLEUCINE BIOSYNTHESIS IN STREPTOMYCES RIMOSUS

I. Horváth, A. Szentirmai, J. Zsadányi

ДАЛЬНЕЙШИЕ ЭКСПЕРИМЕНТЫ В ОТНОШЕНИИ РЕГУЛЯЦИИ БИОСИНТЕЗА ВАЛИНА И ИЗОЛЕЙЦИНА У STREPTOMYCES RIMOSUS

И. ХОРВАТ, А. СЕНТИРМАИ, Й. ЖАДАНЬИ

У ***Streptomyces rimosus*** — подобно другим микроорганизмам — вследствие перевивок временно повышается синтез энзимов, принимающих участие в биосинтезе изолейцина и валина. Этот усиленный синтез валин ещё более повышает. Защитный эффект от спонтанного усиления синтеза энзима может быть достигнут одновременным прибавлением валина, лейцина и изолейцина.

Уже описанное индукционное действие α-кэтомасляной кислоты имеет место в течение всего времени культивирования; репрессировать можно одновременной дачей валина и лейцина, один валин оказывает частичную репрессию.

Первый общий энзим биосинтеза валина и изолейцина, ацетогидрокси-кислота-синтетаз, может быть подавлен валином.

"EARLY, NON-VIRION" ANTIGENS IN HERPES SIMPLEX VIRUS INFECTED TISSUE CULTURE CELLS

I. DETECTION OF "EARLY" ANTIGENS WITH COMPLEMENT FIXATION TEST

L. GÉDER, L. VÁCZI, É. GÖNCZÖL, E. JENEY, F. LEHEL

«РАННИЕ», «НЕВИРИОННЫЕ», АНТИГЕНЫ В КЛЕТКАХ ТКАНЕВОЙ КУЛЬТУРЫ, ЗАРАЖЕННОЙ ВИРУСОМ HERPES SIMPLEX

I. ВЫЯВЛЕНИЕ «РАННИХ» АНТИГЕНОВ РЕАКЦИЕЙ СВЯЗЫВАНИЯ КОМПЛЕМЕНТА

Л. ГЕДЕР, Л. ВАЦИ, Е. ГЁНЦЁЛ, Е. ЙЕНЕИ, Ф. ЛЕХЕЛ

В клетках БС-Ц-1, Хэла и в фибробластах человеческого эмбриона, зараженных вирусом *Herpes simplex* (1—8 $ТЦД_{50}$ по клеткам), можно выявить вирус-специфический ранний антигенный компонент. Присутствие 10 μг/мл цитозин-арабинозида не подавляло его образования. Антиген термолабилен, чувствителен к обработке эфиром и повторному замораживанию-оттаиванию, не может быть осажден 100 000 *g* в течение 1 часа.

Из 8 кроличих иммунных сывороток против *Herpes simplex* 3 реагировали с ранним антигеном, но не было обнаружено связи между комплементсвязывающими титрами антител против раннего антигена и против вируса. Среди 6 человеческих реконвалесцентных сывороток ни одна не реагировала с ранним антигеном.

"EARLY, NON-VIRION" ANTIGENS IN HERPES SIMPLEX VIRUS INFECTED TISSUE CULTURE CELLS

II. INTRACELLULAR LOCALIZATION OF "EARLY" ANTIGEN WITH INDIRECT IMMUNOFLUORESCENCE TECHNIQUE

L. GÉDER, L. VÁCZI, E. JENEY, É. GÖNCZÖL, F. LEHEL

«РАННИЕ», «НЕВИРИОННЫЕ», АНТИГЕНЫ В КЛЕТКАХ ТКАНЕВОЙ КУЛЬТУРЫ, ЗАРАЖЕННОЙ ВИРУСОМ HERPES SIMPLEX

II. ОПРЕДЕЛЕНИЕ ВНУТРИКЛЕТОЧНОЙ ЛОКАЛИЗАЦИИ «РАННЕГО» АНТИГЕНА С ПОМОЩЬЮ НЕПРЯМОЙ ИММУНФЛУОРЕСЦЕНТНОЙ ТЕХНИКИ

Л. ГЕДЕР, Л. ВАЦИ, Е. ЙЕНЕИ, Е. ГЁНЦЁЛ, Ф. ЛЕХЕЛ

Иммунфлуоресцентным методом было показано, что в БС-Ц-1 клетках и в фибробластах куриного эмбриона, зараженных вирусом *Herpes simplex* (по клеткам 4,5 $ТЦД_{50}$), спустя 5 $^1/_2$ — 8 часов после заражения появляется вирус-специфический ранний антигенный компонент. Этот антиген концентрируется чаще всего в периферической части клеточного ядра. 10 μг/мл цитозин-арабинозида не подавляет образование антигена.

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