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VOLUME II

Edited
by
GY. J. WEISZFEILER

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MAGYAR
TUDOMÁNYOS AKADÉMIA
KÖNYVTÁRA

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OF THE HUNGARIAN ACADEMY OF SCIENCES

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K. BALÁZS and B. LÁNG

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FOREWORD

The present year-book of the Microbiological Research Group publishes the results achieved in our Institute in 1967. Margarita Tálas's paper deals with investigations on the mode of action of oncogenic viruses. The book also contains the material of the conference on the question of *interferon* where Soviet, French and Hungarian research workers give an account of their investigations connected with the production of *interferon* in vivo and in vitro, as well as with its application on human beings. Three contributions describe the analyses of the specific allergy caused by BCG, the variability and the antigenic structure of mycobacteria. Two further studies report on the work started in the field of industrial microbiology: the mode of action of cellulase, some questions of the production of cellulase, and the improvement of the digestibility of unicellular algae by cellulase enzyme.

Prof. Dr. Gy. J. Weiszfeiler

Director of the Microbiological
Research Group of the Hungarian
Academy of Sciences

CONTENTS

EXPERIMENTAL INVESTIGATION OF SOME ASPECTS OF CELL TRANSFORMATION BY VIRUSES (ON THE MODEL OF SIMIAN VIRUS 40 AND POLYOMA VIRUS)[1]

by

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In recent years a number of viruses inducing tumors in animals were found to be able to change (transform) cells in tissue cultures. Biological implications of this phenomenon lay, first of all, in the fact that such virus-transformed cell lines induced development of tumors when inoculated into the appropriate host. Thus, in the hands of research workers there was a model of oncogenesis in vitro permitting to study at length the stages of interaction of oncogenic virus with the cell, to investigate a number of aspects of viral carcinogenesis and thereby to elucidate the role of viruses in the etiology of human malignant diseases.

The process of cell transformation under the effect of DNA-containing oncogenic viruses of Papova group (Melnick 1962) — polyoma virus and simian virus 40 (SV40) — was studied with particular intensity in recent years. The SV40, a latent agent in monkeys, causing tumors in hamsters (Eddy et al. 1961, Deichman and Prigozhina 1962, Girardi et al. 1962) and transforming human cells in vitro (Koprowski et al. 1962), was found as a contaminant in vaccines against poliomyelitis and adenovirus diseases (Sweet and Hilleman 1960, Magrath et al. 1961, Gerber et al. 1961). Another virus of this group, polyoma, is interesting, first of all, because of its extremely broad spectrum of oncogenicity: it induces tumors in seven species of rodents (Stewart et al. 1960, Negroni 1963). Moreover, polyoma virus-induced tumors are characterized by extremely wide morphological

[1] This paper presents the results of studies carried out at the USSR AMS Institute of Poliomyelitis and Viral Encephalitides, Moscow (Director of the Institute — Prof. M. P. Chumakov, Full Member of the USSR AMS) together with M. P. Chumakov, A. N. Mustafina, G. I. Avgustinovich, N. M. Shestopalova, V. N. Reingold, M. B. Korolev, V. Ya. Karmysheva and T. I. Zavodova; in the Virus Department, Karolinska Institute, Stockholm (Head of the Virus Department — Prof. Sven Gard); and in the Microbiological Research Group of the Hungarian Academy of Sciences, Budapest (Director of the Group — Prof. Gy. J. Weiszfeiler, Corresponding Member of the Hungarian Academy of Sciences).

[2] The author's name in previous publications is M. Ya. Chumakova.

varieties. In mice polyoma virus induces adenocarcinomas of mammary and sebaceous glands, pleomorphic tumors of preauricular, submaxillar, hypoglossal glands, epithelial thymomas, sarcomas of kidneys, bones, subcutaneous connective tissue, tumors of the stomach, lungs, etc. (Stewart 1960).

The following aspects of the problem were studied by us: (1) possibility of in vitro transformation of cells of different origins by oncoviruses; (2) dependence of transformation upon the dose of the virus inoculum; (3) morphological characteristics of transformed cell cultures; (4) biological properties of transformed cells in vivo; (5) the fate of virus in the transformed cells.

Materials and Methods

Viruses. 1. SV40, strain A-426, was received from the National Institutes of Health, Bethesda, USA. 2. Polyoma virus SE-2510 strain was obtained from I. S. Irlin (N. F. Gamaleya Institute of Epidemiology and Microbiology, Moscow).

Cell cultures. In the studies the following cultures were used: diploid strains of human embryo skin-muscle cells derived and propagated according to Hayflick's method (Hayflick and Moorhead 1961), diploid strains of hamster embryo cells, cultures of cells from tumors induced by SV40 and polyoma virus, as well as primary and continuous cultures of mouse, hamster and chick embryo cells, and primary kidney cell cultures from M. rhesus and Cercopithecus aethiops monkeys. All cell cultures were prepared by trypsin digestion procedure (Dulbecco and Vogt 1954) and grown in Parker's medium 199 and Eagle's medium with 10–20% bovine or calf serum.

Virus titration was performed by hemagglutination test, by the cytopathic effect, by the plaque method (Chumakova et al. 1963).

Virus antigen was determined by means of the MAP-test (Rowe et al. 1959) and immunologic test in white rats (Chumakova et al. 1964).

Light, luminescent and electron microscopy as well as karyotype analyses were performed by methods described by Chumakov et al. (1964).

Results

Transformation of human diploid cells by SV40

Characteristics of SV40-infected cell cultures before transformation. Skin-muscle (SM) cell strains of human embryo were infected with different doses of SV40 (Chumakov et al. 1964). Monolayer cell cultures were infected

at the level of 4–6 passages. The highest virus concentration was 3 plaque-forming units (PFU)/cell, successive doses were 10-fold lower. Passages of the culture were carried out every 3–4 days.

In the first days after virus inoculation infected and control cell cultures showed no morphological differences from each other and consisted of spindle cells of fibroblast type with oval nuclei and 2–4 round nucleoli (Fig. 1A).

Later, at 2–3 weeks, alongside with increase of extracellular and intracellular viruses (Figs 2 and 3) inoculated cultures exhibited partial cyto-

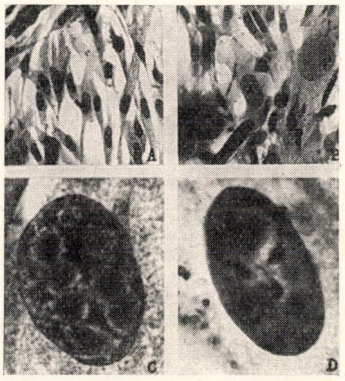


FIG. 1. Human embryo cell culture
A — Control culture consisting of typical spindle-shaped fibroblast-type cells. Object-glass ×40; B — Transformed culture consisting of polymorphous cells; 26th passage after inoculation with undiluted SV40 virus. Object-glass ×40; C — Numerous oxiphilic intranuclear inclusions in tissue culture cells 3 weeks after inoculation. Object-glass ×90; D — Feulgen — positive staining of intranuclear inclusions. Object-glass ×90

pathic effect the extent of which varied with different cell strains. Sometimes the viral cytopathic effect resulted in the destruction of the entire cell population. In other cases the diploid cell strain survived despite marked increase of synthesis of intracellular virus (for SM-26-1 strain from 3 to 30 PFU/cell, Fig. 4).

Cytological examination (Karmysheva et al. 1963) at this stage revealed some changes in inoculated cell cultures. Beginning with the 2nd passage after inoculation, occasional cells of sublines inoculated with the two highest doses of the virus (SM-26-0 and SM-26-1 sublines, 3 and 0.3 PFU/cell, respectively) were found to form small oxiphilic masses, and in subsequent passages numerous oxiphilic DNA-containing inclusions were formed in

cells of these cultures (Figs 1C, D). By the 17th passage after inoculation these inclusions were observed in 0.4% of cells. Early in the infection, an increase in the size, deformation and enhanced pyroninophilia of nucleoli were observed.

In early passages after inoculation specific viral fluorescence of the nuclei was observed in 3–8% of cells only. In nuclei and nucleoli fluorescence was diffuse or in aggregates of fluorescent mass resembling by shape and

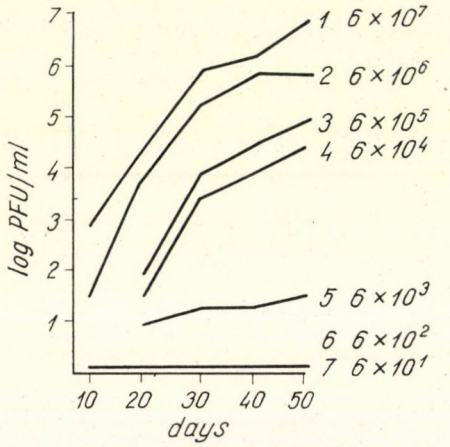


FIG. 2. Accumulation of SV40 in the fluid from SM-15 cell culture
1–7: curves indicating virus accumulation in cultures inoculated with indicated amounts of virus; ordinate: virus titer; abscissa: days after inoculation with SV40

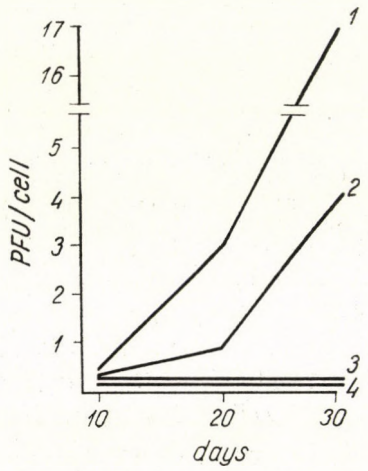


FIG. 3. Accumulation of intracellular SV40 in SM-15 cells
1–4: see Fig. 2

distribution intranuclear inclusions (Fig. 5). Occasional nuclei showed almost total fluorescence. In such cells fluorescence of the cytoplasm could be observed.

Karyotype of infected cultures at this period did not differ from the controls (Mustafina 1966).

By means of electron microscopy (ultrathin sections) early and marked changes in the nucleus of human cells infected with SV40 could be revealed

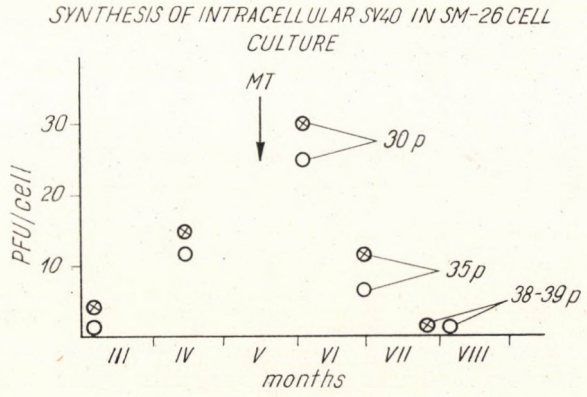


FIG. 4

⊗ — SM-26-0 — cell subline inoculated with undiluted virus
○ — SM-26-1 — cell subline inoculated with first dilution of virus
MT — morphological transformation
p — passage of cells

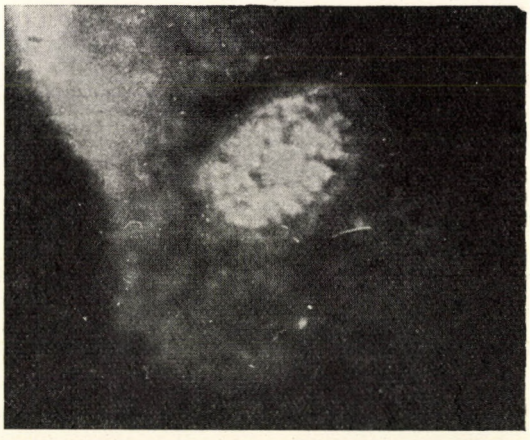


FIG. 5. Localization of viral antigen in cells infected with SV40. Direct fluorescent antibody method. Object-glass ×90

(Shestopalova et al. 1963). These changes consisted in chromatin degeneration (change in the pattern of osmiophilia) and in the appearance in the nucleolus of osmiophilic roughly granulated areas. Virus particles were found in the cell nucleus either as scattered aggregates (Fig. 6) or as virus crystals. Density of virus particles was on the average 480 particles per 1 square micron.

In the cytoplasm of virus-affected cells no normal cellular organelle were found. The endoplasmic reticulum was detected only in some areas

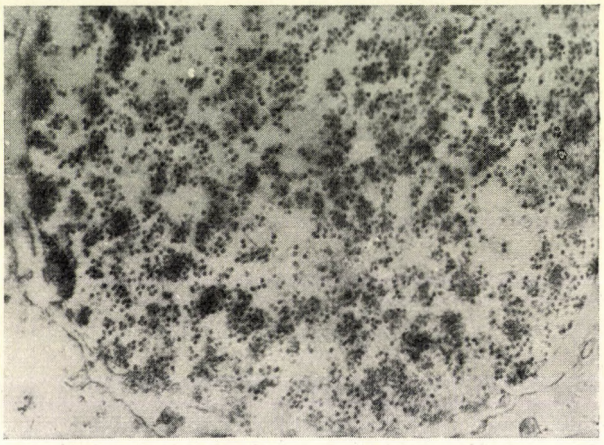


FIG. 6. Electron micrograph of a part of the nucleus. Human diploid cell culture 8 days after inoculation with SV40. Aggregates of virus particles in the nucleus are visible. ×55 300

in the form of membranes. Mitochondria were severely deformed. The integrity of the cytoplasmic membrane was broken. No Golgi apparatus could be found in such cells. These degenerative changes indicated death of human cells under the effect of SV40 multiplication.

Characteristics of transformed cultures. Morphological changes in the pattern of growth of infected human diploid cells (SM-26 strain) occurred in two sublines: SM-26-0 and SM-26-1 inoculated with 3 and 0.3 PFU/cell, respectively. First signs of transformation, namely rapid change of pH of the growth medium towards acid side and appearance of areas of growth of epithelium-like cells, were observed after 10 weeks of cultivation of virus-infected cells (21st passage of SM-26-0 subline and 22nd passage of SM-26-1 subline).

Cytological examination showed that in transformed cultures (Fig. 1B) there was an increase in the number of large cells with giant nuclei exceed-

ing by 4–10 times the average volume of nuclei in control cells. The number of giant nuclei in transformed cultures was 17%, in controls 1%. Such nuclei frequently had irregular blade shape and contained numerous (up to 10), frequently deformed nucleoli. Giant nuclei were frequently

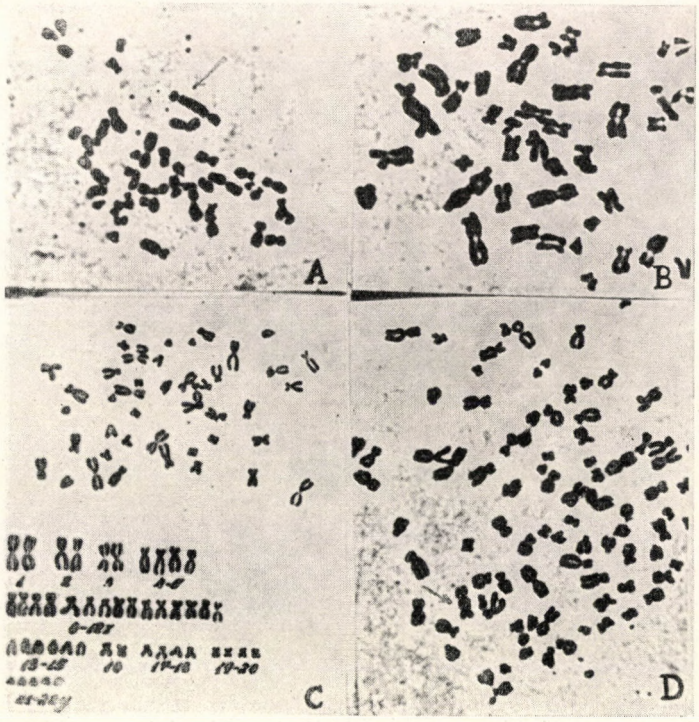


FIG. 7

A, D — Dicentric chromosomes in cells from transformed SM-26-0 human embryo culture; B — Endoreduplication and long subtelocentric chromosome in a cell from the same culture; C — Normal human karyotype

surrounded only by a narrow band of cytoplasm, which indicated the disturbance in the nucleus–cytoplasm relationship. Abnormal mitoses were frequent.

Changes in the karyotype (Mustafina 1966) occurred in two directions: polyploidy and appearance of chromosome aberrations (Fig. 7). Considerable variability of cells with regard to the number of chromosomes (24 numerical variants) and a gradual increase with passages of the number of tetraploid cells were observed. Chromosome aberrations consisted in

appearance of dicentric chromosomes of various configurations, chromosome breaks, associations of satellites of acrocentric chromosomes and other changes.

Using electron-microscopic methods, formations never found in cells of control culture, were detected in dividing cells of the transformed culture: paired canals of the endoplasmic reticulum (Korolev et al. 1966) (Fig. 8).

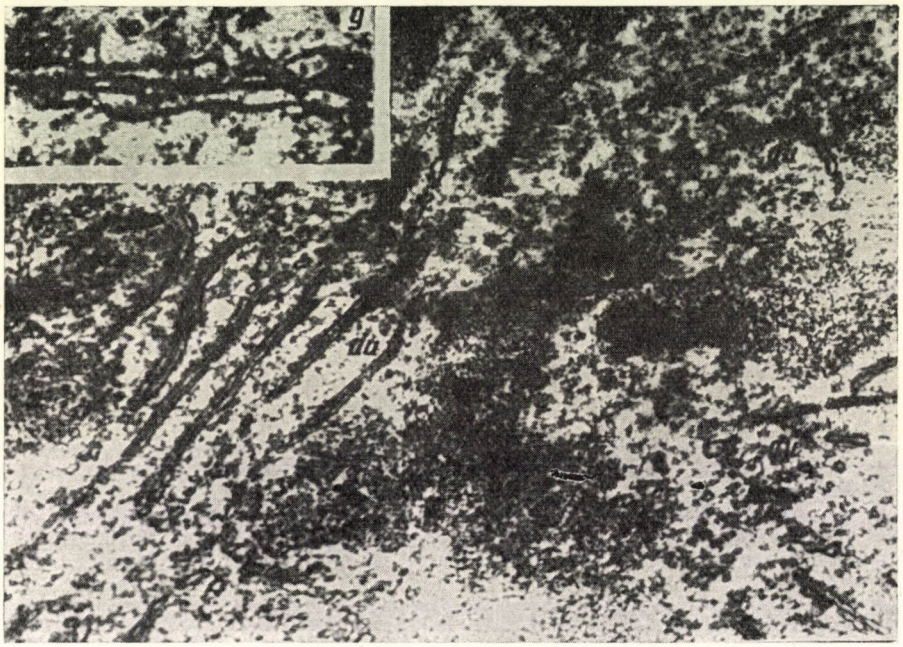


FIG. 8. Paired canals of endoplasmic reticulum in the cell of SM-26-0 transformed human embryo tissue culture (×11 900)

In ultrathin sections these canals looked like seven-layer structures arranged frequently in parallel arrays between chromosomes. The greatest concentration of the paired canals of the endoplasmic reticulum was observed at the metaphase stage. In interphase cells such structures were absent. Multilayer structures similar to these were reported in cells of some tumor tissues (Chentsov 1960). In dividing cells of normal tissues such structures have never been known. The function of these structures was not elucidated.

Besides, in dividing cells of the transformed culture at the 41st passage virus-like particles with a diameter of 370 ± 20 Å were detected in the immediate proximity of chromosomes; this size corresponded to that of

virus particles detected in infected tissue cultures before transformation. Occasional virus-like particles were found in dividing cells containing paired canals of the endoplasmic reticulum. In addition to virus-like particles in the hyaloplasm rounded and demarkated granules of high electron-optic density were sometimes present which appeared to be precursors of mature virus.

In the period before morphological transformation and some time after appearance of the first signs thereof an increase in the synthesis of intracellular virus was observed in both sublines from 3–0.3 PFU to 25–30 PFU/cell. Then in the 35th passage of the cell culture a marked diminution of newly formed virus was observed and in the 38–39th passages, that is 17–18 passages after first signs of morphological transformation, no intracellular infective virus could be detected by the plaque method (Fig. 4). As was mentioned above, however, virus-like particles were found by means of electron-microscopy in cells of a later passage (41st) of this culture.

Cultures of human diploid cells transformed by SV40 (SM-26-1 subline) were carried, on the total, through 74 passages during 8 months of cultivation (with 1 $^1/_2$-month interval during which time the cells were stored at —70 °C). After a stage of crisis the transformed cells died. The crisis of SM-26-0 subline occurred after 5 months of cultivation (observations of A. N. Mustafina).

Sublines 26-3, 26-5 and 26-8, inoculated with 10^3, 10^5, 10^8 lower doses of SV40, respectively, died simultaneously with the control cell culture after 5 months of cultivation with signs of transition of diploid cells into the third phase. Signs of the third phase included appearance of detritis in cell cultures, slowing down of the growth and then its complete cessation. During the entire observation period no signs of transformation were found in 26-3, 26-5, 26-8 sublines inoculated with low doses of the virus.

Transformation of hamster embryo cells by SV40

A suspension of hamster embryo cells was inoculated with SV40 and after 30 minutes of contact with the virus was seeded into flasks. At 20 hours unadsorbed virus was washed off the cells. Transfers of the cell culture were made every 3–4 days (Avgustinovich et al. 1965, Avgustinovich and Chumakova 1965).

The maximum concentration of the virus used for inoculation of hamster embryo cells varied from 0.4 to 10 PFU/cell. Subsequent doses were decreasing 10-fold dilutions down to 10^{-6}.

Cell strains of hamster embryo cultures not infected with the virus consisted of elongated and polygonal cells (Fig. 9). They were maintained successfully in vitro for 1 $^1/_2$–2 months, then went into the third stage of development and died.

As early as 6 days after inoculation with SV40 (in the 1st passage) cells of hamster embryo culture showed a trend to an increase in the number

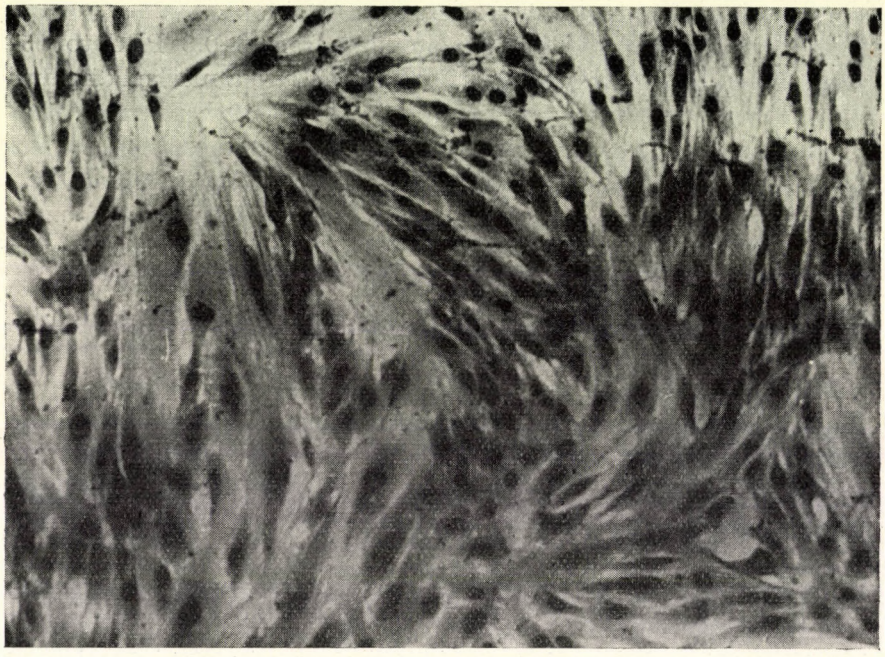


FIG. 9. Normal hamster embryo cell culture. ×113

of nucleoli in the subline inoculated with the highest dose of the virus (Table I). Later, 20–25 days after inoculation, a more rapid acidification of the nutrient medium and appearance of giant cells with deformed nuclei were noted (Table I). At 30–35 days, isles of enhanced cell growth gradually driving out cells of the monolayer could be seen in the culture (Fig. 10).

Minimal virus doses which caused transformation of hamster embryo cell cultures were 1 PFU/10 or 25 cells, respectively, in the two experiments made. Lower concentrations of the virus produced no transforming effect, and tissue cultures inoculated with these doses showed characteristics similar to those of control cell cultures and died simultaneously with the latter with signs of the third phase in 2–2 $^1/_2$ months.

TABLE I

Characteristics of hamster embryo cell culture (number of nucleoli in the cell nucleus and the number of giant cells)

Cell cultures	Per cent of cells with indicated number of nucleoli[1]			Per cent of giant cells
	1–3	4–5	5	
In the 1st passage				
Control	70.3	27.7	2.0	0
SV40-infected[2]	50.0	43.6	6.4	0
In the 2nd passage				
Control	68.8	26.1	5.1	0
SV40-infected	28.6	20.3	36.1	15

[1] per 1000 cells

[2] cell culture infected with the highest dose of SV40

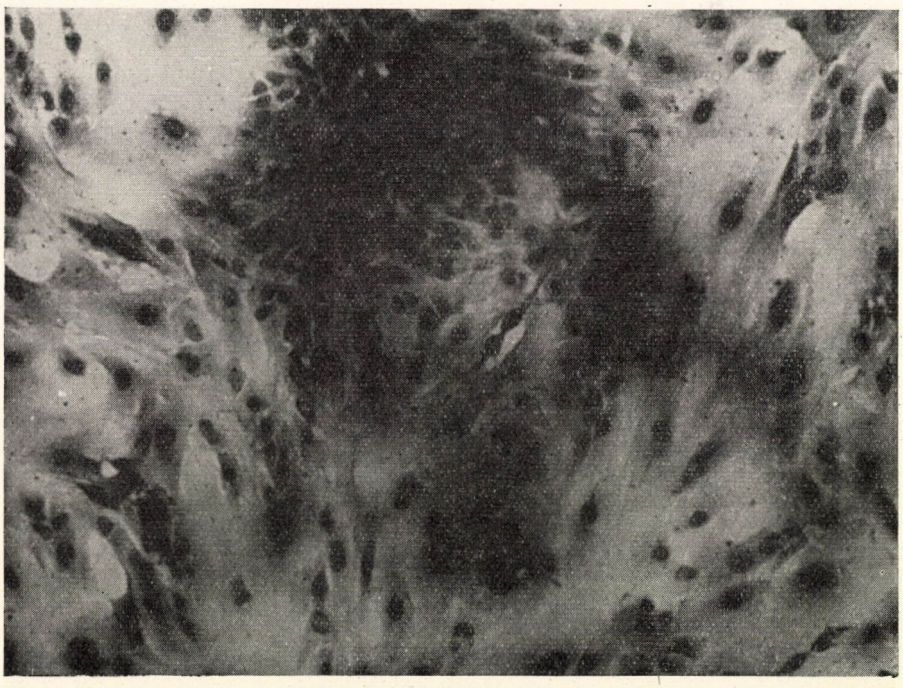


FIG. 10. SV40-transformed hamster embryo cell culture. The area of multilayer growth is visible. ×113

Hamster embryo cell cultures transformed by SV40 had some characteristics similar to those of transformed human embryo diploid cells. They differed from control cell cultures by marked polymorphism of cells, high numbers of giant cells with large deformed nuclei and multiple nucleoli, high mitotic activity. Karyologic studies also revealed marked changes: numerous abnormal mitoses and increased number of cells with polyploid and aneuploid number of chromosomes (Fig. 11).

Infective virus was isolated only within the first three days from the subline inoculated with the maximum dose of the virus. Subsequently

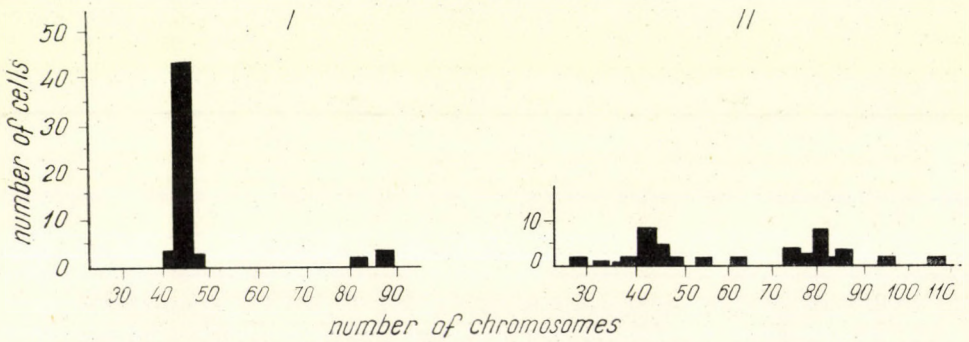


FIG. 11. Histograms of control and (I) SV40-transformed (II) hamster embryo cell cultures

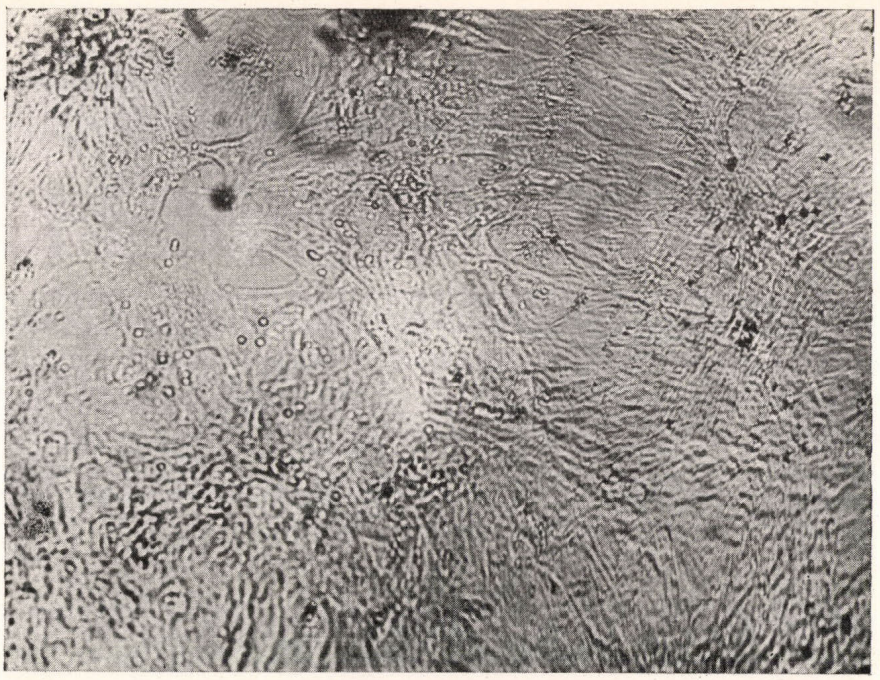
no infective virus could be recovered by means of the cytopathic effect titration.

Viral antigen detected by fluorescence was found in hamster embryo cell culture only during the first six days after inoculation.

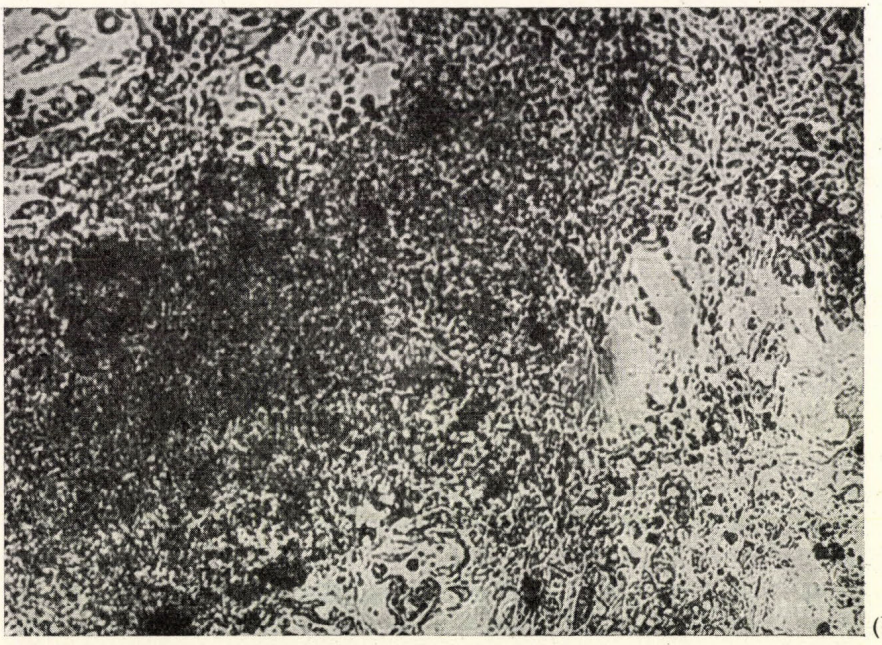
Viral antigen in the transformed cells was determined by means of the immunological test in white rats Wistar (Chumakova et al. 1964). Evidence of high antigenic activity of SV40 gave rise to the development of this test (Chumakova et al. 1963, 1963a). The test consisted in inoculation of white rats with live cells (6–24 million cells per animal) with an adjuvant and in measuring at 10–23 days of the presence of antibody in the blood serum of rats by means of inhibition of plaque formation by SV40. With this method it was possible to detect viral antigen in all transformed cultures during the period of their cultivation (Avgustinovich et al. 1965a).

Transformation of green monkey heart and lung cell cultures by SV40

Attempts were made to produce transformation of cell cultures by SV40. For this purpose cell cultures of those organs of green monkey were selected in which SV40 produced minimal destructive changes.



(a)



(b)

FIG. 12. Normal (a) and SV40-transformed (b) monkey lung cell culture

Transformation under the effect of SV40 was obtained in heart and lung cell cultures from green monkeys (Avgustinovich and Chumakova 1965).

First signs of morphological transformation in green monkey heart cell cultures were noticed 30 days after inoculation. During all this time the cell monolayer was maintained without transfer.

Definite foci of transformation in green monkey lung cell culture were observed 90 days after inoculation (the last 30 days the cell culture being maintained without transfer).

Some characteristics of transformed green monkey lung cell cultures were studied. Transformed cultures (Fig. 12) were capable of multilayer growth (a sign of disturbance in the process of contact inhibition), exhibited considerable polymorphism of cells, increased number of giant cells and amount of abnormal mitoses.

Infective virus was regularly recovered from transformed cultures. Fluorescent microscopy procedures also revealed regular presence of viral and tumor antigens in the transformed culture.

Neoplastic properties of in vitro transformed cells of hamster embryo

Morphological and chromosomal changes of cell cultures under the influence of oncogenic viruses as well as the capacity of transformed cultures of practically unlimited growth in vitro do not constitute sufficient criteria for the statement that transformation in vitro is accompanied by acquisition of neoplastic properties by the cells. At the present time the question whether or not transformation in vitro is accompanied by malignization may be solved only by transplantation of transformed cells to a genetically compatible host or, even better, to autochthonous one. In such cases the influence of the so-called transplantation antigens is excluded.

Because of the foregoing, experimental examination of oncogenic properties of in vitro transformed human and simian cells is difficult. The system of hamster cells is a convenient model for investigation of this question (Black and Rowe 1963b).

SV40-transformed hamster embryo cell strains as well as control embryo cultures were implanted to 3–6-week old hamsters into the subepithelial layer of the cheek pouch or under the skin of the back. Virus-transformed cultures in doses of 10–100 cells produced palpable tumor in hamster in 35–100 days after implantation (Fig. 13). The duration of the latent period depended upon the number of inoculated cells. In animals receiving 1–3 million cells from non-infected cultures of hamster embryo no tumors were found during 6 months of observation.

Histological examination of three tumors performed by A. P. Savinov showed the tumors to be fibrosarcomas.

Tumor cells were readily transplantable in vivo and in vitro. Cell cultures prepared from in vivo transplantable tumors did not differ from the original transformed culture by the number of giant cells, chromosomal

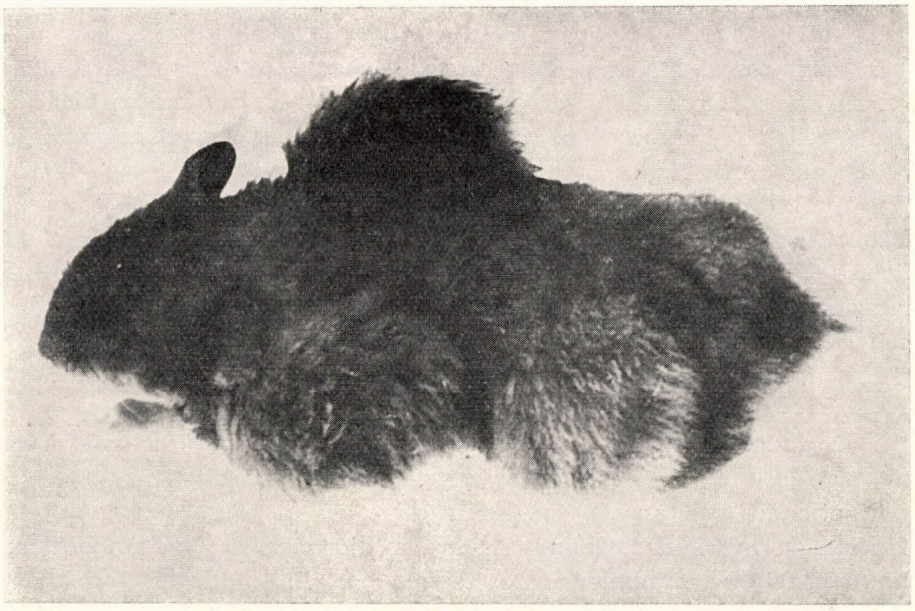


FIG. 13. Tumors in hamster after inoculation of 100, 1000 and 10 000 hamster embryo cells transformed by SV40 in vitro

characteristics and some other properties. Thus, it has been shown experimentally in the system of hamster embryo cells and SV40 that transformation of cells in vitro by the virus was accompanied by acquisition of oncogenic properties by the cells (Avgustinovich et al. 1965, Avgustinovich and Chumakova 1965).

Isolation of oncogenic virus from in vitro transformed cells and tumors

As was shown above, the oncogenic virus causing transformation may persist in transformed cell culture for a long time (green monkey lung cells, human embryo cells) or disappear (hamster embryo cells). In the latter case numerous attempts to detect the infective virus by means of conventional methods of virus isolation from disrupted cells were unsuccessful.

In some systems, for example tumors of mice induced by polyoma virus, even application of a number of stimulating effects such as X-irradiation, ultraviolet irradiation, exhaustion of cells did not result in isolation of the infective virus (Habel and Silverberg 1960, Winocour and Sachs 1961). The phenomenon of disappearance of oncogenic virus from the cell transformed by it is explained at present by irreversible integration of virus information with the cellular information.

In some cases, however, it is possible to achieve the synthesis of the infective virus from the so-called "virus-negative tumors" by placing the intact cells under study into a cell culture susceptible to oncogenic virus (Sabin and Koch 1963, Black and Rowe 1963a, Enders 1965, Black 1966). This method of mixed cell cultures suggested by Gerber and Kirschstein (1962) was used by us in some modification (Chumakova and Avgustinovich 1965) for isolation of SV40 from primary and transplantable in vivo hamster tumors and from transformed in vitro hamster embryo cells as well as for isolation of polyoma virus from transplantable in vivo and in vitro tumors of mice.

Mixed culture method for isolation of oncogenic virus. Virus-transformed in vitro cell culture or tumor tissue were treated with trypsin for production of cell suspension. After counting of cells and appropriate dilution in nutrient medium (Eagle's medium with 5% bovine serum) the tumor cell suspension was added to the indicator cell culture susceptible to the destructive effect of the oncogenic virus. Indicator cells for isolation of SV40 were green monkey kidney cells and for isolation of polyoma virus mouse, embryo and kidney cells (Fig. 14). The cells under test and indicator cells were allowed to contact in suspension for 18 hours at 4 °C or the cells under test were added to the monolayer of indicator cells. As a routine, we introduced 1 million of tumor cells into a flask monolayer cell culture or mixed with 5–10 million indicator cells suspended in nutrient medium.

After 3–4 days of combined growth of cells, a portion of cells from the mixed culture was transferred into a fresh indicator cell culture, another portion was disrupted and tested for the cytopathic effect of virus in tissue culture as well as in hemagglutination test and MAP-test (for polyoma virus) and in immunologic test in white rats (for SV40) (Chumakova et al. 1964). In this manner every passage of mixed culture was tested. In parallel immunologie tests in animals, intact transformed or tumor cells were studied and passages of transformed cells in vitro and in vivo were made (Fig. 14).

Tumor cells, as a rule, grew more rapidly than did normal indicator cells, therefore it was not possible to detect the cytopathic effect of the virus in such cultures with prevalence of tumor cells. Owing to this, we had to resort to indirect methods of detection of virus antigen — to immunologic tests in animals, i.e. to determination of virus-specific antibody in the animal blood serum in response to inoculation of the material under study. After detection of virus antigen the appropriate samples of cells from mixed cultures were broken by 3 cycles of freezing and thawing and

SCHEMA OF ISOLATION OF THE ONCOGENIC AGENT FROM THE TUMOR

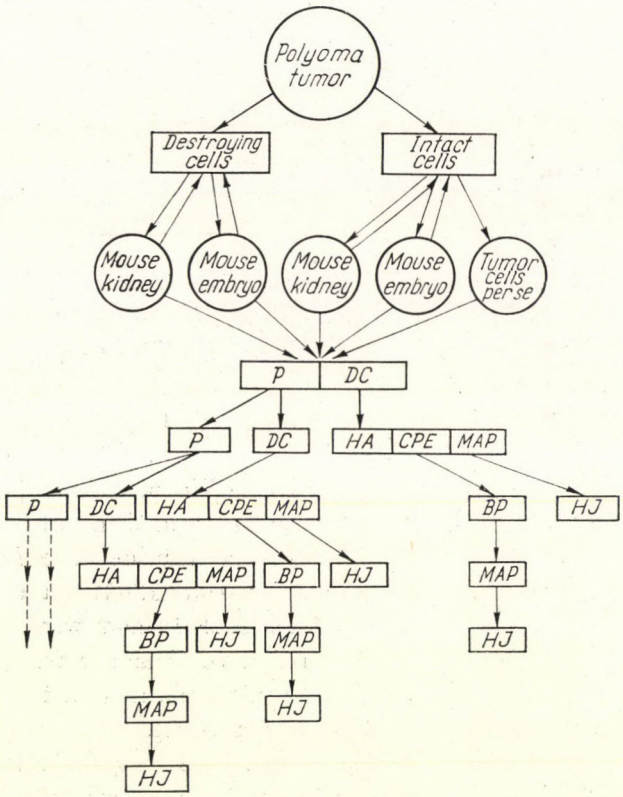


FIG. 14

P — passage of mixed culture; DC — destruction of cells; HA — hemagglutination test; MAP — mouse antibody production test; CPE — cytophatic effect test; HI — hemagglutination-inhibition test; BP — blind passage

inoculated into susceptible cells for longer observation, detection of the cytopathogenic effect, isolation and identification of the infectious agent.

Throughout all the experimental work on isolation of oncogenic viruses special precautions were observed to prevent contamination of cell cultures with laboratory virus strains, and careful control of animals and indicator cell cultures was performed in order to exclude spontaneous virus carrier state.

Comparison of methods for virus isolation. Two methods for isolation of oncogenic virus from tumor cells and in vitro transformed cells were compared: isolation of virus from disrupted cells and from intact cells grown together with indicator cells (Chumakova and Avgustinovich 1965, Tálas 1967). Results of this comparison are presented in Table II.

From primary hamster tumors induced by SV40 it was possible, as a rule, to isolate the infective virus in the usual manner: by inoculation of disrupted tumor cells into a cell culture susceptible to the virus. In some cases, however, the infective virus could be detected only by means of mixed culture procedure (tumors Nos 4 and 7).

Two transformed lines of hamster embryo cells were obtained by inoculation of the cell monolayer with SV40 (10 PFU/cell). Morphological transformation occurred on the 3rd week after inoculation with the virus. Infective virus could not be isolated from these transformed lines of hamster embryo cells (HT-SV40-1 and HT-SV40-2) by the conventional procedures of isolation from disrupted cells beginning with the 3rd passage after transformation. By the mixed culture procedure the infective virus was recovered in the 5th, 7th and 9th passages of HT-SV40-1 line and in the 2nd, 4th, 7th, 10th, 20th passages of HT-SV40-2 line (Table II).

Polyoma virus was isolated from two tumors[1] originally induced by polyoma virus (SE-1956-11c strain) in A/Sn strain of mice. Cells of the primary tumor were cloned in tissue culture and then underwent 13 passages in vivo. The last passage in vivo was in hybride mice AxABA/2/F_1 and AxCBA. The tumors were removed for tests 15–17 days after detection.

In the course of some passages SESO-c8 tumors were considered to be "virus-negative" since neither the infective virus nor antibody to polyoma virus in the blood of tumor-bearing mice could be detected by the CPE test or plaque formation test (Hellström 1963).

Infective polyoma virus was detected in passages of mixed culture with addition of fresh indicator cells: in the 1st and 3rd passages of MN-Py-1

[1] SESO-c8 tumors were kindly given to us by Dr. H. Sjögren (Karolinska Institute. Stockholm). In our studies these tumors were designated as MN-Py-1 and MN-Py-2,

TABLE II

Comparison of effectivity of two methods of virus isolation from transformed virus cells in vitro and from mouse and hamster tumors

Tumors or transformed cells		Number of isolates from 1×10^6 cells	
		intact a	disrupted b
Primary hamster tumors induced by SV40			
PHN-SV40-1[1]		2	1
PHN-SV40-2		0	0
PHN-SV40-3		4	1
PHN-SV40-4		3	0
PHN-SV40-5		4	2
PHN-SV40-6		7	4
PHN-SV40-7		5	0
PHN-SV40-8		4	4
PHN-SV40-9		0	0
PHN-SV40-10		3	5
Hamster embryo cells transformed in vitro by SV40			
HT-SV40-1	5p, 7p, 9p[2]	+[3]	0
HT-SV40-2	2p, 4p, 7p, 10p, 20p	+	0 (2p+)[4]
Mouse tumors induced by polyoma virus			
MN-Py-1[5]	13 p in vivo	2	0
	14 p in vivo	1	0
MN-Py-2	13 p in vivo	1	0
	14 p in vivo	0	0

[1] primary hamster neoplasm induced by SV40
[2] hamster transformed cells
[3] virus-positive passage, number of agents not tested
[4] 2 pandssage, virus-positive
[5] mouse neoplasm induced by polyoma virus

tumor cells and in the 2nd passage of MN-Py-2 tumor cells. Originally in all cases the viral antigen was demonstrated in the MAP-test in mice. After the 14th passage of tumor cells in mice polyoma virus was isolated only from MN-Py-1 tumor. In these cases tests with disrupted cells were completely negative (Table II).

Certain correlation was found between the number of isolates and the number of intact tumor cells tested (Fig. 15). Infective agents could be isolated only when at least 100 000 tumor cells were inoculated into the

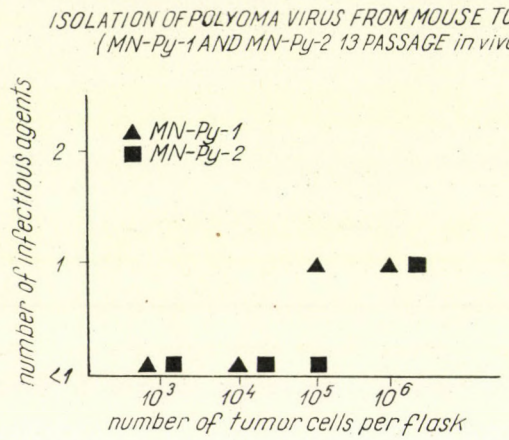


FIG. 15. Isolation of polyoma virus from mouse tumors

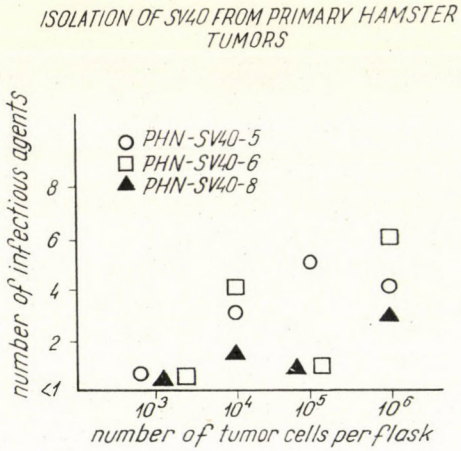


FIG. 16. Isolation of SV40 from primary hamster tumors

flask indicator culture (MN-Py-1 tumor). Since 10 such flask cultures were used, 1 million tumor cells were tested simultaneously and in such tests two infective agents were isolated (one each from two tumors).

Similar correlation was found in isolation of oncogenic virus from primary hamster tumors induced by SV40: with an increase in the number of simultaneously tested cells the number of isolates increased (Fig. 16).

All the isolates were identified either as SV40 or as polyoma virus. Investigation of properties of the isolates revealed no characteristics distinguishing them from strains of SV40 or polyoma virus inducing tumors or cell transformation.

Thus, comparison of the two methods for isolation of the oncogenic SV40 and polyoma virus from in vitro transformed and tumor cells of hamsters and mice in all cases demonstrated superiority of the mixed culture method: combined propagation of intact tumor cells and a culture of cells susceptible to oncogenic virus. From some tumors, however, infective SV40 and polyoma viruses could not be isolated even in tests with intact cells (Table II, PHN-SV40-2, PHN-SV40-9 and MN-Py2-14p tumors).

Isolation of SV40 from hamster tumor cells passed in vivo and in vitro. The influence of conditions of passage of tumor cells upon the success of isolation of infective oncogenic virus was studied on the model of hamster tumor cells multiply passed in vitro and in vivo.

Continuous lines of tumor cells were derived from hamster tumors primarily induced in vivo by SV40 (Table II). These lines were passed either in vivo in 3–4-week old hamsters or in vitro in tissue cultures.

Isolation of oncogenic virus was done by the mixed culture method testing 10 million tumor cells (1 million cells per flask indicator cell culture, 10 flasks).

From PHN-SV40-4 and PHN-SV40-5 tumors transplanted in vivo it was possible, as a rule, to isolate 1 to 6 infective viruses using 10 million cells. The exception was the 3rd passage of HN-SV40-4 tumor with which virus isolation tests were negative (Table III).

As has been shown above, in primary PHN-SV40-2 and PHN-SV40-9 tumors the infective SV40 could not be detected by any of the methods used (Table II). However, in two in vivo passages of PHN-SV40-9 tumor three infective agents were detected (Table III). In the course of 25 passages in vivo the PHN-SV40-2 tumor remained "virus-negative".

Passages of the same tumor cell lines in vitro exerted almost no effect on the results of infective virus isolation (Table III). In some cases of in vitro passages it was possible to isolate a bit more infective agents from 10 million tumor cells tested (PHN-SV40-4 and PHN-SV40-5 tumors). However all attempts to isolate the infective virus from the "virus-negative" PHN-SV40-2 tumor, even using cell passages in vitro, were unsuccessful. Three virus agents were isolated from initially "virus-negative" PHN-SV40-9 tumor as in passages in vitro as in vivo. All infective agent isolates were identified as SV40.

TABLE III

Isolation of SV40 from hamster tumor cells after passages of cells in vivo and in vitro

Tumors	In vivo		In vitro	
	No. of passage	No. of agents[1]	No. of passage	No. of agents
PHN-SV40-4	1	2	5	5
	3	0	10	4
	5	3	15	7
	7	7		
	10	5		
PHN-SV40-5	1	5	7	6
	3	4	20	5
	5	6	28	10
	7	4		
	11	6		
PHN-SV40-9	5	0	5	0
	10	1	15	2
	15	2	20	0
	20	0	30	1
PHN-SV40-2	5	0	5	0
	10	0	10	0
	15	0	15	0
	20	0	20	0
	25	0	30	0

[1] Number of isolates from 1×10^6 tumor cells

Thus, the above experiments demonstrated insignificant increase in the number of virus isolates in passages in vivo and in vitro of primary hamster tumors induced by SV40. The isolation rate was slightly higher with cell passages in vitro than in vivo. On the whole, considerable variability of the results of infective virus isolation from tumor cells was observed from passage to passage.

Changes in the sensitivity of hamster tumor cells to repeated inoculation with SV40 in the course of passages. It is known that virus-transformed lines of cell cultures and tumor cells may have different susceptibility

to repeated inoculations with oncogenic virus. The causes determining the extent of this susceptibility have not been elucidated completely.

Hellström J. et al. (1964) found certain correlation between the response of tumor cells to superinfection and their karyotype (tumors of mice induced by polyoma virus). Gershon and Sachs (1963), however, revealed no such correlation in the same system of cells.

A number of research workers observed changes in the relationships between the tumor cell and superinfecting virus. Thus, Winocour and Sachs (1962) and Gershon and Sachs (1963) found that tumor cells susceptible to reinfection acquired resistance in passages in vivo. They also found derivation of resistant clones from susceptible ones but never the contrary. Hellström et al. (1962), however, observed both transition of susceptible clones into resistant ones and resistant into susceptible ones. Gershon and Sachs explained this discrepancy in the observed results by the fact that they worked with "virus-negative" tumors, while Hellström and his collaborators studied cell clones spontaneously yielding infective virus.

We studied changes in the susceptibility of populations of SV40-induced hamster tumor cells to repeated inoculation with SV40 in passages in vivo and in vitro. The tumors were characterized by different isolation rate of the infective virus.

Tumor cell cultures containing 1.5×10^5 cells were inoculated with 1×10^5 PFU of SV40. After 3 hours of adsorption at 37 °C the virus was washed off and Parker's medium 199 with 2% bovine serum was added. Samples were tested for virus every 24 hours. Each sample consisted of 5 tube cultures infected with the virus. Before tests for the total virus (intracellular and extracellular) the cultures were frozen and thawed 4 times. During the test no changes of the nutrient medium in the cultures were made. The virus was titered by the plaque method in green monkey kidney cell culture.

The results of these tests are presented in Fig. 17. In passages of PHN-SV40-4 and PHN-SV40-5 tumor cells regularly yielding infective virus (Table III) a steady trend for increasing resistance of the cell population to SV40 was observed. This trend was particularly manifest in passages of the cells in vitro (the difference in sensitivity of PHN-SV40-4 cells in the 1st and 15th passages reached 2 log in titers of newly synthetized virus, Fig. 17a).

Increased resistance of the cell population to reinfection was also observed with PHN-SV40-9 tumor cells but on a lower level (the difference between the 10th and 20th passages in vivo was 1.5 log and between 15th and 30th passages in vitro 1.3 log in titers of newly synthetized virus, Fig. 17c).

Sensitivity of "virus-negative" PHN-SV40-2 tumor cells to SV40 remained almost unaltered during passages in vivo and in vitro, being intermediate among other cell lines tested with respect to titers of newly synthetized virus (Fig. 17d).

The amount of newly synthetized SV40 virus in most sensitive cell lines 4 days after inoculation was $10^{3.6}$ PFU/ml and in most resistant cells $10^{1.4}$ PFU/ml.

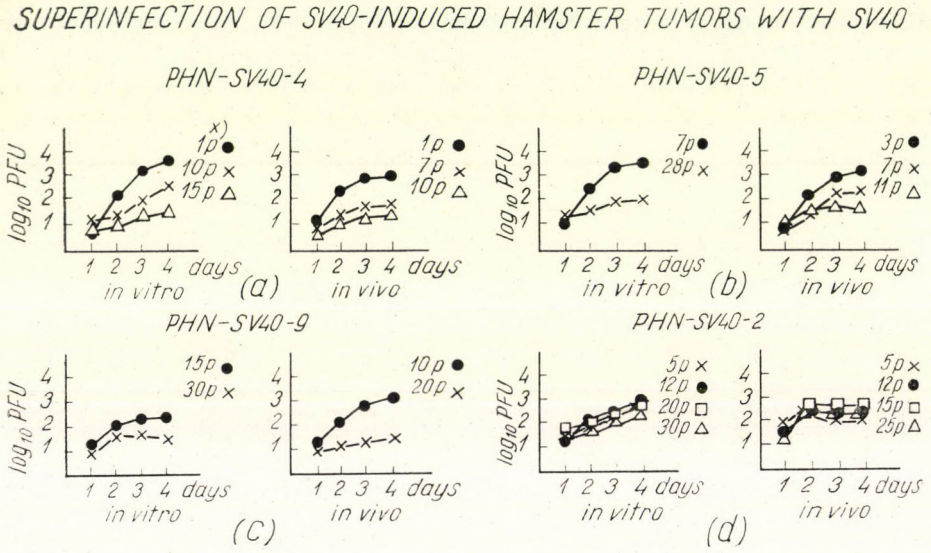


FIG. 17. Superinfection of SV40-induced hamster tumor cells with SV40

These observations of changes in the sensitivity of uncloned tumor cells to superinfection in the course of in vivo and in vitro passages revealed, first, predominant selection of resistant cells and, second, certain correlation between the extent of cell sensitivity and isolation of the infective virus, namely, with the increase in the number of passages and resistance of cells there was a slight increase in the number of oncogenic virus isolates.

Discussion

The interaction of oncogenic virus and cell in vitro may proceed in various forms and terminate either by death of cells due to virus multiplication or by transformation — alteration of the cell under the effect of virus. Frequently

both processes occur in the same cell population. The outcome of infection depends both on the properties of the cell and on conditions of virus–cell interaction.

Cells of different organs of the same animal react in different ways to inoculation with oncogenic virus. Inoculation of green monkey kidney cell cultures with SV40 results in degeneration of the cell population due to virus multiplication; however, the same dose of the virus inoculated into green monkey heart and lung cell cultures led in our experiments to chronic infection and transformation of cells.

In primary cultures of human embryo skin-muscle cells synthesis of SV40 occurs without marked lytic effect (Shein and Enders 1962a); cytopathic changes were observed only in the 2nd passage of cells at 17–23 days. However, in prepuce cells of a 4-day-old baby, SV40 produced the typical cytopathic effect 2 days after inoculation (Petursson et al. 1966).

The cause of different forms of oncogenic virus-cell interaction in cultures susceptible to the lytic effect of the virus may lie in the dose of the virus inoculum. By changing virus concentration, in a number of cases either transforming or lytic effect can be obtained. This was demonstrated in mouse cell culture infected with polyoma virus (Sachs 1962) and in green monkey cell cultures (Ponten 1964) and human embryo cell cultures (Mustafina 1966) inoculated with SV40. As a rule, reduction in the virus dose led to chronic infection in the cell population thereby favouring cell transformation.

The portion of cells undergoing transformation under the effect of DNA-containing viruses (polyoma virus and SV40) is extremely low. Even under conditions of multiple infection of one cell only about 1% of cells is transformed (Sachs et al. 1962, Stoker 1962, Black and Rowe 1963). Investigations of clonal cultures showed that low percentage of transformation cannot be explained by genetic inhomogeneity of the cell population since in clonal and mixed cultures the per cent of transformation was similar (Macpherson and Stoker 1962). It was suggested that low rate of transformation was associated with different physiological conditions of cells, i.e. that the virus could affect the cell only during a very short time in its development. Recently this hypothesis was confirmed experimentally. The susceptibility of hamster fibroblast cells of BHK-21 line to the transforming effect of polyoma virus increased when the cells were undergoing the mitotic cycle and approximately doubled in the end of interphase (Basilico and Marin 1966).

We made attempts to determine the minimum infective dose of SV40 capable of causing cell transformation in vitro. It was found that for human

embryo diploid cells the minimum transforming dose was 0.3 PFU/cell, and for hamster cell cultures a dose 10 times lower also caused transformation.

Visible morphological transformation of human diploid cell populations was observed 10 weeks, green monkey heart cell cultures 30 days, green monkey lung cell cultures 90 days, and hamster embryo cells 20–25 days after inoculation. Similar data were obtained by other investigators with human diploid cells (Koprowski et al. 1962, Rabson et al. 1962) and hamster cells (Black and Rowe 1963, Shein et al. 1963).

Transformation of human diploid cells by SV40 occurs slightly faster if by the time of inoculation the cells have undergone some passages in vitro or entered the third phase of their development. A possible explanation of this fact may consist in the presence in such cell cultures of chromosomal aberrations which are targets for the transforming effect of the virus (Jensen et al. 1963, Todaro et al. 1963), as well as in slow growth of these cultures conducive to selection of transformed cells (Jensen et al. 1963).

Transformation of individual cells occurs much earlier than can be seen in the entire population. SV40 may produce some effect on first division of human cells and cause chromosomal aberrations (Wolman et al. 1964); similar observations were made with polyoma virus in hamster cell cultures (Stoker and Macpherson 1961).

Cytological studies at the cellular level indicated that various forms of virus-cell interaction may be observed in infected cell cultures even before the period of morphological transformation of the cell population. In some cells marked cytopathic changes were seen: formation of inclusions, degenerative changes in the nucleus, absence of normal cellular organelles, deformation of mitochondria, breaks in the integrity of cytoplasmic membranes and similar changes leading to the death of cells. Other cells exhibited hyperthrophy and hyperplasia of the nuclei, disturbance of the cytoplasmic ratio, pleomorphism, changes in the karyotype, i.e. cytological changes such as those observed in tumor cells in vivo.

Registration of visible morphological transformation was possible when transformed cells occupied primary position in the cell culture. At this period the transformed cell population was characterized by disturbance of contact inhibition of motility of cells and mitoses (Stoker and Macpherson 1961, Vogt 1963). In all of our transformed cell cultures both phenomena were observed: isles of multilayer disoriented cell growth and increased rate of mitoses. These two phenomena, however, seem to be able to exist independently of each other, since in a number of cases in human and hamster cell systems we observed oriented cell growth in the absence of

contact inhibition of mitoses in the first stages of transformation. Similar observations were made also by Shein and Enders (1962a) in human kidney cell culture transformed by SV40.

In human and hamster cell cultures transformed by SV40 marked changes in the karyotype were observed which involved both changes in the number of chromosomes and their morphology. Almost all possible modifications could be observed (Mustafina and Karmysheva 1965). No specific changes characteristic only of SV40-transformed cell lines were revealed. The frequently observed endoreduplication of chromosomes might be the cause of appearance of polyploid cells (Cooper and Black 1963). In the process of cultivation the karyotype of transformed cells changes all the time (Mustafina 1966).

It is known that prolonged cultivation of cells in vitro even without any known carcinogenic factors (chemical or viral) may lead to transformation or malignization of cells (Earle 1943, Sanford 1958, Evans et al. 1958). This was demonstrated by the example of cultivation of normal mouse kidney line A/Sn (Chumakova et al. 1962). Here the only significant criterion of malignity of cell lines was their capacity to produce tumors in the appropriate host.

In some instance attempts were made to express the property of transplantability of cells in morphological characteristics in vitro (Vogt and Dulbecco 1963, Weisberg 1964). However for the system polyoma virus–hamster cells relationships between transplantability of cells, on the one hand, and morphological characteristics (Stanners et al. 1963) and cell karyotype (Defendi and Lehman 1965), on the other, could not always be revealed.

On the basis of the transplantability property of cells it was established that the process of transformation by viruses does not parallel the process of malignization of cells. It was shown in hamster embryo cells–polyoma virus system that virus transformation of cells may undergo a number of stages. Transformed cultures at different stages of transformation possessed transplantability of various degrees (Vogt and Dulbecco 1963). Cells recently transformed in vitro by polyoma virus as well as primary tumors in hamsters induced by polyoma virus possessed low transplantability. However, highly transplantable cells could be selected from these cell populations by a single passage in vivo (Sanford et al. 1959, Defendi and Lehman 1965).

Hamster cell cultures transformed by SV40 in our experiments possessed high transplantable activity in first passages after morphological changes. It was discovered in some experiments that inoculation of even 10 cells

from transformed culture induced tumors in hamsters. In this case malignization of cells occurred at a rather early stage of the development of transformed cell population.

All attempts to transplant human cells transformed by SV40 into heterogenous organisms were unsuccessful (Shein and Enders 1962b, Pontén et al. 1963, Ashkenazi and Melnick 1963). Limited survival of the cells was observed only in green monkey brain (Rabson et al. 1962) and in cheek pouches of hamsters treated with cortisone (Moyer et al. 1964). More definite proofs of malignant properties of SV40-transformed human cells were obtained in experiments in patients with disseminated forms of neoplasms (Jensen et al. 1964). In these cases intradermal inoculation of SV40-transformed cells caused formation of nodules in one allogenic and two autologous patients. Proofs of growth of transformed cells in vivo were obtained by detection of chromosome markers and by means of SV40-specific complement fixation test. Morphologically, the nodules were undifferentiated sarcomas which regressed in 8–12 days. Neoplastic growth in vivo was achieved only by using transformed lines which had undergone many passages in vitro after transformation and were in the stage of crisis. Thus, these experiments demonstrated once again that in vitro transformation may be accompanied by malignization of cells and that these two processes may be spaced in time.

The process of cell transformation by polyoma and SV40 viruses may be accompanied by disappearance of infective virus particles. According to virus-genetic conception of carcinogenesis (Zilber 1946, Zilber and Abelev 1962), this fact is interpreted as integration of virus and cell information, as a state similar to lysogeny in the system of "bacterial cell–moderate phage". At present this conception receives more and more experimental confirmations.

Most of the evidence of the existence of viral genome in the transformed cell was obtained by means of immunological procedures revealing the presence of virus-dependent products in the cell. In "virus-negative" tumors and cells transformed by polyoma virus the virus-specific complement-fixing antigens were found (Habel 1965). Similar antigens were detected in SV40 tumor cells (Black et al. 1963, Rapp et al. 1964, Habel et al. 1965). These findings are interpreted as a constant influence of viral genome in the virus-transformed cell. The presence of transplantation antigens specific for viral genome was demonstrated in tumors of mice and hamsters induced by polyoma virus (Habel 1961, Sjögren et al. 1961, Sjögren 1964), in cells transformed in vitro by polyoma virus (Defendi 1963) as well as in tumors induced by SV40 (Defendi 1964, Koch and

Sabin 1964). Using the test of resistance to transplantation as a possible genetic marker, Ting (1964) also obtained evidence of possible presence of viral genome in "virus-negative" tumors induced by polyoma virus.

A number of investigations in recent years established that under appropriate conditions it was possible to induce complete infective virus from tumors considered "virus-negative" before application of these methods. Induction of the infective agent was possible by means of growing tumor cells together with cells susceptible to oncogenic virus (Gerber and Kirschstein 1962). Using this mixed culture method (overlay technique), it was demonstrated that viral genome was constantly present in the majority of hamster tumor cells induced by SV40, causing under appropriate conditions the synthesis of infective virus particles (Gerber 1963, 1964, Sabin and Koch 1963, Black 1966).

Our comparison of two methods for isolation of oncogenic virus from tumors induced by polyoma and SV40 viruses and from transformed cells also demonstrated that the mixed culture method gave better results. By this method the infective polyoma virus was isolated from SESO-c8 tumor which had been considered "virus-negative" in the course of 13 passages in vivo.

At the present time it is difficult to say what does happen when the mixed culture method is used. Detection by this method in some cases of extremely little synthesis of virus particles cannot be excluded. Besides, in combined growth of tumor and virus-sensitive cells conditions may arise for induction of infective virus by genome of the virus present in "masked" state. Evidence obtained recently is in favor of induction theory: synthesis of infective virus in mixed cultures depends upon viability of tumor cells under study and their direct contact with indicator cells and may be enhanced by addition of cell fusion factor (Gerber 1966).

Thus, facts of isolation of infective virus from long-term passed tumor or in vitro transformed cells alongside with the evidence of the presence in cells of new virus-specific antigens and most recent experimental data on synthesis in tumor cells of messenger RNA of the virus (Fujinaga and Green 1966) confirm the hypothesis that genetic material of the virus in one or another form is constantly present in the transformed cell. Another question is, what is the role of this genetic material and whether its presence is necessary for maintaining the state of transformation and malignization or its mission is over after it had played the role of starting mechanism in the process of transformation of a normal cell into a malignant one. This question remains open so far. We have only separate facts at our disposal. No correlation was established between the presence of complement-fixing and trans-

plantation antigens and oncogenic properties of cells. In a number of systems it was shown that the presence of viral genome in transformed cell may affect the state of differentiation of the cell (Black et al. 1966) as well as determine its morphology (Temin 1960, Shein et al. 1963). In the system of mixed cultures of chick fibroblasts and hamster tumor cells transformed by Rous sarcoma virus non-infective genome was not only transmitted to tumor cells but was also responsible for their sarcomatous transformation (Sarma et al. 1966).

Transformed cells and tumors induced by polyoma and SV40 viruses constitute mixed populations always varying with respect to cell karyotype and susceptibility to virus superinfection. By analogy with lysogenic bacteria it could be suggested that resistance of tumor cells to superinfection may be used as an additional proof of integration of at least part of viral genome with the cell. However, investigation of clonal cultures of tumor cells induced by polyoma virus revealed no correlation between resistance of tumor cells and spontaneous isolation of virus determined by hemagglutination and plaque formation (Hellström 1963). Later, refined karyologic studies demonstrated the existence between the karyotype of clonal cells and their sensitivity (Hellström et al. 1964), confirming the evidence that the cell karyotype may determine the cell phenotype under certain circumstances (Vogt 1959). Clonal lines differing in their sensitivity to superinfection were found to be similar with respect to transplantation resistance phenomenon (Sjögren 1964). The discovered correlation of susceptibility of tumor cells to polyoma virus and vaccinia virus may probably indicate the interest of interferon in different resistance of cells to superinfection (Hellström and Hellström 1963).

In our passages of uncloned hamster tumor cells in vivo and in vitro a steady increase in the resistance of the cells to reinfection with SV40 was observed. In a number of cases the mixed culture method yielded a greater number of infective agents per 10 million of passages tumor cells than from primary tumors. However the isolation rate of virus varied so much from passage to passage that it would be quite unsubstantiated to suggest that the increased resistance was associated with selection of cells containing virus information.

The causes of predominant selection of resistant cells as well as the mechanism of resistance of tumor cells are not sufficiently clear. The available facts indicate that the immunity in tumor cells apparently differs in its mechanism from that to superinfection in lysogenic bacteria. Evidence of isolation of resistant clones from sensitive ones in the absence of a new virus infection (Gershon and Sachs 1963) serves as additional proof to this.

Conclusions

1. Transformation of human embryo diploid cells, green monkey heart and lung cells and hamster embryo cells in vitro was induced by SV40. Visible morphological transformation of the population of human embryo cells was detected in 10 weeks, of green monkey heart and lung cells at 30 and 90 days, respectively, and hamster embryo cells at 20–25 days after inoculation.

2. It was shown that, with other conditions equal, the outcome of oncogenic virus–cell interaction depended upon the dose of the virus inoculated. The minimal dose of SV40 capable of inducing transformation of human embryo cell culture was 1 PFU/3 cells and with hamster embryo cells 1 PFU/10–25 cells in the population.

3. Homotransplantation experiments demonstrated that transformation of hamster embryo cells is accompanied by acquisition of malignant properties by cells. Both transformation and malignization processes in this system were closely spaced in time.

4. In human embryo cells in vitro there occurs accumulation of SV40 particles from 1 to 25–30 PFU/cell and their release from cells. Morphological transformation of the population was detected at the period of maximum content of intracellular virus. In further passages of transformed human embryo culture a marked reduction of the amount of intracellular virus was observed, down to levels undetectable by the plaque method in 38–39th passages of the infected culture (7.5 months of observation).

Infective SV40 virus could be regularly recovered from transformed cultures of green monkey lung cells. No multiplication of SV40 in hamster embryo cells was observed.

5. Specific viral antigen determined by the immunofluorescence technique was found in SV40-transformed human embryo cells in the period of virus multiplication. In hamster embryo cells the viral antigen could be determined by the immunofluorescence only within the first 6 days after inoculation. However, by means of the immunological test in animals the viral antigen could be detected in transformed hamster cells throughout the period of cultivation.

6. Cytological analysis revealed early changes in human embryo cells inoculated with SV40 consisting in formation of oxiphilic DNA-containing inclusions, increase in the size and pyroninophilia of the nucleoli. In the transformed population there increased the number of large cells with giant nuclei in which nucleus–cytoplasm relationship was distorted, abnormal

mitoses appeared, cell karyotype was changed markedly (polyploidy and chromosomal aberrations).

Increase in the number of nucleoli, appearance of abnormal mitoses and change in the cell karyotype was also characteristic of hamster embryo cells transformed by SV40.

7. Electron-microscopic studies revealed that SV40 was accumulated in the nucleus of human embryo cell and the latter undergoes a number of changes (deformation of mitochondria, breaks in the integrity of the cytoplasmic membrane, lack of Golgi apparatus, etc.) which lead to the death of the cell.

In dividing cells of transformed human embryo culture seven-layer formations were found arrayed in parallel between chromosomes: paired canals of endoplasmic reticulum similar to multilayer structures of cells in some tumor tissues.

8. The system of tumor cells growing together with normal cells sensitive to oncogenic virus was shown to be favourable for induction of complete infective virus from "masked state". Our comparison of two methods for virus isolation from polyoma tumors of mice, SV40 tumors of hamsters and hamster embryo cells transformed in vitro by SV40 in all instances demonstrated better results by the mixed-culture method.

Modification of the mixed-culture method including application of the immunological test in animals was conducive to detection of infective virus particles.

9. Passing of tumor cells in vitro or in vivo was found to exert some influence of the frequency of induction of infective virus particles from tumor cells. Some increase in the number of oncovirus isolates from tumors in passages was observed as well as isolation of infective virus from primarily virus-negative tumors.

10. In passages of SV40 tumor cells of hamster in vitro and in vivo a marked trend for predominant selection of cells resistant to superinfection with oncogenic virus was established. The difference in titers of newly synthesized virus (after superinfection) between the 1st and 15th passage of the tumor in vitro was of the order of 2 logs (PFU).

11. Variations in results of isolation of the infective virus from tumors from passage to passage did not permit to associate an increase in the resistance of tumor cells with selection of cells containing virus information.

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ROUND-TABLE CONFERENCE ON INTERFERON AND ONCOGENIC VIRUSES

held on March 28 and 29, 1967

Organized by the Microbiological Research Group of the Hungarian Academy of Sciences and Co-ordination Committee 53

Participants

Prof. Dr. R. Manninger, Prof. Dr. J. Baló, Prof. Dr. Gy. Ivánovics, Prof. Dr. L. Váczi, Prof. Dr. Gy. J. Weiszfeiler (Chairman), Dr. E. Farkas, Dr. F. Fornosi (Secretary), research workers of the Microbiological Institute of the Medical University of Szeged, Microbiological Institute of the Medical University of Debrecen, Microbiological Research Group of the Hungarian Academy of Sciences, National Health Institute and Phylaxia Institute; Dr. Ch. Chany, Dr. E. Falcoff and Dr. Rebecca Falcoff from France, Prof. Dr. Z. Ermolieva, and Dr. N. Furer from the USSR.

Agenda:

March 28, 1967

1. Prof. Dr. Gy. J. Weiszfeiler (Budapest): Problems of studying oncogenic viruses and interferon (opening address)
2. Dr. E. Falcoff (Paris): Mass production of human interferon for therapeutic trials
3. Prof. Dr. Z. Ermolieva and Dr. N. M. Furer (Moscow): Experimental and clinical studies on interferon and interferon inducers
4. Dr. N. M. Furer, N. V. Pokidova, G. E. Weissberg, B. M. Nemirovskaya, V. P. Kuznetsov, N. A. Zhukovskaya and Prof. Z. V. Ermolieva (Moscow): Experimental studies of polysaccharides-interferon inducers
5. Dr. Gresser et al. (Paris): The effect on Friend's leukemia of interferon formed in the brain of mice (read by Dr. C. Chany)
6. Dr. I. Mécs (Szeged): Studies of the action mechanism of interferon
7. Dr. Gy. Hadházy, Prof. L. Váczi, Dr. L. Gergely and Dr. F. Tóth (Debrecen): Comparative studies relating to the interferon-formation capacity of normal and leukemic leucocytes
8. Dr. L. Géder, Prof. L. Váczi, Dr. F. Lehel, and Dr. E. Jeney (Debrecen): The significance of virus-induced early antigens in oncological research

9. Dr. I. Béládi, R. Pusztai, Dr. I. Mucsi and M. Bakay (Szeged): Production of interferon in chick embryo fibroblast cells inoculated with oncogenic adenoviruses
10. Dr. Margarita Tálas, Dr. Gy. J. Weiszfeiler, L. Bátkai and Helga Batka (Budapest): Production of interferon by polyoma virus in cultures of peritoneal macrophages and embryonal cells of hamster
11. Dr. I. Rostóczy and Dr. I. Mécs (Szeged): The effect of alkylating agents on viruses

March 29, 1967

Discussion of the lectures and co-ordination of further research work.

In his opening address, Prof. Dr. Gy. J. Weiszfeiler referred to the theoretical and practical problems of interferon research. He emphasized the importance of studying the interrelation of the effect of oncogenic viruses and interferon formation. He recalled the talks of March 11, arranged jointly by the Oncological Society and Co-ordination Committee 53, as a result of which the Microbiological Research Group of the Hungarian Academy of Sciences was asked to provide, within the scope of Principal Task 53 (Microbiological Research), the co-operative work connected with the study of oncogenic viruses; the Research Group agreed to it. This Conference was held with a view to accomplish the co-ordination tasks.

Dr. E. Falcoff gave a summary of the latest results connected with interferon research. His investigations, in which he aimed at a clarification of the mechanism of interferon formation, were of special interest. Simple methods have been developed for producing human interferon in leucocytes and amniotic cells, as well as for the titration of interferon. The observation (by Dr. Gresser et al.) that in Friend's leukemia the prolonged administration of interferon lengthens the survival period of the animals deserves attention. From the weight of the spleen of leukemic mice, the in vivo efficiency of interferon may be inferred. As a method, this provides a novel possibility of titrating interferon activity in vivo.

The reports of the Soviet authors (Prof. Dr. E. Ermolieva and Dr. N Furer) arose great interest. The results, achieved with interferon produced in human diploid cells and leucocytes, and with interferon inductors (UV virus, prodigiosan polysaccharide of bacterial origin) applied locally in various diseases of the eye and skin, are encouraging. The participants of the Conference are looking forward to further success in the application of the method to influenza. Experiences with the determination of the physico-chemical properties of interferon, and with the purification and concentration of interferon are of great importance and very useful. Results

connected with the determination and practical application of interferon inductors are promising.

The reports of the Hungarian authors* partly dealt with interferon formation, partly with the correlations of interferon and oncogenic viruses. The trend of further research work is determined by those results from which the conclusion may be drawn that during the formation of RNA virus-induced interferon the RNA synthesis of the virus does not cease (Dr. I. Mécs). The formation of interferon in chicken embryo fibroblast cultures has been observed also in case of infection with the type-12 adenovirus of oncogenic properties (Dr. I. Béládi). In addition to these, the effect of alkylating agents on interferon production has been studied (Dr. I. Rostóczy).

Reports of the research workers of the Microbiological Institute of the Medical University of Debrecen, according to which more interferon is produced in the leucocytes of leukemic patients than in those of healthy individuals, were heard with interest (Dr. Gy. Hadházy). Formation of the early antigen of the herpes simplex virus, and the occurrence frequency of antibodies to early antigen were studied in serological analyses and by means of immuno-fluorescence in the blood sera of tumorous and healthy individuals. It appears from the findings that antibodies can be demonstrated to be more frequent in tumorous patients than in healthy individuals. In tissue culture it was possible to isolate the formation of three antigens of the herpes simplex virus (Dr. L. Géder).

The Microbiological Research Group of the Hungarian Academy of Sciences (Mrs. Dr. M. Tálas) has found interferon production in the hamster's monocytes and chicken embryo fibroblasts after infection with polyoma virus. The problem is important in respect to the relationship between oncogenic viruses and interferon.

The lectures were discussed on 29th March. On the basis of this debate the following resolution was formed by the Conference. The French and Soviet guest researchers have spent another week in Hungary and during that time presented the methods employed by them to the laboratories of the Microbiological Research Group of the Hungarian Academy of Sciences; this is of extreme importance in respect of further progress in research work.

* Published: Mécs, I.: *J. gen. Virol.* **1** 25 (1967)
Hadházy, Gy., Gergely, L., Tóth, F. D. and Szegédi, Gy.: *Acta Microbiol. Hung.* **14** 391 (1967)
In press: Géder, L., Jeney, E., Gönczöl, É. and Lehel, F.: *Magyar Onkologia*
Rostóczy, I.: *Acta Microbiol. Hung.*

Resolution

The participants agreed that the Conference was most useful presenting scientific achievements, as well as determining the further tasks.

The studies of the French researchers (Falcoff, Chany and Gresser) relating to the mechanism of interferon formation are of theoretical importance, the methods of interferon production and in vivo titration are significant in respect to the improvement of laboratory techniques.

The accomplishments of the Soviet researchers (Ermolieva and Furer) are noteworthy in disclosing the prospects of the clinical-practical and prophylactic uses of interferon and the interferon stimulants; in the determination of the physico-chemical properties of interferon, and the chemical definition of interferon stimulants which are of importance in both theoretical and practical respects.

Hungarian researchers have achieved encouraging initial success in clarifying the mechanism of action of interferon, the interrelations between various oncogenic viruses, resp. transformed cells and interferon production, and early antigen formation (Microbiological Research Group of the Hungarian Academy of Sciences, Microbiological Institute of the Medical University of Szeged, Microbiological Institute of the Medical University of Debrecen).

For further increasing the successfulness of interferon research, the participants of the Conference consider necessary the solution of the following problems, and the realization of the following research plans:

1. For a more uniform evaluation of interferon preparations produced in the various laboratories, it is expedient to supply all laboratories with identical interferon preparations of relative high and stable titer. Comparisons should then be made everywhere with this preparation that is to be regarded as an international reference standard. As the first step, the French researchers have supplied the laboratories represented at this Conference with their preparations of human-leucocyte lyophilized interferon.

2. Taking into account available methods and equipment, and considering the far-reaching opportunities of research work, studies in the following subjects should be given priority at present:

(a) the mechanism of action of interferon;

(b) induction of interferon in vitro an in vivo (e.g. with viruses, polysaccharides, bacteria, endotoxins);

(c) relationship with oncogenic viruses, correlations between cell transformation and interferon formation, early antigen formation;

(d) the practical efficiency of interferon in the prophylaxis and therapy of virus diseases.

3. The participants of the Conference were pleased to learn that research work concerning interferon and oncogenic viruses has been started in Hungary (Microbiological Research Group of the Hungarian Academy of Sciences, Microbiological Institute of the Medical University of Debrecen, Microbiological Institute of the Medical University of Szeged, Virus Vaccine Distribution Department of the National Health Institute, Institute of Veterinary Hygiene of the Hungarian Academy of Sciences, Phylaxia Institute and that progress of a satisfactory rate can be ensured by international co-operation. It would be desirable that Hungary join in the studies of the practical applicability of interferon. But for such experiments sufficient quantities of interferon ought to be produced in Hungary, and suitable clinical material made available for trials.

4. At this Conference the researchers have discussed thoroughly and exchanged their experiences, whereby the basis for establishing close co-operation between Soviet, French and Hungarian researchers has been created. Mutual further exchange of information and, for this purpose, organization of periodic consultations (restricted conferences) are considered necessary. The next conference can be held 2 years hence, and the French participants have agree to arrange and convene it.

5. The participants deem it desirable that the papers and reports presented at the Conference and not published so far, be published in English in the periodical publication of the Microbiological Research Group of the Hungarian Academy of Sciences.

Finally, the participants of the Conference express their appreciation and thanks for having arranged the Conference, and suggest to Co-ordination Committee 53 that the proceedings summing up the conclusions of principles be mailed to:

(a) the foreign and home participants (institutes) of the Conference;

(b) all Hungarian institutions whose information on the problem of interferon, and the correlated problems of interferon and oncogenic viruses, is considered desirable.

MASS PRODUCTION OF HUMAN INTERFERON FOR THERAPEUTIC TRIALS

by

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The potential therapeutic use of interferon or interferon-like substances in viral diseases is based on several observations.

Interferon can be detected in the blood and tissues of patients and animals early in the course of viral disease (either natural or experimentally induced) and before the appearance of antibody.

Interferon inhibits the multiplication of antigenically unrelated viruses both in vitro and in vivo. Since at the onset of viral infection only a fraction of the total number of susceptible cells are infected, administration of interferon at an opportune moment may delay or inhibit the evolution of the disease, thus permitting full mobilization of the host's defenses.

In order to evaluate the therapeutic usefulness of interferon in viral diseases of man, it is necessary to produce an interferon of high biologic activity and in large quantity. Since interferon is specific for the species, cells derived from human tissues must be utilized. Of several different possible cell types we have investigated leucocytes and placental tissues. In this communication I will confine my remarks to the results obtained utilizing human leucocytes.

Proteins derived from blood have been utilized in the therapy of various diseases, and blood transfusion itself is a well-accepted procedure. Leucocytes may be easily separated from whole blood. Gresser has previously shown, and has been confirmed recently by others, these cells under appropriate conditions produce large amounts of interferon. We have attempted therefore to define a number of the optimal experimental conditions for the "en masse" production of interferon in vitro. Our results suggest the feasibility of producing interferon on a large scale for use in clinical trials. We will also present the results of experiments pertaining to the purification of interferon and the physico-chemical characterization of the active moiety.

Table I summarizes our basic technique for the preparation of interferon from leucocytes.

TABLE I

Preparation of interferon (human leucocytes)

1	Citrated blood (different donors) + dextran sediments 1 h — 25 °C
2	Upper $^2/_3$ harvested and centrifuged
3	Leucocytic sediment inoculated with parainfluenza virus I (M 100 : 1) — 37 °C — 3 h
4	Lysis of erythrocytes ensues, recentrifugation and resuspension of leucocytic sediment in nutritive medium
5	Incubation 22 h — 37 °C
6	Centrifugation. Supernatant treated at pH 2 24 h and adjusted pH 7
7	Ultracentrifugation. Filtration
8	Various biologic tests: i.e. activity, sterility, etc.

Citrated blood from several donors is mixed (without taking into consideration the blood type). After sedimentation of most of the erythrocytes by means of dextran, the leucocyte rich plasma is centrifuged and the cellular sediment inoculated with parainfluenza virus I (Sendai) at a multiplicity of 100. During infection the contaminant erythrocytes are agglutinated by the virus and then completely lysed. After incubation for 3 hours, the leucocytes are washed, resuspended in a medium containing human serum and incubated at 37 °C for 22 hours. The medium is then centrifuged and the pH of the supernatant adjusted to 2 and then to 7 prior to ultracentrifugation and filtration. The filtrate constitutes our interferon preparation.

Table II illustrates the optimal conditions for the production of interferon from leucocytes in this system.

TABLE II

Optimal conditions of interferon production

Number of leucocytes/ml of suspension	5×10^6
Multiplicity of infection	100
Time of incubation	22 h
Concentration of human sera in the nutrient medium	5%
pH	7.4—7.5
Temperature of incubation	37 °C

In the point of view of the possibility of utilizing interferon in clinical trials we have also developed a method of semi-purification of interferon.

This technique can be easily adapted to different volumes of interferon and provides high yields of biologically active material. Techniques of purification previously described utilizing CM cellulose or CM Sephadex in chromatographic column are not suited for large-scale production of interferon for several reasons.

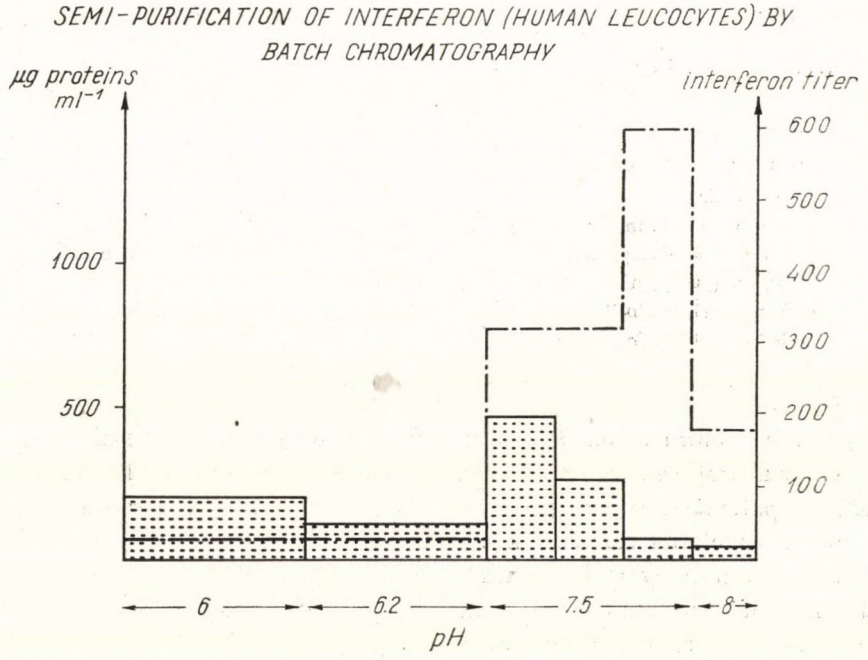

FIG. 1

1. The narrow relationship between the volume of the sample and the volume of the resin
2. The necessity of slow elution
3. The dilution of biologic activity of the initial preparation.

Batch chromatography, the technique which we will describe, provides a partial purification (30–40-fold) and avoids these 3 limitations of column chromatography.

Crude interferon is adsorbed to carboxymethyl CM Sephadex in a beaker containing a magnetic agitator. The resin is separated by centrifugation and washed in various phosphate buffers with increments of pH and molarity. By this technique, most of the extraneous protein is eliminated.

Figure 1 summarizes the result of batch chromatography. The broken line represents titer of interferon. The full line represents the amount of

proteins in each fraction. Under these conditions the biological activity of interferon has been increased 40-fold with a loss of only 20% of the initial total interferon.

Table III illustrates some of the physico-chemical properties of this semi-purified interferon.

TABLE III
Physico-chemical properties of interferon
(human leucocytes)

Molecular weight	25 000
Iso-electric point	6.75
Density in cesium chloride gradient	1.17
Actinomycine (1 γ/ml)	action inhibited
Trypsin (200 γ/ml)	destroyed
RNA'ase (100 γ/ml)	not destroyed
DNA'ase (100 γ/ml)	not destroyed

We have administered the unpurified interferon to several patients with known viral and suspected viral diseases, considered to be incurable. In all, 28 patients have received interferon for prolonged periods of time

11 acute myeloblastic leukemia
7 acute lymphocytic leukemia
2 cases of herpes zoster in leukemic patients
7 new-borns with cytomegalic disease
1 new-born with generalized herpetic infection.

Table IV contains the list of 11 patients with acute myeloblastic leukemia. Our preparation of crude interferon was essentially well tolerated by these patients, even when a total of more than 3 liters were administrated in the course of one year (patient No. 11).

As to the survival of patients with leukemia treated with interferon, we cannot at this time draw any conclusion.

In 7 new-borns the intravenous injections of 40 ml per day was well tolerated for 3 weeks. In some of the other 20 patients chilly sensations were experienced 30 minutes after injection of interferon. This constituted the only un-toward reaction. In only one instance was administration of interferon for a prolonged period of time accompanied by any evidence of a significant allergic reaction. It is possible that partial purification of interferon will eliminate the pyrogens produced by viral infected leucocytes.

TABLE IV

Administration of interferon to 11 patients with acute myeloblastic leukemia

Patient	Age (years)	Duration of treatment (days)	Total amount of interferon (ml)	Survival (months)
1	55	80	270	> 11
2	59	15	180	9
3	67	3	90	> 8
4	59	30	620	> 8
5	19	130	1280	> 7
6	14	50	660	> 9
7	11	16	330	7
8	15	310	2280	16
9	12	360	2200	> 12
10	18	400	2000	16
11	6	400	3620	14

Interferon inoculated intravenously (10–40 ml), daily or twice weekly

Although we have observed any case of serum hepatitis in our study, this danger remains. It is possible, however, that the techniques employed to separate the inducing virus from the interferon preparation have also eliminated potential contaminating viruses.

As to the therapeutic value of interferon it is too early to form any opinion. The preliminary results seem encouraging since the 7 new-borns with cytomegalic disease have survived and this disease is usually fatal. Another new-born with generalized infection due to herpes simplex virus seemed to improve transiently after receiving interferon.

We must however also refrain at this time from commenting on the effect of interferon on patients with leukemia; although the survival of some of these patients have been prolonged.

The preliminary clinical trials, however, show that intravenous administration of interferon derived from human leucocytes is feasible and without danger. It is hoped that these studies will form the basis for further trials with preparations of interferon of greater potency and greater purity.

EXPERIMENTAL AND CLINICAL STUDIES ON INTERFERON AND INTERFERON INDUCERS

by

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Ten years have elapsed since the discovery of interferon by Isaacs and Lindenmann. Since then many scientists, working at institutes in various countries, have studied the biosynthesis of interferon in cell culture and in animal organisms as well as the purification and physical-chemical properties of interferons; they have also explored the mechanisms of interferon production and the mechanism action. Research workers have thoroughly examined the spectrum of the antiviral action of interferon and its effect in experimental infections of animals, and made the first steps in its clinical trial.

Many interesting and laborious investigations have been carried out and much has been elucidated. In view of the extreme complexity of the problem, however, a number of its aspects still remains to be solved.

As soon as interferon was discovered it gave rise to great hopes for its clinical application. Its wide spectrum of antiviral action, absence of toxicity, and antigenic properties, absence of emergence of resistant forms in its application all these properties made it possible to assume, that it might prove a promising preparation for the clinical use.

Now it has been established, that interferon can be used in two ways against viral infections: firstly, by exogenous interferon application and secondly, by the stimulation of interferon production in the organism.

In the course of the last years we obtained interferons in chick embryo in tissue culture and in animal organisms not only by means of various infective and inactivated viruses (UV influenza viruses) but also with biologically active polysaccharides. Thus after the introduction of prodigiosan, interferon was obtained in tissue culture as well as in chick embryo while in allantoic fluid interferon accumulated in high titers.

Active interferon has been obtained from various strains of diploid human cells, obtained by R. I. Rapoport and on Wi-38 strain. Various myxoviruses, arboviruses and prodigiosan have been used for the stimulation of interferon production.

The activity of the diploid interferon in assay on diploid cells by the inhibition of the cytopathic effect of Semliki Forest virus reaches 250 units.

A still more active interferon has been obtained from human leucocytes by the method of E. Falcoff, R. Falcoff, H. Fournier and Ch. Chany.

In our experiments the leucocytes, after contact with the Newcastle disease-virus for 3 hours, were introduced into Eagle's medium or into 199 medium (t = 37 °C), where the interferon-production took place. After 22 hours the leucocytes were removed by centrifuging; in the remaining medium, containing interferon and virus, the inactivation of virus was carried out by acidification by bringing the medium to pH 2.0.

The interferon activity was determined on diploid human cells and varied within 400–600 units per ml in assay by inhibition of the cytopathic effect of Semliki Forest virus.

In in vivo tests for treatment of experimental herpetic keratitis of rabbits we used rabbit interferon and UV-viruses. The infection of the cornea was affected by a 10% suspension of mouse brain, infected by the virus of *Herpes simplex*. The investigated preparation were introduced by instillation in the rabbits' right eyes, the left eyes serving for control. In each experiment in the eyes of two rabbits placebo was introduced (medium, on which interferon was prepared).

When introducing different UV-virus and rabbit interferon, a considerable delay was observed in the development of experimental herpetic keratites as compared with the control, clinical recovery resulted within 10 to 12 days. By the same time the rabbits, that received placebo, showing remnants of inflammation intensive infiltration and oedema were observed even some rabbits had a severe ulceration of the cornea.

After the reduction of inflammation (20–25 days) there remained stable opaqueness of the cornea. Figure 1 shows the eye of a rabbit, treated with interferon for 12 days—the eye is normal, no signs of inflammation. Figure 2 is the left control eye of the same rabbit, the eyes are heavily affected.

Figure 3 shows the eye of a rabbit treated by UV-virus, the eye is normal, while on Fig. 4 the control eye of the same rabbit is seen heavily affected.

In clinical trial of interferon in eye and skin viral diseases, as well as in influenza prophylaxis, the above mentioned inducers and interferons produced by human leucocytes and diploid human cells were used.

UV-virus and leucocytary interferon were used for treatment of patients suffering from various forms of herpetic keratitis. UV-virus has been

applied to 30 patients. The preparation was introduced by way of frequent instillations, 6–8 doses daily. The treatment proved to be effective in case of all patients with superficial forms of keratitis. It is noteworthy, that UV-virus was effective in two cases of deep keratitis when previous treatment by kerecide (5'-iodo-2'-deoxyuridine) gave no effect.

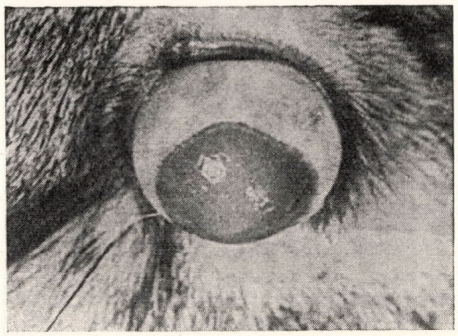

FIG. 1

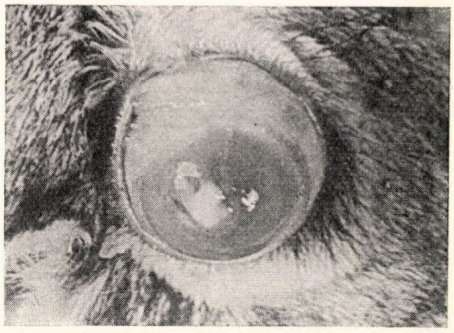

FIG. 2

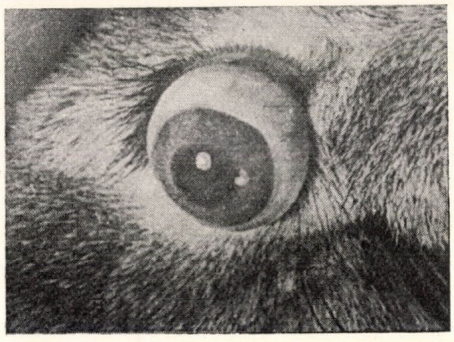

FIG. 3

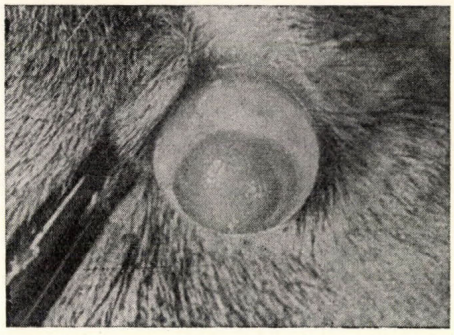

FIG. 4

Clinical recovery of patients, treated with UV-virus, came after 7–14 days, i.e. approximately within the same period as when treated by 5'-iodo-2'-deoxyuridine. A similar curative effect was observed with the first patients who received leucocytary interferon; however in view of the still limited number of patients treated so far, no final conclusions can be drawn as yet.

In a skin disease clinical diploid and leucocytary interferon as well as UV-virus-inducer of endogenous interferon, were applied to 255 patients. As placebo was applied medium, used to prepare interferon and UV-virus. The interferon was applied in the form of a 50% ointment, which was

administered to the affected areas 3–4 times daily and also used for lotions and gurgling mixtures depending on the localization of the affected areas.

All the preparations proved effective for treatment of cases of herpes labialis and herpes progenitalis (caused by the *Herpes simplex* virus), by disease caused by the *Herpes zoster* virus and in the treatment of aftous stomatitis.

Improvement in the condition of the patients was observed during the very first days after interferon application: the blisters dried up, hyperemia, pain and itching diminished and the periods of recovery diminished to $^1/_3$ or $^1/_4$ of the original length of time.

A noteworthy fact is, that the pain, caused by *Herpes zoster* virus disappeared already after 3 or 4 days' treatment, whereas patients treated by other methods sometimes felt pain even several months after the skin disease disappeared.

In cases of *Herpes simplex* especially effective results were obtained by the use of leucocytary interferon: in some cases recovery was observed on the second day after the treatment began.

In all cases the remission period became much longer up to 6 months (with patients who suffered from monthly inflammations).

In the treatment of a disease, presumably of virus etiology, among others, of pemphigus epithalization began much quicker, then when placebo was applied on affected areas of the same patients, with patients, suffering from Gallopo acrodermatitis a reduction of painful sensations and inflammation, and disappearance of peelings were observed, on the average, within 7–10 days, whereas treatment by any other means proved completely ineffective.

Thus treatment of local virus infections by interferon and UV-virus has produced an undoubted therapeutical effect.

The prophylaxis of UV-virus and the polysaccharide prodigiosan in influenza was carried out in six organized collectives.

Altogether 3190 people were examined. In three collectives 471 people received UV-virus and 324—placebo (physiological solution). In the other 3 collectives 1002 people were treated with prodigiosan (a bacterial polysaccharide) and 1393 were left for control.

A dose of 0.25 ml of the preparation was administered intranasally by a sprayer once daily with 3–4 days intervals. Observations for incidence of disease in experimental and control groups were carried out beginning with the second day after the introduction of the preparation and up to the 5th day after its last application.

The summary effectiveness coefficient in respect of inner control was 3.0 and in some collectives it amounted up to 4.4 and 5.3.

In the prophylactic by prodigiosan in an epidemiological experiment the summary coefficient of effectiveness was 2.0. In some collectives, however, it fluctuated from 1.9 to 9.0.

Thus, such interferon production inducers as UV-virus and prodigiosan resulted in a definite 2.5–3 fold statistically proved reduction of influenza incidence.

The application of leucocytary interferon for influenza prophylaxis with children of preschool age in closed collectives also produced good results. According to data, obtained by clinicists (N. M. Zlatkovskaya) the incidence of influenza ceased immediately in three groups, while in the control groups of the same collective, a great number of sick children was registered (up to 40%).

Thus, even the use of native preparations of human interferon proved effective in local application for the treatment of eye and skin virus diseases.

Promising results have been obtained in influenza prophylaxis by application of inducers of endogenous interferon production.

Through the common efforts of scientists from all countries of the world we must soon get highly active, purified interferon preparations and powerful inducers of endogenous interferon-production for the fight against many virus infections.

TABLE I

Interferon human in the treatment of viral dermatosis
(190 patients)

Disease	Leucocytic interferon		Diploid interferon		Other methods of treatment	
	Amelioration	Recovered	Amelioration	Recovered	Amelioration	Recovered
	Duration (days)		Duration (days)		Duration (days)	
Herpes simplex	1—2	3— 4	2	3	7—10	
Herpes zoster	2—3	6—10	3	8—9	20—40	
Stomatitis aphthosae	2	6— 7	2	5—7	10—15	
Acrodermatitis Gallopo	3—4	8—10	—	—		

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EXPERIMENTAL STUDIES OF POLYSACCHARIDES-INTERFERON INDUCERS

by

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The works of Isaacs, Cox and Rotem, Kleinschmidt and Murphy, Youngner and Stinebring, Rytel, Shope and Kilbourne, Ermolieva and Furer have shown that cells of animals in vitro and in vivo produced interferon not only in response to adsorption of viruses, but also to introduction of foreign RNA, various bacteria, bacterial endotoxines, the polysaccharide statolon, the antibiotic helenine, the polysaccharides acetoxan, prodigiosan and polysaccharides obtained from several actinomycetes.

Prodigiosan obtained from *B. prodigiosum* by Ermolieva and Waissberg is a lipopolysaccharide of high molecular weight (4.7×10.6). It has been found to contain glucose, mannose, galactose, a small amount of aminosugar, a few amino acids and about 15% of lipides.

After parenteral introduction in animal organisms this non-toxical polysaccharide (the toxic dose 300 time exceeds the effective one) stimulates in the organism protective reactions intensifies the activity of hypophysis and the surrenal cortex (A. I. Braude) of phagocytary activity of the reticuloendothelial system and of the phagocytary activity of leucocytes. It also causes a change in the relationship of blood proteins in favour of gamma-globulins, the stimulation of immunogenesis etc. All these enhances the resistance of the organism to a number of pathogenous effects.

Interferon production in tissue culture was studied by introducing prodigiosan in a 48-hour cell culture of chick embryo (100–200 μg/ml of polysaccharide). After an incubation period of 24–48 hours the interferon activity was 20–30 units in plaque reduction assay against vaccinia virus.

In in ovo tests, when from 100 to 1000 μg of prodigiosan was introduced in the allantoic cavity of a 13-day old chick embryo, an accumulation of interferon, within the limits of 100 units after 48-hour incubation, was obtained in the allantoic fluid.

In chick embryo prodigiosan not only induced interferon production but also intensified its formation in subsequent infection of the embryo by influenza virus; a synergide effect was observed in such cases.

For all interferons, obtained by us in cell culture as well as in chick embryo and in animals, methods of purification and concentration were developed.

In 1964 we proposed a method of purification of chick allantoic interferon, obtained through the challenge of chick embryo by different mixoviruses. This method includes various ways of purification proposed by Lampson and Merigan and is based on preliminary concentration of native

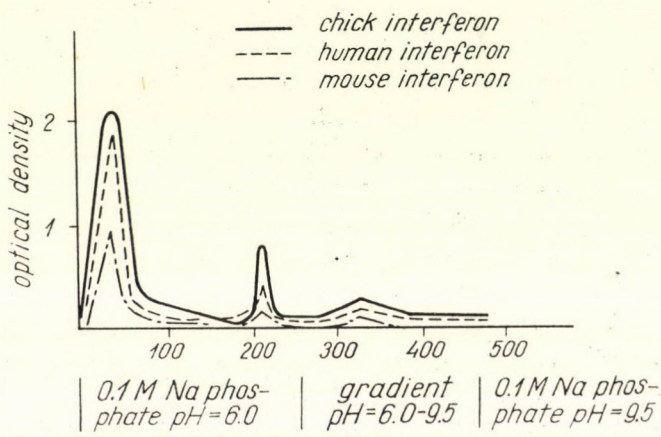

FIG. 1

interferon by zinc acetate and subsequent chromatography of CM Sephadex with pH gradient from 6.0 to 9.5, 0.1 M phosphate buffer.

In column chromatography of interferon zinc complex we employed C-50 Sephadex, which has a greater permeability than C-25 Sephadex, and therefore facilitates the separation of interferon from inert proteins. The degree of purification reaches 6000.

Later on we employed this method for purification of mouse interferon, obtained in vivo in mouse serum by different inducers of interferon production, as well as for purification of interferon, obtained from human leucocytes.

The data received from these investigations showed that chick, mouse and human interferons, obtained in different conditions, possess similar chromatographic profiles, as is shown in Fig. 1.

The determination of molecular weights of all these interferons was made on G-100 Sephadex columns, calibrated by proteins with known molecular weights (gamma-globulines, serum albumines, carboxipeptidase,

chemotrypsine, trypsine, RNA-ase). The dependence between the logarithm of the eluate volume was shown by a straight line (Fig. 2).

As shown in Fig. 2, after prodigiosan was introduced in the chick embryo, various molecular species of interferon were secreted into the allantoic fluid. Thus, 4 hours after the introduction of prodigiosan there an active fraction of 80 000 m. w. was discovered, whereas after 10 hours already two protein fractions appeared, which proved to be 65 000 and 35 000, respec-

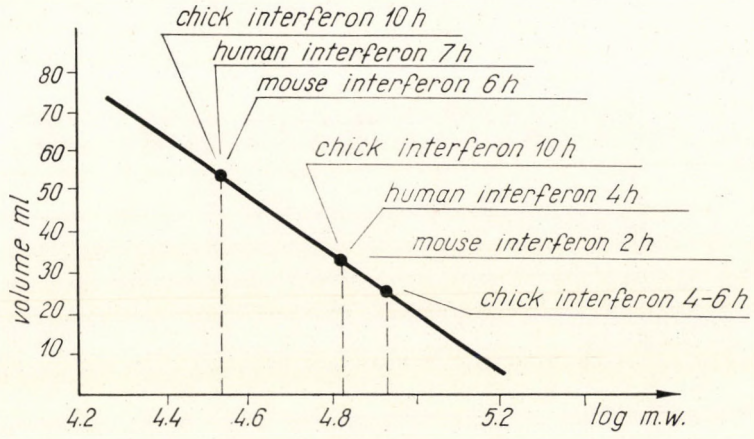

FIG. 2

tively. 24 hours after the introduction in the allantoic fluid interferon of 25 000–30 000 molecular weight was found to prevail.

In mouse serum, 2 hours after infection by NDV or prodigiosan introduction interferon of 65 000 molecular weight was found only, whereas after a longer period the bulk of antiviral activity was found in the fraction of 35 000 m.w., but interferon of higher molecular weight was simultaneously found in the blood.

The presence of interferon of higher m.w. 2 hours after the introduction of several inducers is explained by Youngner and Stinebring that directly after the introduction of the inducers "preformed" heavy interferon appears in the serum, while later on, interferon is formed in the cells of the reticuloendothelial system. The result of our experiments shows, that interferon produced in ovo and in leucocytes during the first hours is also of higher molecular weight. It is possible, that in animal organisms, too, interferon is not preformed, but its synthesis and activation take place in several stages, beginning with a higher molecular weight.

In in vivo experiments we investigated the effect of endogenous interferon during the process of viral infections in animals.

For interferon inducers, we tested NDV and influenza viruses, prodigiosan, acetoxan and polysaccharides, obtained by V. A. Ziganov from various actinomycetes.

The assay of interferon production showed, that the highest concentration in serum was induced by intravenal or intraperitoneal injection of NDV. The interferon level in the mouse serum was 5–10 fold lower after prodigiosan introduction than after NDV. However, in protection of mice from virus

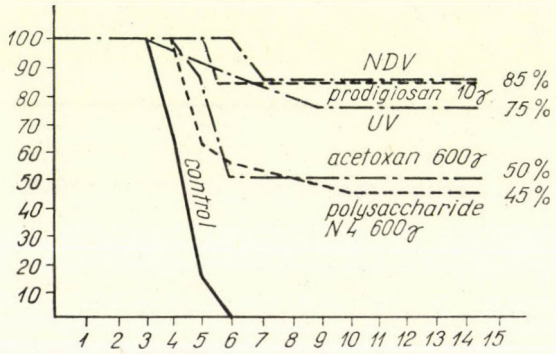

FIG. 3

infection the intraperitoneal introduction of prodigiosan proved equally effective as NDV (Fig. 3).

After the mice were challenged with 1000 ID EMC virus (i/p) 100% control mice died on the 6th day, while after prophylactic injection of NDV or prodigiosan (10–20 μg) up to 85% of the animals survived. After the injection of influenza UV virus, 75% of the animals survived.

When 600 μg of acetoxan or 600 μg of various polysaccharides, obtained from actinomycetes, were injected the percentage of survival was 50% and 40–60%, respectively (Fig. 3).

After an intraperitoneal injection of 10 μg of prodigiosan at different intervals before the mice were challenged by EMC-virus, 80% of survival was observed even when prodigiosan was introduced 72 hours before the virus challenge. It is noteworthy, that the injection of prodigiosan simultaneously with the virus protected 45% of the mice, whereas 100% control mice died.

When using inducers of endogenous interferon production for parenteral introduction, it is highly desirable to employ inducers of non-viral etiology, of non-protein nature without antigenic properties.

It is possible, that the means of protection of the organism from a number of virus infections may be found among biologically active polysaccharides, enhancing various protective properties of the organism, are not antigenic and at the same time act as inducers in endogenous interferon production.

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PRODUCTION OF INTERFERON IN CHICK EMBRYO FIBROBLAST CELLS INOCULATED WITH ONCOGENIC ADENOVIRUSES

by

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Since the discovery of the interferon by Isaacs and Lindenmann (1957) it seems to be a general characteristic of living cells of many animal species the production of interferon as their response to virus infection. Of the oncogenic viruses the polyoma virus was first described as interferon inducer (Allison 1961). Later in the cells infected with Rous sarcoma virus (Bader 1962), and SV40 (Diderholm 1963) interferon production was also observed. Meanwhile the adenovirus was considered to be unable to induce interferon (Larke 1966).

We found the occurrence of interferon production in chick embryo fibroblast cells inoculated with human adenoviruses (Béládi and Pusztai 1967). The human adenoviruses do not grow in these cells, neither structural viral antigen nor infective virus could be demonstrated (Levinthal et al. 1966). The interferon induced by adenoviruses has similar properties like the interferon elaborated in chick cells by other viruses (Béládi and Pusztai 1967).

The adenovirus types were grown in HeLa or H Ep-2 cells. Their infectivity was determined also in these cells. The $TCID_{50}$ was calculated using the formula of Reed and Muench.

Sindbis virus was grown in primary chick embryo fibroblast cells used for challenge. The infective titer of Sindbis virus was estimated by plaque method on monolayers of primary chick embryo fibroblast cells.

The production and assay of interferon were carried out as described previously (Béládi and Pusztai 1967). The titer of the interferon was expressed as the reciprocal of the dilution to give a 50% reduction of the control plaque count.

Several adenovirus types were tested for their ability to produce interferon in chick embryo fibroblast cells. Table I shows the results of the experiment using some adenovirus types.

It can be seen that all types induced interferon only the titers are somewhat different ranging from 32 to 256. In this experiment the inter-

TABLE I

Titers of interferon produced in chick embryo cells inoculated with different types of adenovirus

Types used for induction	Titers of interferon[2]
3	256
3[1]	128
4	128
7	64
10	32
16	256
27	128

[1] Virus-strain isolated and grown only in HeLa cells

[2] Reciprocal of dilution giving 50% reduction in plaque count of Sindbis virus

feron stimulated by type 10 had a low titer (32), but else this type capable also of inducing interferon with a titer of 128. Of the types tested, the so-called "weakly oncogenic" types (types 3 and 7) behaved similar to those of other types. One strain of type 3, isolated in HeLa cells from patient suffering from pharyngo-conjunctivitis fever was also examined. The purpose of testing this strain, passed only in HeLa cells, was to prove that adenovirus strains which do not contain adenovirus-SV40 hybrids are also capable to induce interferon.

The experiment with the "highly oncogenic" adenovirus types (types 12 and 18) showed that these types, as well as the others also induce interferon. The results are summarized in Table II.

TABLE II

Induction of interferon by oncogenic adenoviruses

Adenovirus types	Titers of interferon[1]
Type 12	256
Type 18	128

[1] Reciprocal of dilution giving 50% reduction in plaque count

Further the production of interferon in chick cells inoculated with type 12 was studied following the time of incubation.

It can be seen that the production of interferon started 4 hours after the infection and its titer increased up to 24th hour of the incubation.

Employing different $TCID_{50}$ of type 12 for inoculation (about 1.2×10^7 cells) could be stated that the titer of the interferon produced depends on the quantity of the $TCID_{50}$ inoculated.

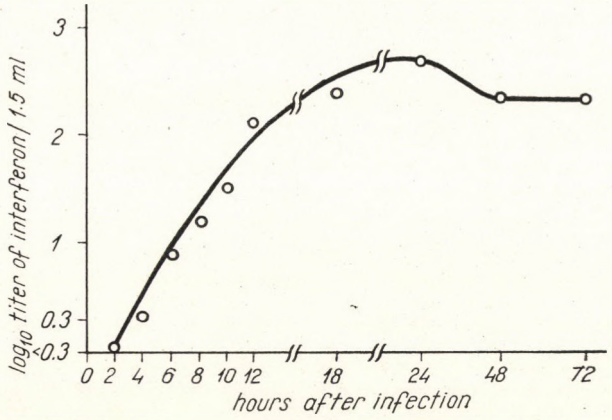

FIG. 1. Interferon production in chick embryo cells inoculated with adenovirus type 12

TABLE III
Production of interferon in chick embryo cells inoculated with different $TCID_{50}$ of adenovirus type 12

$TCID_{50}$	Titer of interferon[1]
$10^{5.5}$	512
$10^{4.5}$	64
$10^{3.5}$	<4

[1] Reciprocal of dilution giving 50% reduction in plaque count.

The $10^{5.5}$ $TCID_{50}$ induced an interferon with a titer of 512, at the same time the $10^{3.5}$ $TCID_{50}$ is less than 4.

Treating the adenovirus types 5 and 12 with 2× crystalline trypsin resulted in the loss of interferon inducing capacity of the virus (Béládi and Pusztai 1967). While the trypsin-treated virus had the same infective titer as the untreated one, the data obtained suggested that the trypsin

sensitive penton antigen (Ginsberg et al. 1966) may be responsible for the interferon induction.

Type 12 kept at 56 °C for different periods was tested for induction. The results are illustrated in Fig. 2.

The virus treated for 2.5 minutes produced interferon at a lower extent and after 3 minutes the interferon inducing capacity was totally destroyed. The infectivity decreased parallely, 3-minute-treatment inactivated the virus. In contrast to this the virus inactivated by ultraviolet irradiation induced interferon like the non-inactivated one.

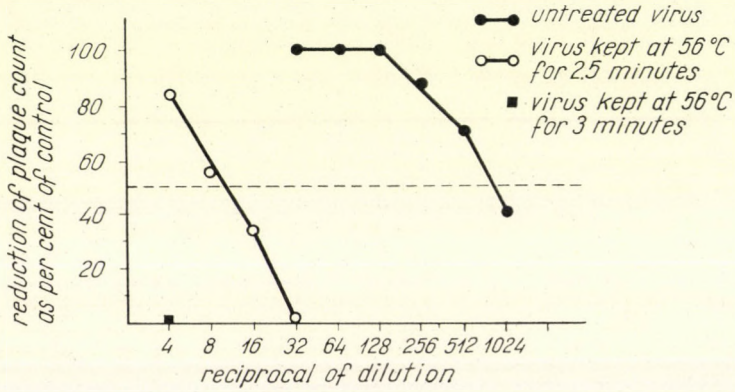

FIG. 2. Effect of heat on the interferon induction of adenovirus type 12

Other cells than chick embryo fibroblast (mouse and hamster embryo fibroblast and BHK_{21} cell strain) were also studied for interferon production infecting them with type 12. No interferon could be demonstrated in any of the investigated cells.

It has been shown by Levinthal et al. (1966) that in the type 12 infected hamster and mouse embryo cells the tumor antigen is formed in a considerable large proportion of the cells, however, tumor antigen was found in very few of the chick embryo cells. On the basis that the interferon inhibits the formation of the tumor antigen (Oxman and Black 1966) a correlation between the interferon and tumor antigen formation in the different cells infected with oncogenic adenoviruses may be assumed. In the cells where no interferon production takes place, the formation of the tumor antigen can occur, whereas in the cells, e.g. chick embryo cells, the interferon is responsible for the inhibition of tumor antigen formation.

In summary, the oncogenic adenovirus types (3, 7 and 12) induce interferon in chick embryo fibroblast cells like the other adenovirus types.

The penton antigen of the adenovirus is supposed to be responsible for the induction, as the trypsin treatment abolishes the capacity of the virus to induce interferon. Also the heat (56 °C for 3 minutes) renders the virus unable for induction. Other cells than chick embryo cells do not produce interferon infecting them with type 12. A correlation is indicated between the interferon and the tumor antigen formation in the type 12 infected cells.

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PRODUCTION OF INTERFERON BY POLYOMA VIRUS IN CULTURES OF PERITONEAL MACROPHAGES AND EMBRYONAL CELLS OF HAMSTER*

by

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The role of interferon in the process of malignization of cells under the effect of spontaneous viruses has been studied little so far.

Some reports noted direct effect of exogenous interferon on the process of cell transformation in vitro under the influence of SV40 (Todaro and Baron 1965), Rous sarcoma virus (Traub and Morgan 1967), on T-antigen production (Oxman and Black 1966) as well as on development of tumors in mice under the influence of SV40 (Atanasiu and Chany 1960). All these studies reveal that inoculation of exogenous interferon reduced the transforming or malignizing activity of the viruses, sometimes even after adsorption of the virus on cell.

As regards endogenous interferon, a number of viruses (e.g. poliomyelitis, vesicular stomatites virus) have been known to exhibit a certain relationship between the extent of virulence of virus mutants, their capacity to block synthesis of the cell RNA and the capacity to stimulate interferon production (Ho and Enders 1959, Wagner and Huang 1966, Aurelian and Roizman 1965). As a rule, cytocidal viruses blocking the cell RNA synthesis are poor inducers of interferon, whereas activation of intracellular interferon under the effect of viruses leads to virus survival in the cell, to latent virus infection (Philipson and Dinter 1963; on the model of foot-and-mouth disease virus and calf kidney cell culture). This fact is also of interest from viral carcinogenesis point of view, since in latent infection favourable conditions may arise for integration of viral into cellular information.

A relationship has also been discovered between the extent of oncogenicity of polyoma virus variants and their capacity to stimulate interferon synthesis. Thus, Friedman et al. (1963) found that polyoma virus variant possessing the highest oncogenicity had lower capacity for production of endogenous interferon; Gotlieb-Stamansky et al. (1966) experimenting with two other strains of polyoma arrived at a contrary conclusion: in

*Report delivered at the Conference on "Prospects of the development of virology", Moscow, June, 1967.

their hands high production of endogenous interferon was correlated with the high oncogenic activity of the strains.

This paper presents results of experiments concerning stimulation of interferon production by polyoma virus in two cell systems: peritoneal macrophages and hamster embryo cell culture. We failed to find in literature any reports on interferon production in hamster cultures by polyoma virus despite the fact that this system is a convenient model for studies of trans-

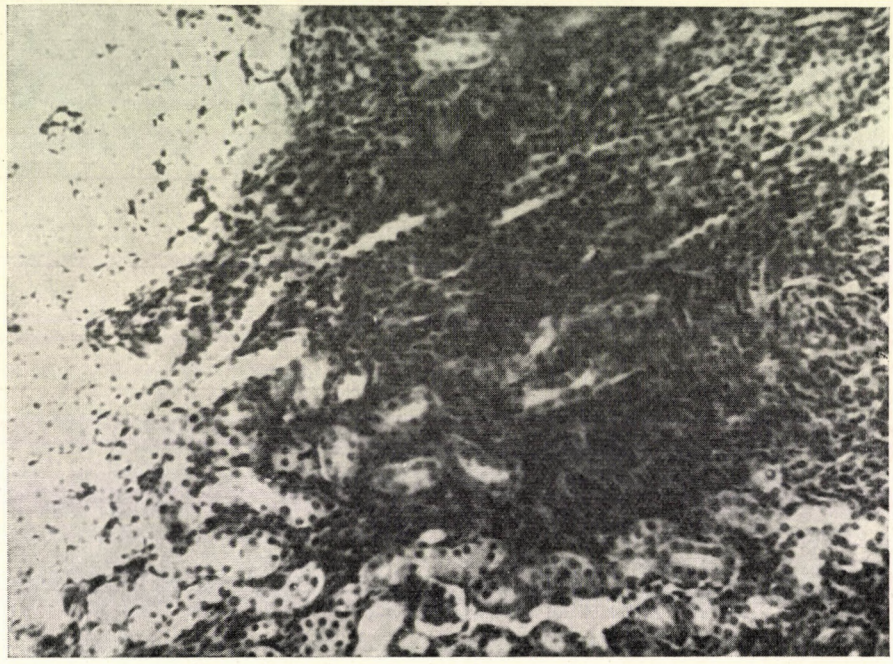

FIG. 1. Kidney tumor in a 14-day old hamster (×80). Dr. E. Vince's preparation

formation process. Interferon production by polyoma virus in mouse embryo cell cultures was reported Allison (1961).

Experiments were carried out with a highly oncogenic Toronto strain of polyoma virus (McCulloch et al. 1959) causing 100% death of hamsters at 7–15 days after inoculation with development of diffuse kidney sarcomas (Fig. 1). The strain was obtained from Prof. M. P. Chumakov.

A few words about cell systems. The culture of peritoneal macrophages was obtained after mobilization of macrophages in peritoneal exudates of hamster by means of 2% starch. Macrophages containing peritoneal exudates were harvested at 3–4 days after inoculation of starch and seeded

into bottles in amounts of 3–5 million cells per 1 ml with 30% calf serum in Eagle's medium or medium 199 of Parker. Polyoma virus was added either in macrophage culture at 3–4 days of growth. The hamster embryo cell culture was prepared by conventional methods

We found that polyoma virus could multiply in macrophage culture. The maximum HA titers were achieved at 3–5 days (Fig. 2). Two to three weeks later morphological transformation of cells was observed with the formation of areas of multilayer growth (Fig. 3). In a number of cases areas of multilayer growth were observed also in control cultures but in lower numbers (Fig. 4).

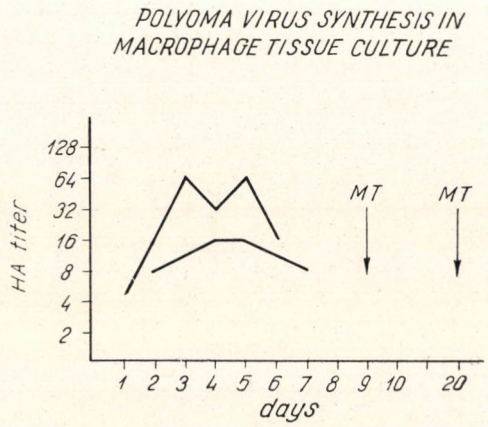

FIG. 2. Polyoma virus synthesis in macrophage tissue culture. MT — morphological transformation

It was also found that in macrophage cultures under the influence of polyoma virus a substance was formed which blocked synthesis of Chikungunya virus or synthesis of vesicular stomatitis virus in hamster cell culture. Later this substance with inhibitory properties was identified as interferon. Titration of the inhibitor was performed by blocking the synthesis of the indicator virus (yield reduction test; Sreevalsan and Lockart 1962): different dilutions of interferon specimen were added to hamster embryo cultures (primary or continuously developed by us in Budapest); after 4 or 18 hours of incubation Chikungunya or vesicular stomatitis virus were added (100 PFU/ml) and 18 hours later the amount of virus was determined by the plaque method in chick fibroblast culture.

The synthesis of Chikungunya virus was completely inhibited in cells previously treated with cultural fluid obtained from peritoneal macrophage cultures infected with polyoma virus (Fig. 5). Marked inhibition of virus

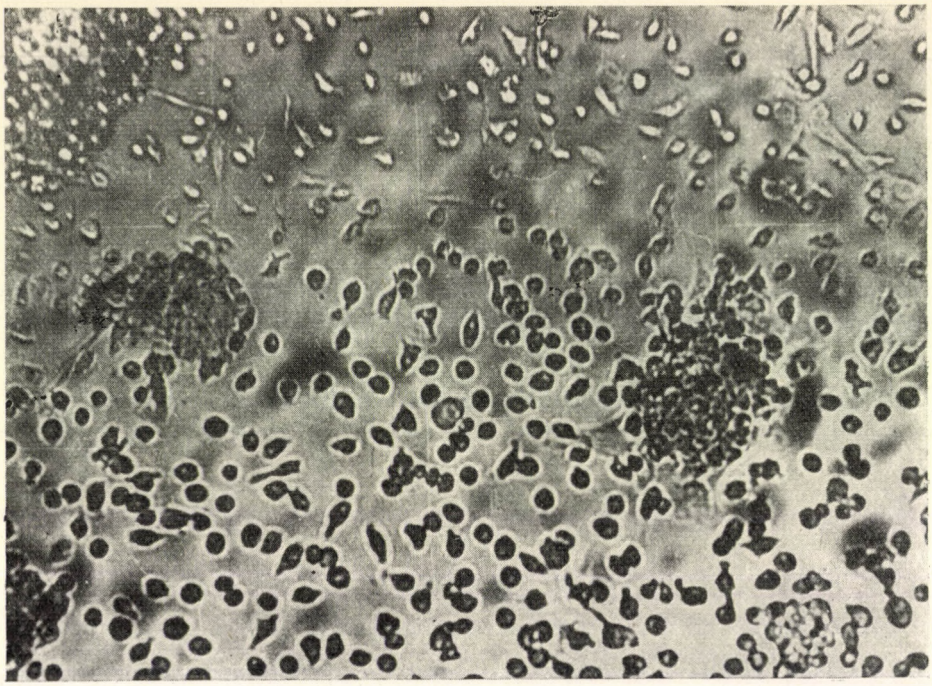

FIG. 3. The areas of multilayer growth in hamster macrophage tissue culture after polyoma virus inoculation

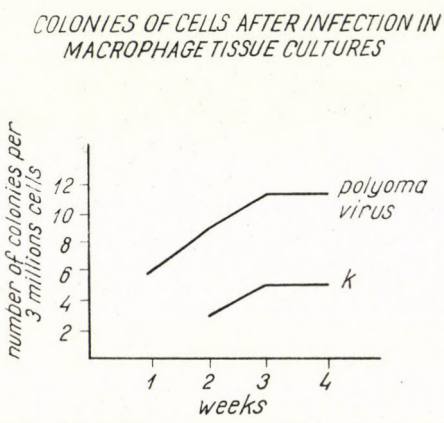

FIG. 4. Colonies of cells after infection in macrophage tissue cultures

synthesis was also observed in cultures treated with uninfected macrophage tissue culture fluids (Fig. 5). These experiments were repeated several times and we established that hamster macrophage cells produce interferon not only under the effect of polyoma virus but also "spontaneously" in response to mobilization with starch and subsequent cultivation. Both inhibitors possessed similar properties of interferon-like substance (Tálas 1967).

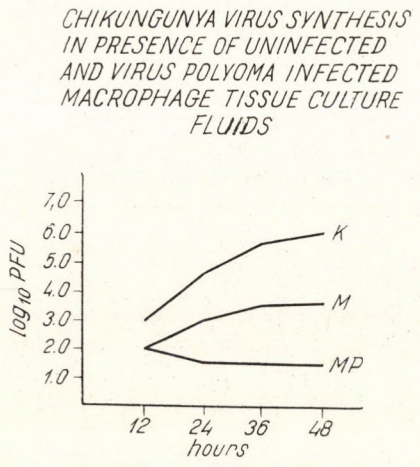

FIG. 5. Chikungunya virus synthesis in the presence of uninfected and polyoma virus infected macrophage tissue culture fluids. Chikungunya virus synthesis: in control hamster embryo culture (K), in hamster embryo cultures treated with uninfected macrophage tissue culture fluids (M) and with polyoma virus infected macrophage tissue culture fluids (MP)

In our experiments mobilized macrophages had higher interferon-producing activity than macrophages normally present in the body. Since in other cell cultures interferon production without stimulation by virus was not observed, this phenomenon may be considered to be peculiar for macrophagal cells. The fact of interferon production in non-infected macrophages may serve, in addition, as a proof in favour of the hypothesis of pre-existence of interferon in body cells.

In hamster embryo tissue cultures under the effect of polyoma virus a virus inhibitor is also formed, its synthesis occurring in parallel to an increase of polyoma virus hemagglutination titer without demonstrable cytophathic effect (Fig. 6). Chikungunya virus synthesis was inhibited only when tissue cultures had been treated with supernatants from

polyoma-virus-infected homologous tissue cultures and never after treatment with fluids from control uninfected cultures (Fig. 7).

We studied some properties of the resulting virus inhibitors in order to find out whether they were interferons. The following results were obtained:

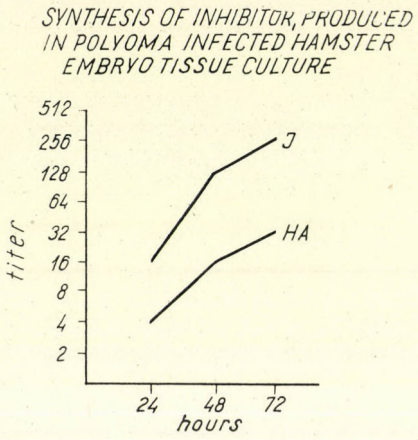

FIG. 6. Synthesis of inhibitor produced in polyoma infected hamster embryo tissue culture. I — interferon titer; HA — polyoma virus hemagglutination titer

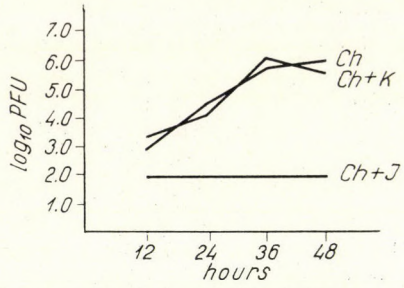

FIG. 7. Chikungunya virus synthesis under the influence of the inhibitor produced in polyoma virus infected hamster embryo tissue culture: in control hamster embryo tissue culture (Ch), in hamster embryo tissue culture treated with uninfected (Ch + K) and with polyoma virus infected homologous tissue culture fluids (Ch + I)

1. The inhibitor was species-specific: it did not affect Chikungunya virus synthesis in chick embryo cultures.

2. The inhibitor suppressed synthesis of several different viruses — Chikungunya, Newcastle disease, vesicular stomatitis in hamster cell cultures.

3. The inhibitor was stable at pH 2 for 5 days.

4. The titer of the inhibitor did not decrease upon dialysis against Earle's solution or phosphate buffer for 24 hours.

5. The titer of the inhibitor did not decrease after ultracentrifugation at 100 000 g for 1 hour.

6. The titer of the inhibitor was not affected by treatment with RNA-ase and DNA-ase or polyoma virus antiserum.

7. The inhibitor was destroyed by treatment with trypsin.

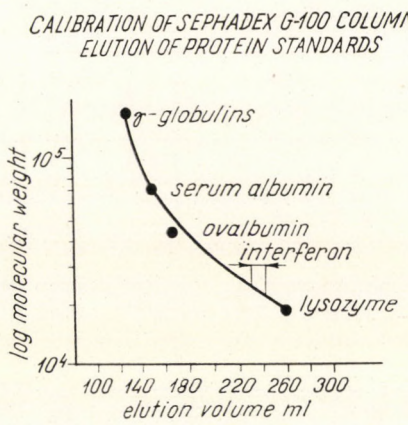

FIG. 8. Calibration of Sephadex G-100 column by elution of protein standards

This evidence concerning the properties of the inhibitor indicates that the virus inhibitor stimulated by polyoma virus in hamster cell cultures belongs to the group of interferon-like proteins (Isaacs 1963).

One of the important properties of interferons is their molecular weight. Determinations of molecular weight of hamster interferon were performed, using fraction collector LKB with uvikord. A column of 35×360 mm in size with Sephadex G-100 was used for chromatography, the eluate was 0.1 M Na phosphate buffer, pH 7.2, the rate of elution being 25 ml/h. The column was previously graduated by protein labels: human gamma globulin with m.w. 160.00; bovine albumin with m.w. 69 000, egg albumin with m.w. 44 000, lysozyme with m.w. 17 500. The graph shows relationship between molecular weight of protein and volume of the eluate (Fig. 8).

A crude preparation of interferon centrifuged at 5000 rpm was used for chromatography. The highest biological activity of interferon was found in fractions, the sum volume of the eluate of which was 232 and

243 ml (Fig. 9). It was thus determined that molecular weight of hamster interferon lies within the range of 21 500–24 000 (Fig. 8). Thus molecular weight of interferon induced by polyoma virus in hamster embryo cell cultures is similar to that of other interferons of viral origin (Fantes 1966).

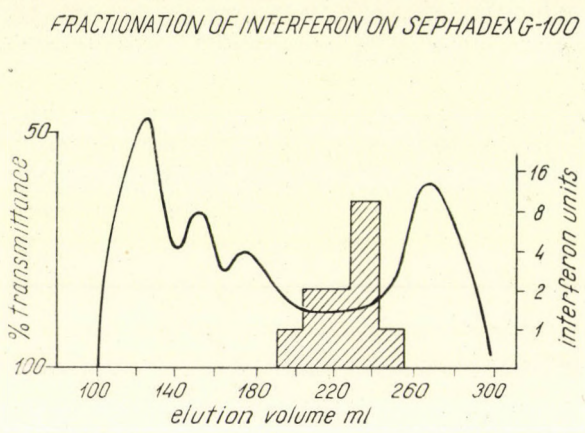

FIG. 9. Fractionation of interferon on Sephadex G-100
▨ — biological activity of interferon

Summary

It was found that a highly oncogenic strain of polyoma virus during multiplication in peritoneal macrophage cell culture and in hamster embryo cell cultures induced production of highly active interferon physico-chemical and biological properties of which corresponded to those of other interferons described. It was also found that hamster peritoneal macrophages produced interferon without virus effect, in response to irritation.

Addendum

In the April issue of the Journal of Experimental Medicine (1967), T. J. Smith and R. R. Wagner reported that uninfected peritoneal macrophages of rabbit also produce interferon after mobilization with glycogen.

Acknowledgements

The authors express their sincere thanks to Dr. Ch. Chany, Dr. Rebecca Falcoff and Dr. E. Falcoff for the helpful advice they have given. The authors are further indebted to Dr. E. Vince for the morphological investigation on hamster tumors, and to Anna Balázs and Magdolna Mázsár for their technical assistance.

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MUTATION OF THE PIGMENT-PRODUCING PROPERTY OF THE MYCOBACTERIA

by

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It was long ago when yellow pigment-producing saprophytic mycobacteria were first observed. The pigment of *Mycobacterium phlei* is composed of carotinoids as demonstrated by Chargaff and Lederer (1935), Ingraham Steenbock (1935), Tuzian and Haxo (1952), Goodwin and Jamikorn (1956). In the course of an earlier work (Weiszfeiler and Kalinina 1936) we observed that *M. tuberculosis* strains may give rise to pigment-producing colonies by mutation. The resulting mutant (M 15) maintained the pigment-producing capacity when serially transferred in culture media or passaged in animals. It was as early as in 1944 (Weiszfeiler and Morosova) that we succeeded in isolating, together with virulent *M. tuberculosis*, pigment-producing strains from the lungs of two fatal cases of tuberculosis. These strains (2703 and 350), which had originated from the non-pigment-producing strain by mutation, were not saprophytes. They grew slowly. Their origin from the tubercle bacterium was evidenced by their immunogenicity against tuberculosis and their failure to be cultivated at temperatures other than 37 °C.

We have further examined four strains of *M. tuberculosis*. These have originally been virulent but their virulence declined while the strains were cultivated in vitro for years. The strains N and Bovinus 8 formed both pigment-producing and non-producing colonies after having been reisolated from guinea pigs. In the strains AK and K_6 the pigment-producing colonies arose in the course of in vitro transfers.

In the mycobacterium strains of monkey origin which were differentiated by us (Karassova and Weiszfeiler 1965) as a new species — *M. simiae* — from other mycobacteria, appearance of the pigment-producing capacity has also been observed, namely in the case of two non-pigment-producing strains (Nos 3, 64). The pigment-producing variants arose after reisolation from mouse passage (strain No. 3) and in vitro (strain No. 64), respectively. The chromogenic variants Nos 3 and 64 readily propagated even at room temperature. In the cultures of a yellow pigment-producing variant (No. 47) white colonies appeared, the mutation arose in inverse direction.

TABLE I

Mycoabacterial strains with mutational change of the pigment production

Original strains				Mutation occurred	Strains with new properties			
Species	Sign	Virulence	Pigmentation		Virulence	Pigmentation	Saprophytic growth (+24 °C)	Amidases
M. tuberculosis human	AK	+	—	in vitro	—	+	—	3
	K_6	+	—	in vitro	—	+	+	3, 5, 6, 8
	M15	+	—	in vitro	—	+	—	
	N	+	—	in vivo	—	+	—	
	2703	+	—	in vivo	—		—	
	350	+	—	in vivo	—	+	—	
M. tuberculosis bovine	Bov. 8.	+	—	in vivo	—	+	—	3, 5, 6
M. kansasii	232	±	photochr.	in vivo	±	+	—	3, 5
Atyp. scotochrom	918	—	+	heat action	—	—	—	—
M. simiae	3	+	—	in vivo	+	+	+	5, 6
	64	+	—	in vitro	+	+	+	1, 3, 5, 6
	47	±	+	in vitro	—	—	+	—

M. kansasii, which is of human origin and does not produce pigment in darkness, developed a scotochromogenic variant (i.e. a variant producing pigment in darkness as well) when passaged in guinea-pigs. The same observations have been made by Tacquet and also by Runyon.

In the course of our experiments carried out about reactivation of mycobacteria after various injuries as action of heat, phenol and aceton, we submitted a slowly-growing, scotochromogenic strain of human origin (No. 918) to 100 °C for 60 minutes (Weiszfeiler and Karassova 1966). From this suspension no growth could be observed on Löwenstein medium, indicating that the culture had practically been killed. When the same suspension was incubated for 198 days in a fluid medium that contained haemolyzed blood and then subcultered, white colonies were obtained. The heat-treated pigment-producing strain No. 918 was inoculated into new-born mice as well. After 99 days non-pigment-producing colonies could be cultivated from the organs of these mice. The pigment-producing capacity was lost in the course of reactivation.

As shown by a number of our experiments, passage in newborn mice favorize the reactivation of the reproductive capacity of the *M. tuberculosis* strains that had been injured seriously in their viability by heat, acetone

or phenol and it occurs in the same time hereditary changes of the properties in the bacteria.

The properties of the studied strains are represented in Table I.

Some questions arise concerning the genetic character of the pigment production. Is this character reversible? How frequently does it occur? Is it the result of a plus or a minus mutation? Another question has been posed concerning the nature of the pigment-producing atypical strains of human origin which, named scotochromatic strains, have represented a serious practical problem most recently: might these strains belong to the pigment-producing mutants of the tubercle bacterium? It is easy to study the development of the pigment-producing capacity, for the appearance of yellow colonies among white ones is readily recognizable. Taking into account that the pigment-producing mycobacteria grow at the same rate or faster than the original stains, it is highly improbable that a stain isolated from colourless colonies in the course of successive passages in culture media originally contained pigment-producing bacteria in a latent form, as suggested by Hauduroy (1960).

As we have demonstrated, the pigment produced by our strain Bovinus 8 contains beta carotin and zeaxanthine, the empiric formula of which is $C_{40}H_{52}$ and $C_{40}H_{52}O_2$, respectively. Probably, as supposed by Porter and Lincoln (1950) the carotin arises from lycopine, the colourless precursor having the formula $C_{40}H_{64}$, with the aid of a specific dehydrogenase. This view is supported by the observation of Schlegel (1959) who was able to demonstrate lycopin in a non-pigment-producing strain that had developed from the carotin-producing *M. phlei* as well as in the strain propagated in the presence of diphenyl amine.

Pigments other than beta carotin are also present in chromogenic strains, indicating that the pigment production requires more than one enzyme.

The appearance of pigment production may or may not be coupled with simultaneous alteration of other inheritable characteristics. We have demonstrated by the gel precipitation test simultaneous changes in the antigenic structure; moreover, such strains lose their virulence, change their enzyme activity and show sometimes an increased multiplication rate. These changes in inheritable characteristics include several plus and minus mutations. We call this phenomenon plurifactorial mutation (Weiszfeiler). The dissociation phenomena resulting from mutations affecting more than one gene, thus leading to profound alterations in numerous characteristics, we consider as further manifestation of plurifactorial mutation. It may be supposed that in cultures injured in viability by autolysis, a

common phenomenon in aged cultures, by endocellular or extracellular factors and antibiotics acting within the macro-organism, just like in cultures impaired by heat, phenol or acetone, individuals may arise which begin to produce enzymes the biosynthesis of which was repressed in the original strain. These regenerated cells reflect alterations in their deoxyribonucleic and ribonucleic acids which account for the altered processes of biosynthesis.

In conclusion, the pigment production of a mycobacterial strain does not exclude an origin from the tubercle bacterium and cannot serve as a basis for the classification of atypical mycobacteria. We suppose that some of the yellow pigment-producing strains of human origin result from tubercle bacteria through plurifactorial mutations occurring in the organism.

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MATRIX-TECHNIQUE FOR IMMUNODIFFUSION ANALYSIS

by

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Owing to its simplicity and several other advantages, the antigen analysis introduced by Ouchterlony (1958, 1962) is widely used at present. Our report describes a plate technique modified for macrodimensions; in our experience, this can be used to advantage for two-dimensional double diffusion analysis. The introduction of this technique in our laboratories was justified mainly by the consideration that with the original method the reservoirs were emptied soon and their repeated refilling involves the possibility of artificial line doubling, as appears also from references in the literature (Kaminsky 1954). If plexiglass-matrix is used, the increasing of the reservoirs in the third dimension makes possible — with the use of adequately thin agar layers — to avoid the spontaneous emptying of the reservoirs, so refilling becomes unnecessary. We describe this method also because it differs from the plate techniques described in the literature so far (Wadsworth 1962, Hartmann and Tiolliez 1957, Mansi 1958, Yakulis and Heller 1959, Holm 1965).

Description of the matrix-technique

A close-fitting hexagonal glass plate is placed in a Petri dish of 50 mm diameter; on this are placed three stay-plates of 3 by 3 mm size, cut from (1.2 mm thick) slide glass. A circular plexiglass matrix of 45 mm diameter is then placed on these stay-plates so that the stay-plates are between the holes.

Plexiglass matrix

We use matrices made of 4–6 mm thick plexiglass; the diameter is 45 mm, the shape circular, the surface smooth; the plexiglass should be transparent and free from scratches. Depending on the shape and position of

the reservoirs, we use the 7-hole matrix plate of radial arrangement in most cases; but 3- or 5-hole matrices provided with square or oblong reservoirs have also proved good, depending on the requirements of work (Fig. 1).

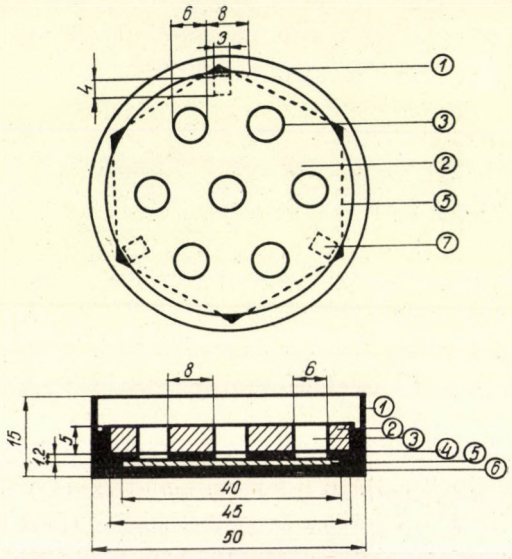

FIG. 1. Plexiglass matrix in Petri dish; top view and section. 1. Petri dish; 2. plexiglass matrix plate; 3. reservoir; 4. second agar layer; 5. hexagonal glass plate; 6. first agar layer; 7. stay-plates of glass

Filling with agar

The matrices and all glass equipment are cleaned and degreased thoroughly. Agar (1% Difco, Bacto agar, 0.45% NaCl, 0.05 M barbiturate buffer, 0.01% Thimerosal, pH = 7.2 to 7.4) is heated to 70–90 °C and placed by means of a pipette between the glass plate and the bottom of the Petri dish so that the level of agar should not rise above the level of the glass plate; agar is then dripped through the central hole of the matrix plate between the latter and the glass plate. Care should be taken that there is no agar on the glass plate in areas that correspond to the reservoirs. Should too much agar flow in, the surplus is removed through the central hole by means of a Pasteur pipette. The plates should then stand at room temperature for 1 hour at least until the agar congeals. Agar cooled down to 40–45 °C is then placed by means of a pipette into the empty space between the margin of the matrix and the margin of the Petri dish, up to the top level of the plexiglass matrix plate. This marginal agar layer

prevents reactant from flowing out of the lateral reservoirs, and fixes the matrix to the Petri dish. After filling the reservoirs the covered Petri dishes are incubated in wet chamber at 37 °C for 8 days. The reservoirs are then flushed with water, and the plexiglass matrix removed carefully; the glass plate with the agar layer on it is lifted, placed in the cover of its Petri dish, and washed in physiological NaCl for 1 day. From the hexagonal

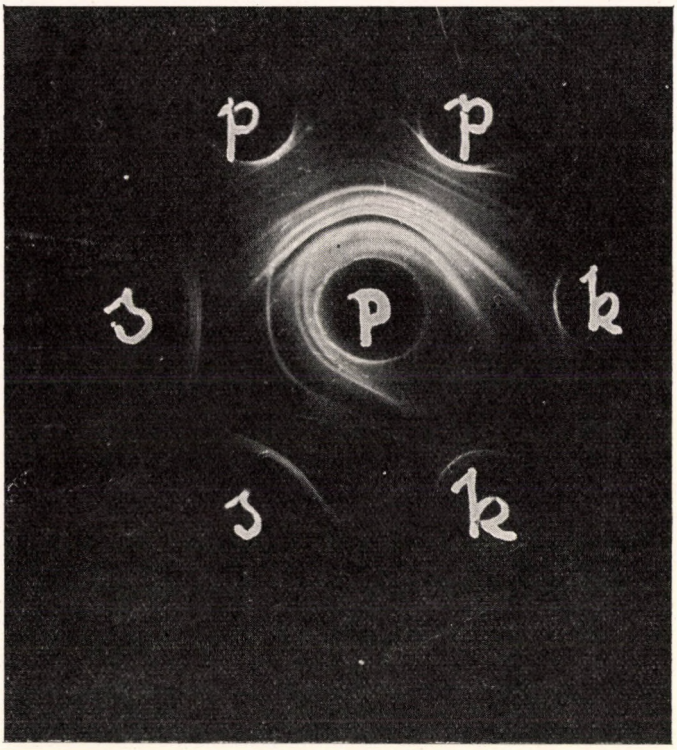

FIG. 2. *Mycobacterium phlei* (p); *M. smegmatis* (s); *M. kansasii* 232 (k); reaction of antigens to anti-*M. phlei* sheep immune serum (p)

plate the agar is pushed over cautiously onto a glass plate of about 10 by 5 cm size, and covered with moist filtering-paper avoiding the formation of air bubbles; then it is dried at 37 °C, and stained in conventional ways with acid fuchsin or some other stain. The slides prepared in this way can be stored as documentary material, and can be photographed (Fig. 2 shows the photograph of a slide prepared with the matrix-technique described here).

The advantages of the matrix-technique

As the reservoirs are relatively large compared to the thickness of the agar layer, there is no need of refilling which involves the possibility of artefacts, accordingly this saves time and work. It is also possible to increase the distances between the reservoirs, which facilitates the separation of components in cases where multi-component protein systems are studied. Within the relatively thin agar layer the neighbouring thinner precipitation lines can be distinguished from one another more easily than in case of thicker agar layers. Washing, drying and staining of thin agar layers is also easier and faster than to work with thick layers. The matrix-technique described here was used for taxonomical studies of mycobacteria based on their antigenic structure (Weiszfeiler et al. 1968).

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COMPARATIVE INVESTIGATION ON SUBJECTS VACCINATED WITH BCG AND INFECTED WITH TUBERCULOSIS USING A BCG TUBERCULIN

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The possibility of a differentiation between tuberculin sensitivity due to BCG vaccination and allergic symptoms caused by infection with virulent tuberculosis bacteria, by the application of tuberculins produced from the BCG strain, was investigated by a number of researchers (Green 1946, Lind 1948, WHO Reports 1961, Magnusson and Bentzon 1961, Kim et al. 1964, Weiszfeiler et al. 1958). Since 1946 a kind of tuberculin from BCG bacteria has been produced in Cuba, the so-called endotuberculin or EBCG. EBCG, also termed as ATB, is a bacillar tuberculin antigen produced through the mechanical disintegration of BCG cultures. The endoantigen, an agent of proteinic nature, is obtained from the bacterial body by grinding with glass beads and diluted subsequently with a 0.5% phenolic solution. The material, freed from the bacterial bodies, is titrated with a Janus green indicator, and the single fractions are compared to PPD-S (international standard, see WHO 1961). The concentrated endotuberculin thus obtained contains 250 TU in each 0.1 ml. The usually applied preparation, containing 10 TU, can be obtained by simple dilution of the concentrated preparation. (See further Dr. M. A. Marcer in Domingo et al. 1947.)

The application of the preparation in its first form was first reported by Heudtlass, Destimone and Tassera in 1946, in the Medical Society of Argentina. This form was further developed by Domingo, by whom the present form of the preparation was elaborated.

Numerous comparative investigations were conducted by R. S. Acosta, M. Amador and others with AT, PPD-S and RT23; their results are somewhat contradictory. According to one of the teams 5 TU of EBCG are equivalent to 5 TU of PPD-S, while, according to some others, 10 TU are considered as equivalent to 2 TU of RT23. According to Acosta, the reactions of the

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EBCG correspond to the reactions obtained with the AT preparations diluted at the ratio of 1 : 50 and 1 : 100.

The authors' comparative studies have been performed on healthy children and on patients of the Childrens' TBC Hospital of Farkasgyepü. The reactions given by EBCG were compared in 260 cases to those of PPD-S, in 321 ca ses to those obtained by the "Human TT" preparation (a purified and liophylized tuberculin made in Hungary),[3] and in 181 cases to AT, in each case on both arms of the same child. The results of the investigations are summarized in Table I.

TABLE I

The average diameters of the tuberculin reactions in different groups

	Group	No. of examined persons	$\bar{x}$ mm	>3 mm %	>9 mm %
EBCG	healthy	260	6.9	77.9	34.4
PPD-S control			8.9	78.6	43.9
EBCG	tuberculotic	321	8.1	84.9	32.8
"Human" TT control			12.9	87.5	73.5
EBCG	healthy	181	7.3	78.5	32.2
AT control			12.4	88.4	75.1
			in mean value		
EBCG examined			7.5	81.5	32.5
Tuberculin control			10.5	84.0	59.0
Total		762			
Difference			40%	3.1%	81.5%

Taken the mean value of the three kinds of tuberculin the average diameter was smaller by 40% in case of EBCG; the percentage ratio of the indurations of 4 mm diameter and more were about the same, but

[3] During repeated field titration tests it could be demonstrated that the indurations obtained by the application of the "Human TT" are equal to those obtained with PPD-S. It may be mentioned as an example that in comparative investigations performed by the authors in 162 patients of the National Korányi TBC Hospital, an average diameter of 15.6 mm was obtained with 5 TU doses of PPD-S, and 15.3 mm with 5 TU of the "Human TT" preparation.

the per cent ratio of the indurations of 10 mm diameter and more was by 81.5% lower in case of the EBCG than in case of the tuberculins serving for comparison. Already in the course of the investigation a working hypothesis could be adopted, according to which the differences in both percentual distribution and the average diameters depend principally on whether the persons whose allergic reactions were compared, had been vaccinated with BCG or developed allergic reactions due to TBC infection. The explanation of these differences are to be found in the specific BCG tuberculin character of EBCG.

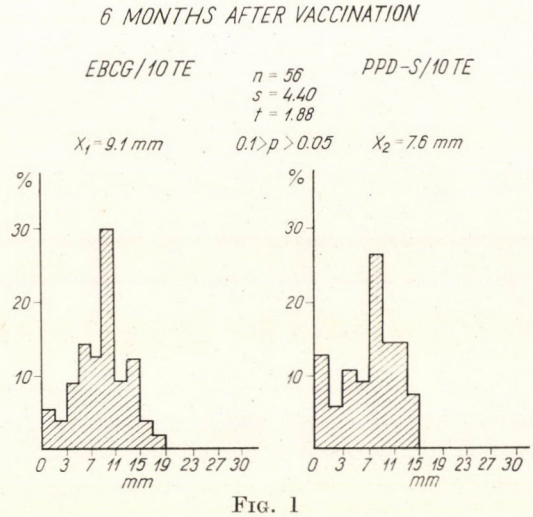

FIG. 1

During further investigations a classification of the examined persons was made. Some groups of the subjects who received BCG vaccination, were separated. Among these subjects 56 were vaccinated 6 months ago, 42 subjects 3 years ago, and 99 children 4 to 6 years ago, all receiving 10 TU of standard PPD-S tuberculin. There were 176 TBC patients who received 10 TU "Human TT" tuberculin each as standard.

The following histograms show the percentage distribution of the indurations developed by the children who were vaccinated 6 months to 3 years ago and received EBCG and PPD-S, respectively (Figs 1–2). It appears from the figure that in case of both groups the reactions from 10 TU doses of the administered EBCG are comparable to those obtained with equally 10 TU doses of PPD-S, which means that no significant difference occurred between the average diameters.

In the following histograms we show the percentage distribution of the indurations developed by patients after receiving EBCG and "Human TT",

respectively (Fig. 3). It can be established that the average diameter of the indurations seen on TBC patients was almost twice as large after 10 TU doses of "Human TT" than after 10 TU of the EBCG. This difference is rather significant.

The following histograms, however, demonstrating the percentage distribution of those vaccinated 4 to 6 years ago, and examined with EBCG, and PPD-S both administered in 10 TU doses, are giving food

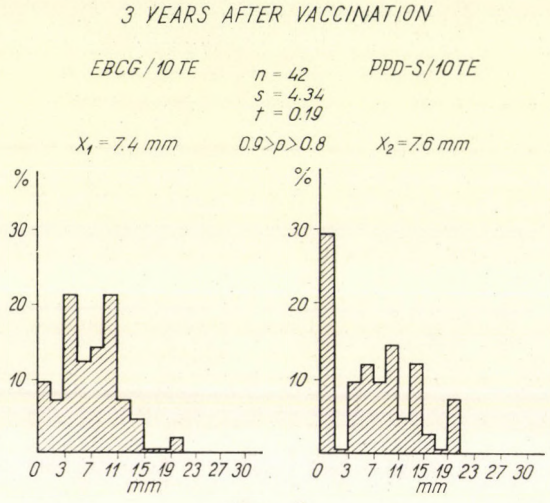

FIG. 2

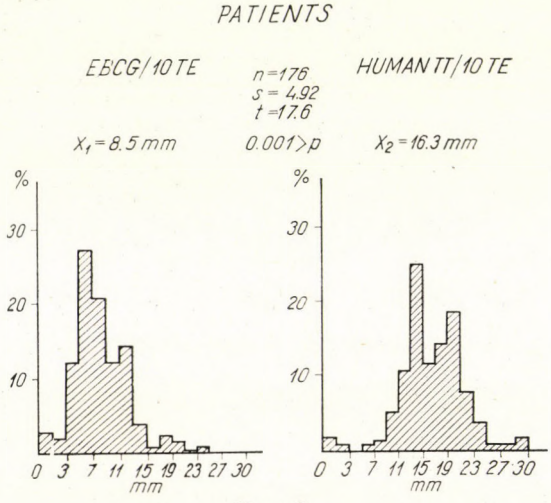

FIG. 3

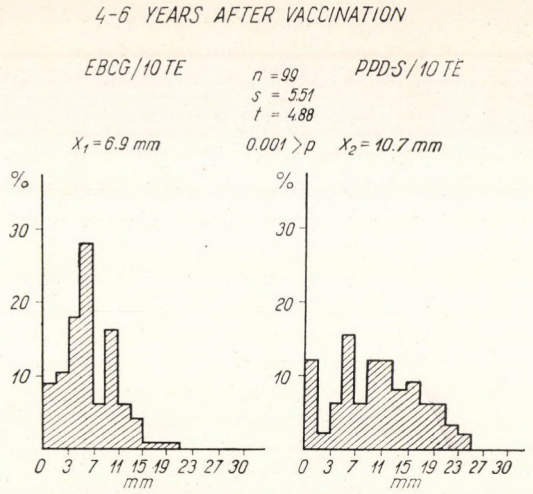
4-6 YEARS AFTER VACCINATION
EBCG/10 TE
n = 99
s = 5.51
t = 4.88
PPD-S/10 TE
X1 = 6.9 mm
0.001 > p
X2 = 10.7 mm
%
30
20
10
0 3 7 11 15 19 23 27 30
mm

FIG. 4

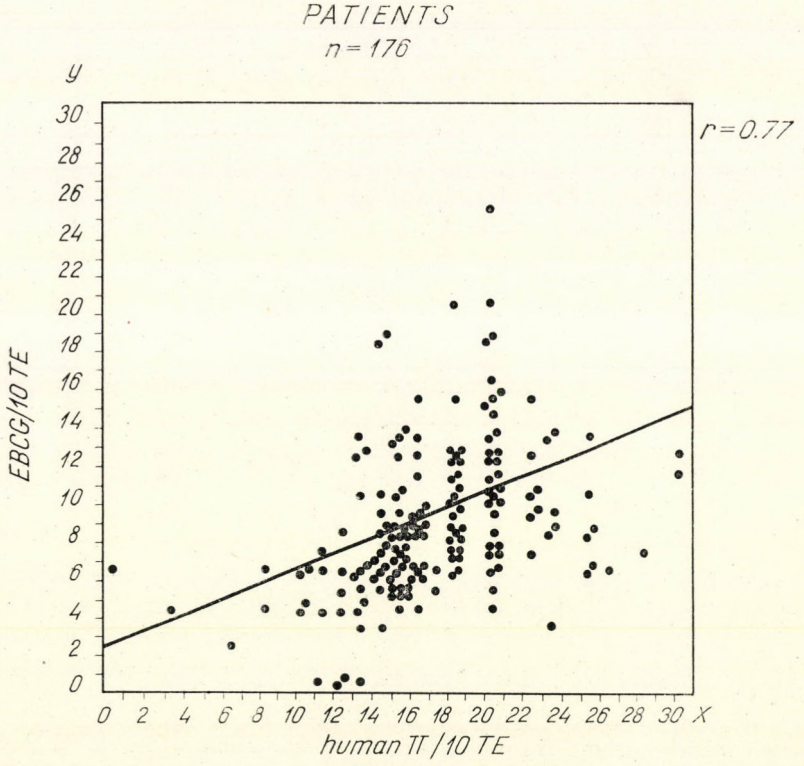
PATIENTS
n = 176
y
r = 0.77
EBCG/10 TE
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 X
human TT/10 TE

FIG. 5

for thought (Fig. 4). It appears that there is a significant difference between the two average diameters, although the subjects of these investigations were a group of vaccinated children. The authors supposed that some of the children underwent tuberculotic superinfection, in which cases the induration after EBCG did not increase while reaction after PPD-S showed a

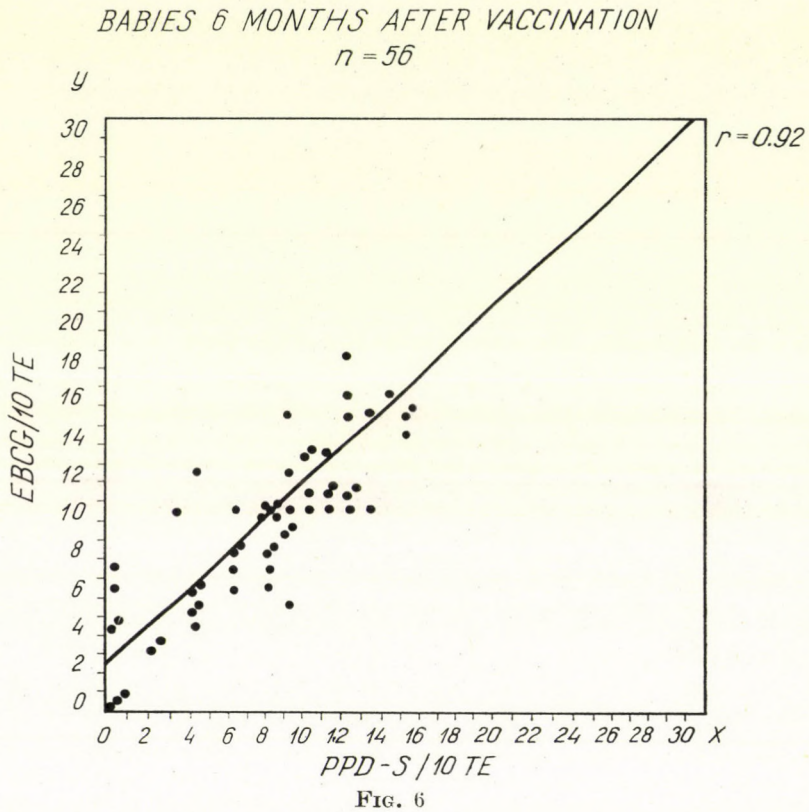

FIG. 6

larger diameter. The following figures demonstrate the correlation of the pairs of values obtained from the two kinds of tuberculin tests performed in patients and in children who were vaccinated 6 months to 3 years, and 4 to 6 years ago (Figs 5–8).

In Fig. 8, related to children who were vaccinated 4 to 6 years ago, the points denoting the pairs of values are grouped along the line of regression. One of the regression lines was calculated on the basis of those pairs of values in which the diameters of the indurations after PPD-S reached a minimum of 10 mm, and was larger at least by 65% than those obtained

after EBCG. It was supposed that this was the group of the superinfected subjects. The pairs of values grouped along the other line of regression were regarded as the group of "purely BCG allergic" persons.

In Figs 9–10, one of the histogram-pairs demonstrates the percentage distribution of the indurations after EBCG and PPD-S respectively in the

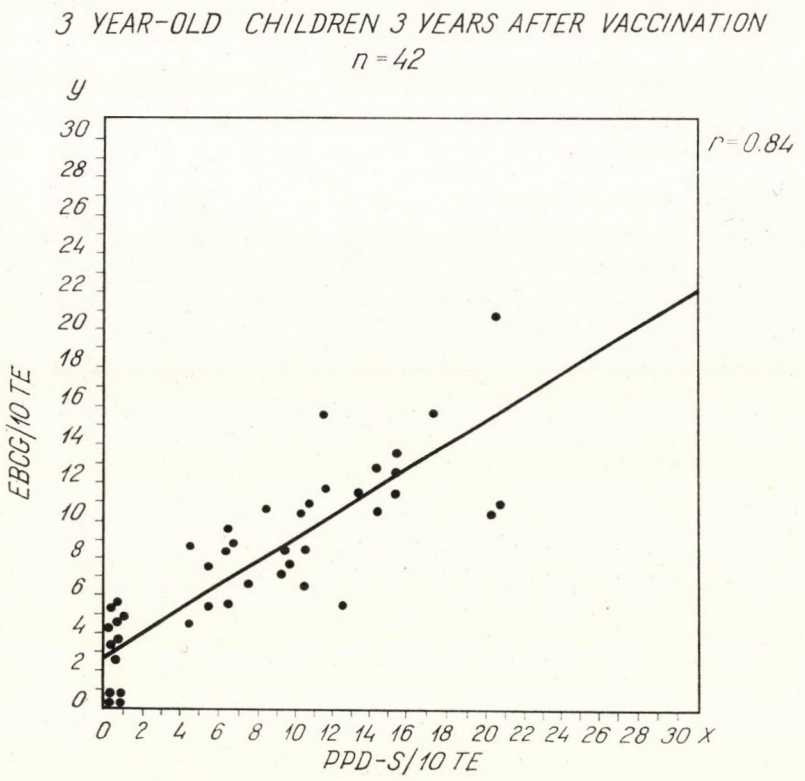

FIG. 7

group regarded as superinfected; the other pair of histogram shows the same data pertaining to the other group, which was supposed to consist of "purely BCG allergic" persons.

It can be established that the average diameters observed in the supposedly superinfected group after 10 TU of PPD-S correspond to the average diameters obtained in patients who had received 10 TU of the "Human TT" tuberculin. At the same time, the average diameter for EBCG of the "purely BCG allergic group" was the same as of the supposedly superinfected group.

It can be observed, furthermore, that with EBCG the average diameter gradually decreases as the time elapsed since the vaccination increases:

(for those vaccinated 6 months ago: 9.1 mm
3 years ago: 7.4 mm
4–6 years ago: 6.9 mm)

This phenomenon was recorded also by Amador and Mendez.

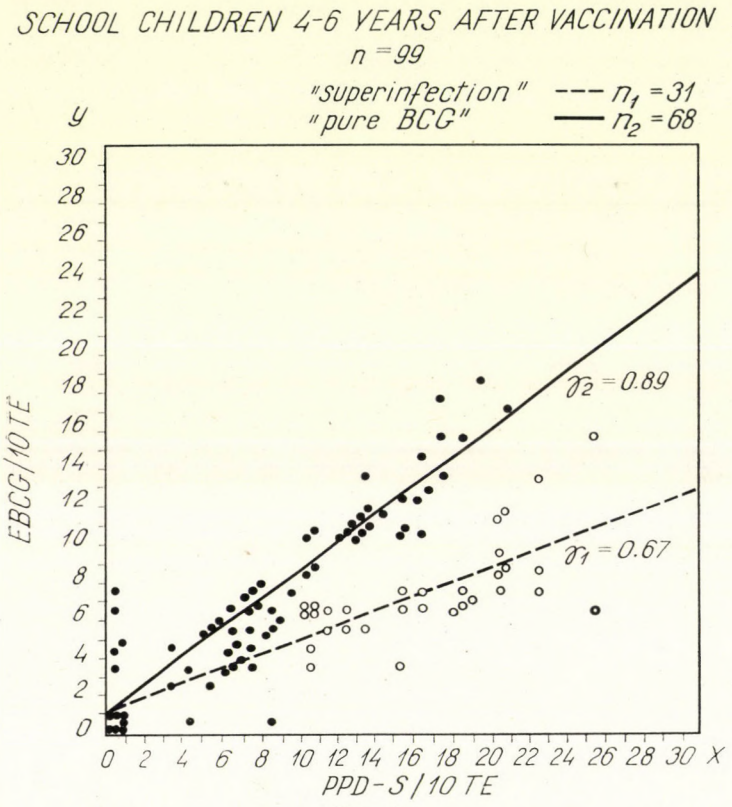

Fig. 8

On the basis of this method it can be supposed that we succeeded in separating groups of children, which were superinfected, from those who were not, but who received vaccination 6 years ago.

The following histogram demonstrates the percentage distribution of the indurations of children, who were vaccinated 4 to 6 years ago, based on investigations with PPD-S; simultaneously the percentage of cases is shown referred to all investigated individuals who were supposedly superinfected (Fig. 11).

From the figure it can be read that the ratio of superinfection would be 31.2%. This would mean a more than 5% annual ratio of superinfection, provided the majority of the cases have been vaccinated 6 years ago. This ratio is, however, markedly higher than can be regarded as likely on the basis of the present rate of infections.

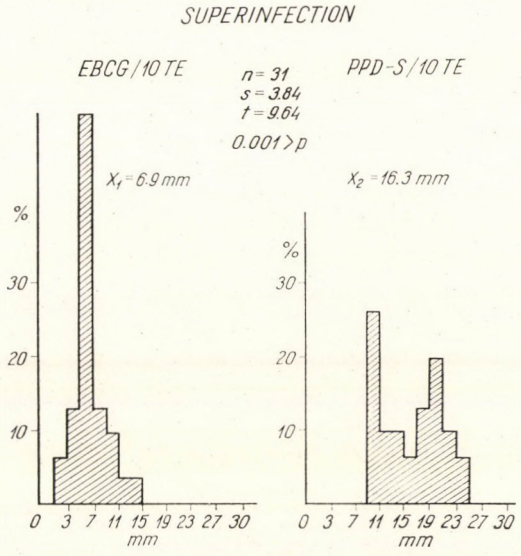

FIG. 9

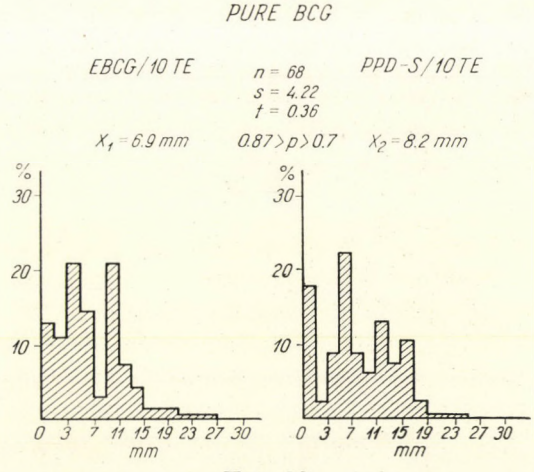

FIG. 10

A much more correct picture can be obtained if only reactions are regarded as being of superinfectious origin, which were obtained with PPD-S, and had a diameter larger than 19 mm. These amount to 11.1% and correspond to a yearly new infection ratio of 1.9%. This value approximates the probability of schooling age. It can be supposed that the group of indurations, which showed diameters of 19 mm with PPD-S and is represented in the figure by hatching demonstrates the BCG allergy preserved as a consequence of a mild course of superinfection, whereas reactions which

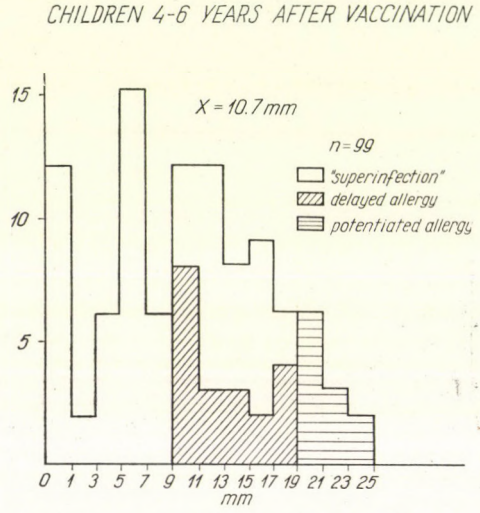

FIG. 11

show diameters of 20 mm and more with PPD-S are amplified by 100% due to more severe superinfections.

It can be established from the data presented in the figure that the reactions obtained with PPD-S and exceeding 19 mm can all be classified in the group of the superinfected cases.

It must be emphasized, of course, that the limited possibilities of the present investigations are far from satisfactory to prove the correctness of the method aimed at the estimation of a superinfection. At the same time, it should be taken into consideration that — according to the established norms published in the international literature — 10 TU of PPD-S are not adequate for the investigation of recent superinfections, as 5 TU of PPD-S, or even lower doses must be applied for this purpose.

In Table II the most important numerical results are summerized.

TABLE II

The comparison of average diameters of reactions on PPD-S or "Human" TT and EBCG by Student's t-test

Examined groups	$\bar{x}$	$\bar{y}$	n	s	t	p	Significant difference
Tuberculose	16.3	8.5	176	4.92	17.6	$0.001 > p$	strong
Vaccinated 6 months ago	7.6	9.1	56	4.40	1.88	$0.1 > p > 0.05$	none
Vaccinated 3 years ago	7.6	7.4	42	4.34	0.19	$0.9 > p > 0.8$	none
Vaccinated 4–6 years ago:							
"Pure BCG"	8.2	6.9	68	4.22	0.36	$0.8 > p > 0.7$	none
"Superinfected"	16.3	6.9	31	3.84	9.64	$0.001 > p$	strong
Total	10.7	6.9	99	5.51	4.88	$0.001 > p$	strong

$\bar{x}$ = average diameter on PPD-S or "Human" TT

$\bar{y}$ = average diameter on EBCG

n = number of cases

s = standard deviation

Summary

The possibility of the demonstration of a specific BCG allergy was investigated in 760 subjects by using EBCG tuberculin.

1. Between the results obtained with children, who were vaccinated 6 months and 3 years ago, respectively, no significant difference was found, whether the investigations had been performed with EBCG or with PPD-S, using 10 TU of both.

2. In case of tuberculous patients, twice as large average diameters were obtained with 10 TU of the "Human TT" as with the same dose of EBCG.

3. The specific features of EBCG justify the hope that perhaps an adequate procedure could be found for the separation of superinfections by using simultaneous reactions with EBCG and PPD-S, or "Human TT"; also for determining that in case of screening tests with 5 TU of "Human TT" what diameters of indurations in children vaccinated more than 3 years before would be indicative of superinfections.

4. EBCG is highly suitable for following up BCG allergy, but screened populations are always mixed in practice. For this reason it is not suitable in itself to mark out the superinfected, nor the diseased individuals. It can be recommended for the checking of the vaccination, or for the longitudinal follow up of vaccination allergy in a specially selected material.

Addendum

After the conclusion of the present work, investigations were published by E. Wisingerova, J. Pokorny, M. A. Bleiker, and V. Nedvedova (Selected papers 1965, Vol. 9, p. 33). These were performed with a native BCG sensitin, the results of which are rather similar to those described above.

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STUDY OF CELLULASE ENZYME ACTIVITY AND CERTAIN FACTORS OF CELLULASE FORMATION BY ASPERGILLUS AND PENICILLIUM STRAINS

by

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Cellulose, which is an important component of the vegetable kingdom, and hence also of food of vegetable origin, cannot be digested by our gastric juices, only the cellulase enzyme is able to decompose it biologically. Cellulase occurs in lower animals, plants, microorganisms. Standard works on this enzyme are the monographs of Siu (1951), Gascoigne, J. A. and Gascoigne, M. M. (1960), Reese (1963) and Flora (1964).

Cellulose may be characterised chemically as β-1,4-polyglucoside, as the linear polymer of D-anhydro-glucopyranose, which may reach in its various complex forms the polymerization degree of 10 000. E. B. Cowling (see in Reese 1963) made a detailed study of cellulose structure and the chemical composition of various types of cellulose. Of all types of native cellulose, the cotton fiber offers the highest resistance to enzymatic action. The cotton fiber contains holocellulose at about 94%, most of which is α-cellulose and only about 5% are hemicellulose. This hemicellulose dissolves in 17.5% NaOH and precipitated again after neutralization. It is composed of relatively short polymers of 10 to 200 polymerization degrees.

It is evident that the dissolved cellulose derivatives are easier to break down than native or amorphous cellulose by cellulolytic enzymes.

Li and King (1963), and King and Smibert (1963) have demonstrated the presence of eight components in the cellulase of *Aspergillus niger* with different specificities based upon the degrees of polymerization of the cellulose and can be divided into two enzyme groups, C_1 and C_x. In the first step, a so-called prehydrolysis takes place where the poly-anhydro-glucose chains are swelling, hydrated under the action of the enzyme system and become accessible thereby to further hydrolyzing enzyme. So in the following step hydrolysis is performed by the enzyme system marked C_x, the β-1,4-glucanases with the β-glucosidase among them. This latter group of enzymes contains the enzymes called formerly cellobiase, gentiobiase.

Leatherwood (1965) immunized guinea-pigs with the cellulolytic enzyme of *Ruminococcus albus*, he studied enzyme inhibition by using the antiserum of the animals, and reached substantially the same conclusion.

Various methods have been applied to determine activity values. Ashan and Norkrans (1953) employed the agar plate-diffusion method and studied the degree of enzymatic effect on the basis of the ring cleared up from cellulose powder. Halliwell (1957) studied the cellulolytic activity of the rumen microorganisms and the culture filtrate of *Trichoderma viride*, using as substrate untreated cotton fibers, dewaxed cotton fibers, cellulose powder, swelled cellulose, hydrocellulose and carboxy-methyl-cellulose (CMC), and choosing as unit 1 ml of the rumen fluid of sheep fed on hay; in standard circumstances this fluid hydrolyses 70% of the cellulose powder. He also determined gravimetrically the quantity of residual undissolved cellulose, and the reducing-sugar values in case of CMC. Toyama (see in Reese 1963) shook strips of filter-paper in an enzyme solution of pH 4 at 40 °C and measured the decrease in diameter. He also applied the agar diffusion procedure combined with a staining method; furthermore employing a third assay he incubated a 4% CMC gel solution at 45 °C and then measured the liquefied layer. Mandels and Reese (1964), Whitaker (1960), Loginova et al. (1966), Flora (1964) made enzyme activity determinations based on the reduction in viscosity of CMC. But no standard assay is in general use even here, different times of enzyme action, temperatures of incubation, concentrations of the CMC solution being applied by the various investigators. In measuring cellulase activities the quantity of reducing sugars that appear under the action of the enzyme is studied by means of dinitro-salicylic acid, orthotoluidine, and Somogyi–Nelson's copper-arsenomolybdate reagents. For determining the glucose formed, the enzymatic method modified by Saifer and Gerstenfeld (in Flora 1964) is also used as a specific reaction. In case of enzyme action on water-insoluble cellulose, the turbidimetric assay is used, in addition to the gravimetric determination; dissolved total carbohydrate is determined by the phenol-H_2SO_4 (in Flora 1964), or anthron assay (Leatherwood 1965).

Until there is no standard method of activity determination and a unit of activity, it is difficult to compare the results obtained by various investigators.

In reports published so far, the conclusions concerning the pH values at which they are optimal the activity of cellulases produced by various factories and various microorganisms show considerable differences.

Halliwell (1957) has found that pH values between 6.6 and 6.8 are favourable to the cellulase effect of the rumen microorganisms, but that the enzymatic process stops at pH 5.9. At the same time he found the pH optimum of the cellulase of *Trichoderma viride* to be at 6.0. In his investigations concerning the cellulase of *Trichoderma viride* Toyama (see in Reese 1963) concluded that the pH optimum is 5.0. For a product of the Worthington Biochemical Corp. (see References) pH 4.0 is indicated as the optimum for the activity of cellulase C_1 originating from *Trichoderma viride*, for C_x activity pH 5.3 in case of a CMC substrate, and pH 4.2 in case of amorphous cellulose substrate. Flora (1964) found that the optimum pH for "hydrocellulase" activity is 4.7 to 4.8 in case of cellulase from *Trichoderma viride*. For a cellulase from the *Trichoderma* species, made by Meiji Seika Co. (see References), the optimum pH value is given as 4.8. For the Takamine cellulase of Miles Chemical Co. (see References) — derived from *Aspergillus niger* — the optimum pH value is 3.5 to 5.0.

The activity of the enzyme decreases as a function of pH value, temperature and storage time, hence the cellulase enzyme is known as a rather unstable enzyme. According to Halliwell (1957) cellulase originating from the rumen microorganisms loses most of its activity when getting into contact with air already at 18 °C, even at 1 °C. The best manner of storage for preserving enzyme activity was with sodium sulphide at 1 °C. Cellulase preparation from *Trichoderma viride* (Worthington Biochemical Corp.) is stable at pH 5.3 to 7.3, at 23 °C for 2 hours. Flora reported that the cellulase of *Trichoderma viride* proved stable for 3 hours at ordinary room temperature and pH 3.8 to 7.0. According to the prospectus of Meiji Seika Co. (see References) the solution of cellulase from *Trichoderma* species is stable at ordinary room temperature for 3 to 4 days. Exceptionally, the Takamine Cellulase (Miles Chemical Co.) is stable even in a solution of pH 2.2 to 3.0 at 37 °C, and shows a very good thermal resistance in a 5.0 pH solution even at 50 °C.

The production of microbial cellulase was studied by Halliwell (1957) and Leatherwood (1965) who investigated cellulase produced by intestinal microorganisms among others; the cellulolytic effect of thermophilic bacteria was studied by Loginova et al. (1966). In his standard work Siu (1951) studied a number of fungus strains in this respect; *Trichoderma viride*, *Myrothecium verrucaria* and *Aspergillus niger* have been considered the most active strains for the hydrolysis of cellulose (I. A. Cascoigne and M. M. Cascoigne 1960, Reese 1963, and Withaker 1960) by most investigators ever since. At present a number of cellulase preparations are being produced by Dutch, Japanese and American firms.

Materials and Methods

Substrates: cellulose powder MN-300,[1] with an average grain size of 10 microns. Carboxymethyl-cellulose (CMC).[2] Water-soluble cellulose derivative was used for the viscosimetric determination of cellulase activity.

The enzymes studied were: Cellulase 36;[3] Takamine Cellulase;[4] Meicelase P;[5] Cellulase Typ I.;[6] Cellulase-Serva;[7] Cellulase 57–59.[8]

Determination of enzyme activity

Carboxymethyl-cellulose assay. This test of enzyme activity is based on the determination of the viscosity decrease of CMC solutions, taking place as a result of enzyme activity. In Hungary this method was employed by Gy. Nagy and K. Egyed. The 1% solution of CMC for daily tests was prepared always freshly. The 0.1 M citrate buffer (pH 3.0 to 5.5) and the CMC solution were pre-incubated at 30 °C. For measuring, 15 ml of the 1% CMC solution and 11 ml of the buffer solution were transfered in a conical flask; the enzyme preparation dissolved in 4 ml of distilled water, or 4 ml of the filtered fermentation broth, or 4 ml of the dilution of the culture medium with distilled water were added. The reaction mixture was then transferred into a modified Ostwald-Fenske viscosimeter, and this was placed into a 30 °C thermostat; the enzyme solution was added, and the time of flow (S_m) measured after 20 min of incubation. Similarly the time of flow of the mixture of the 15 ml CMC solution, 11 ml citrate buffer and 4 ml distilled water omitting the enzyme (S_s), as well as the time of flow with 30 ml distilled water (S_w) were measured. The results were calculated in the term of so-called degrees of degradation according to the formula below

$$\text{degree of degradation} = \frac{S_s - S_m}{S_s - S_w}.$$

Considering that the relationship between the enzyme concentration and the degree of degradation can be regarded as linear only in a relatively

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[3] Rohm and Haas Co., Philadelphia, USA
[4] Chemische Fabriek, Naarden, Holland
[5] Meiji Seika, Ltd., Tokyo, Japan
[6] Sigma Chemical Co., St. Louis, USA
[7] Serva Lab., Heidelberg, Germany
[8] The authors' own preparation from a *Penicillium* species

narrow range, the enzyme concentrations must be chosen in such a manner that the value of the fraction should be between 0.2 and 0.3, so that the degrees of degradation below and over 0.25 be equally represented. Results were illustrated graphically by forming a linear regressive function; the enzyme concentration corresponding to the 0.25 value was determined in this way; this value shows the quantity of the enzyme which is able to decompose 25% of 0.15 g CMC.

Cellulose powder assay. This test is based on the photometric quantitative determination of reducing sugar released from cellulose powder as a result of enzymatic action.

1. In case of enzyme preparations, 50 ml of a 0.1 M citrate buffer (pH 3.0 to 5.5), and of the cellulase to be studied — the latter is rising concentrations — were placed in 100 ml iodine-value flasks; 0.05% cetylpyridinium-bromide was added to the solutions to prevent reproduction of microorganisms. Incubated at 47 °C the reaction starts upon adding 1% of cellulose powder. As a control the initial reduction values were determined with and without enzymes. The tests were repeated with samples taken after 2, 4, 6, 20, 24 and 48 hours.

2. Fermentation medium centrifuged or filtered to remove the mycelium, or in the case of processed liquids 50 ml portions each of the solution to be tested were placed into 100 ml iodine-value flasks, diluted with a 0.1 M citrate buffer (pH 4.5) if necessary; a solid mixture was prepared previously an evaporated suspension of cetylpyridinium-bromide and of 4.5 pH citrate buffer and cellulose powder; and an adequate quantity of this mixture was added to the solution to obtain a reaction mixture corresponding to a 0.1 M citrate buffer, containing 1% cellulose powder and 0.05% of cetylpyridinium-bromide. The reaction mixture was then incubated at 47 °C, a sample was taken immediately after stirring, and the initial reduction value determined by measuring the extinction value against a blank solution omitting the enzyme. The tests were repeated with samples taken after 2, 4, 6, 20, 24 and 48 hours. The extinction value obtained was compared to a standard curve plotted on glucose as a function of time and enzyme concentration, and expressed in micrograms of glucose as the value of enzyme activity.

Reducing sugar assay. Two photometric methods were employed in studying the enzyme activity on cellulose powder, for determining the reducing groups formed in the course of cellulose molecule splitting:

1. Somogyi's copper reagent, and Nelson's arsenomolybdate reagent (as described by Spiro in 1966). It is usually necessary to free the solutions to be tested from disturbing protein and other accompanying

substances, so the solution must be cleared before testing. For this purpose a 4% $ZnSO_4 7H_2O$ solution and a 3.5% $Ba(OH)_2$ solution were used. The barium hydroxide solution was prepared by titrating with it 5 ml $ZnSO_4$ solution, so that 4.8 ml of it should be used up in the presence of a phenolphtalein indicator. For determining the reducing sugar value 1 ml of $ZnSO_4$ and 1 ml of $Ba(OH)_2$ were added to 2 ml of the solution, this was then centrifuged at 6000 g. 2 ml of Somogyi's reagent were added to 2 ml of the supernatant, then kept in a 100 °C water bath for 20 min, and cooled down, after which 1 ml of Nelson's reagent was added. The extinction value of the blue-colour solution was measured by means of a Linson Junior photometer at 540 nm compared to a blank solution omitting the enzyme. The extinction values obtained were compared to a standard curve plotted for glucose, and expressed as a function of time and enzyme concentration in micrograms of glucose as the value of enzyme activity. The standard deviation of this method was 4.7 in the 0–200 μg glucose measuring range.

2. The orthotoluidine assay is described by Bartelheimer et al. (1966) and somewhat modified by Novák (1967). The reagent was prepared by dissolving 2.0 g of thiocarbamide in glacial acetic acid, adding 80 ml of orthotoluidine, and made up to 1000 ml with glacial acetic acid.

Removal of protein was only necessary in case of old fermentation media. A 0.2 ml portion of a 30% trichloroacetic acid solution was added to a 1.8 ml cellulose powder suspension; after 5 min of standing this was centrifuged at 6000 g. A 4.5 ml portion of the orthotoluidine reagent was added to 0.5 ml of the pure solution; this was kept in a boiling water bath for 15 min, after which the extinction of the coloured solution was measured at 630 nm, or 540 nm, respectively compared to a blank solution omitting the enzyme. The standard deviation of this method was 3.5 in the 0–500 μg glucose range. If it is necessary to dilute the coloured mixture, this can be made with glacial acetic acid, and the results of the measurements can be compared to a glucose calibration curve plotted under similar conditions.

Results

Preliminary studies have been made with cellulase preparations commercially available to get information on the C_1 and C_x enzyme systems of a number of available cellulase enzyme preparations, and to investigate the optimum conditions of their activity.

The optimum pH of the activity of the C_1 and C_x enzyme systems have been studied in the pH range of 3.0 to 5.5.

Citrate buffers of proper pH values were used in experimental conditions described above.

The activity values characteristic of the C_x enzyme system were determined by means of viscosimetric studies of carboxymethyl-cellulose. Every measurement variation was performed in 4 parallel experiments. The obtained results were transformed with linear regressive functions with

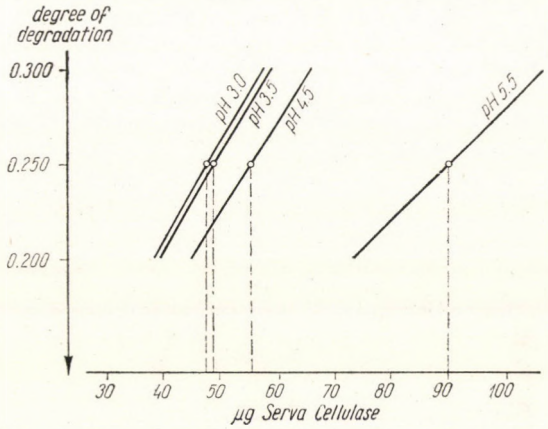


FIG. 1. Viscosimetric activity of Serva Cellulase

the method of the least squares. The following figures show these regressive functions in the range of pH 3.0 to 5.5 for every pH value. The results are shown in Figs 1–2 in the case of Serva Cellulase and Cellulase Typ. I.

Figure 3 shows the quantities in micrograms of the four tested enzyme preparations, representing activity values of 0.25 degradation degree as a function of pH in the pH range of 3.0 to 5.5.

It can be established that all of the four tested cellulase enzyme preparations are least active in the less acidic section of the pH range of 3.0 to 5.5, while the more acidic section of the investigated pH range is more favourable to the enzyme effect. The pH optimum of Cellulase 36 and Serva Cellulase is between 4.0 to 3.5 pH values; that of Meicelase P and Sigma Cellulase Typ. I is in the vicinity of pH 4.5.

The activity of the C_1 enzyme systems was determined on the basis of cellulose powder tests. Most enzyme preparations contain a certain amount of reducing substance as has been shown by control tests. The period of incubation was extended to 48 hours. Cetylpyridinium-bromide inhibited

the reproduction of microorganism, and did not inhibit the enzyme action. The data of measuring were analyzed by means of linear regression calculation, employing the method of the least squares, although the functions had to be shown in some cases transposed to the origo in the coordinate

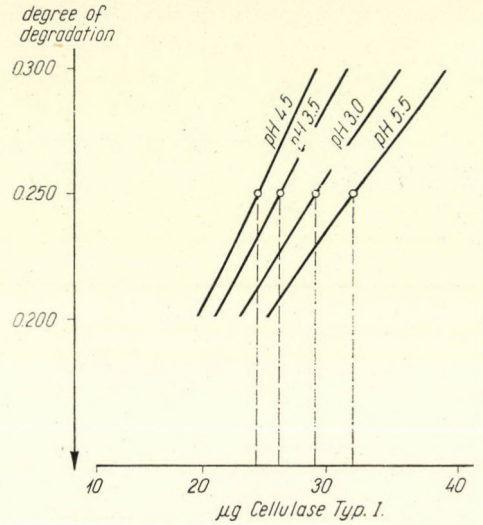


FIG. 2. Viscosimetric activity of Sigma Cellulase Typ I

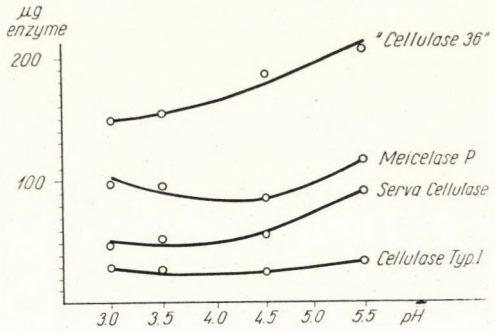
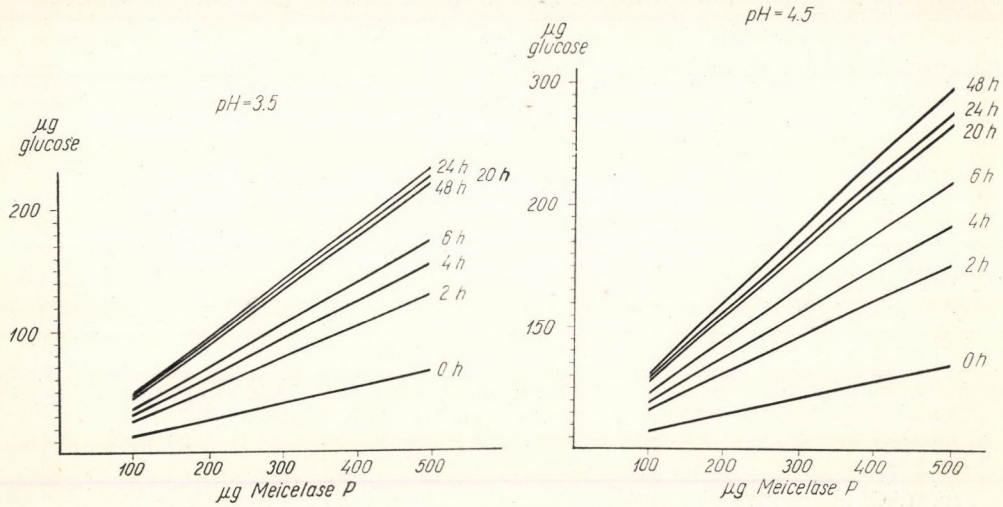


FIG. 3. Quantities in micrograms of the four tested enzyme preparations, representing activity of 0.25 degradation degree value, given as a function of pH, at 3.0–5.5 pH

system. The results of C_1 activity determination of the various cellulase preparations by way of illustration Meicelase P at 3.5 and 4.5 pH, and our product at 4.5 pH are shown in Figs 4–6.

Figure 7 shows the regressive functions obtained after 4 hours of incubation at the optimum pH 4.5 of all preparations for comparing the various

preparations with one another. Enzyme activity is expressed in micrograms of glucose on the ordinate of every figure; enzyme concentration is shown in micrograms on the abscissa.



FIGS 4–5. C_1 activity values of Meicelase P at 3.5–4.5 pH

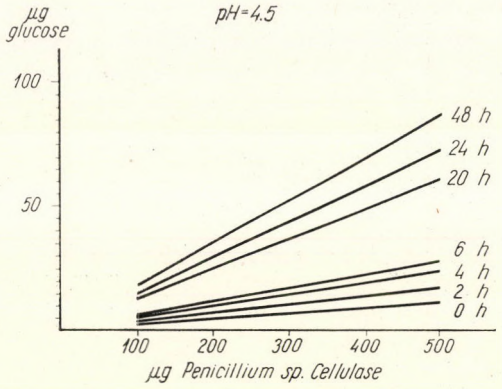


FIG. 6. C_1 activity values of the authors' own cellulase preparation 4.5 pH

It can be also established here, too, that the least favourable for exerting the enzymatic activity is the range of the highest pH. Values about pH 4.5 can be regarded as optimum. Of the preparations tested, the highest activity was found with Meicelase P. Cellulase 36, Takamine Cellulase, and an own enzyme preparation and some own culture filtrate concentrates

were also tested, as well as the stability of activity characteristics of the C_1 and C_x enzyme components during a longer storage at 0 °C.

The storability of enzyme preparations in sealed containers at 0 °C were studied with the carboxymethyl-cellulose and cellulose powder testing methods. After storage of more than one year Takamine Cellulase proved

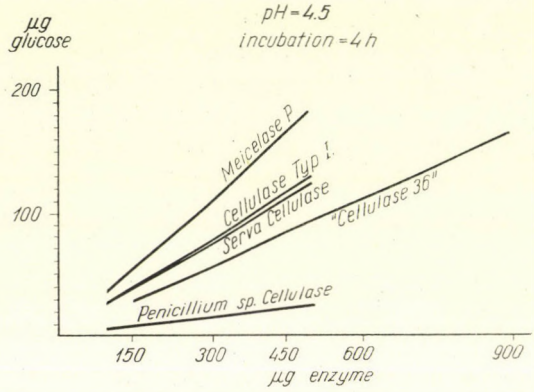


FIG. 7. C_1 activity values of the five tested enzymes at pH optimum after 4 hours of incubation

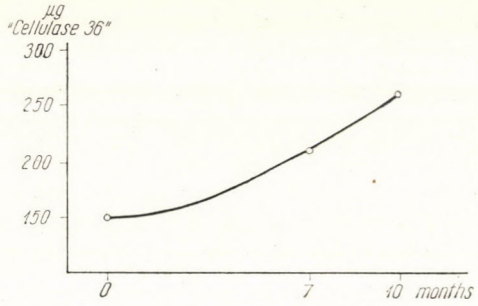


FIG. 8. Viscosimetric activity of "Cellulase 36" stored at 0 °C

to be altogether inactive put to carboxymethyl-cellulose assay, while at the same time the cellulose powder assay showed that about 40% of the initial activity were preserved. The activity values of the C_x system in case of Cellulase 36, stored in the above circumstances, are shown in Fig. 8. This enzyme preparation preserved practically all of its C_1 activity during that time. Testing the authors' own dry cellulase preparation, it was found that its C_x activity decreased rapidly even during storage for one months, while the C_1 activity was preserved at the same time. When samples of

solutions of active ferment concentrates were tested, stored also at 0 °C and kept for such stability tests, it was found that they had lost their C_x activity gradually, but at a lower rate than the authors' own solid cellulase preparation.

So it would seem from the foregoing that the stability of the C_x enzyme system is much lower than that of the C_1 system, not only in their cellulase solutions, but also in the form of solid preparations.

Submersed fermentation was carried out with *Aspergillus niger* and *Penicillium* species to study the conditions of cellulase enzyme production and the factors promoting cellulase formation of an *Aspergillus* strain 1026/5, and of a No. 7.24 strain of *Penicillium* species isolated by K. Egyed.

The *Aspergillus* strain was maintained in nutrient media containing no cellulose or cellulose derivative; the *Penicillium* strain was inoculated at intervals into a fungous nutrient medium, composed of yeast and malt extracts, and of inorganic salts, containing 0.5% cellulose powder and/or 0.5% CMC.

The fermentation of the strains was carried out in 10-litre glass fermentors, with 6 litres of nutrient volume capacity, 150 rpm stirring, 1.5 l/min aeration, at 37 °C temperature. In the first phase the spores of the fungus were inoculated into a inoculum nutrient of the following composition: citric acid 0.2%, corn steep liquor (CSL) 0.2%, glucose 0.5%, KH_2PO_4 0.1%, yeast extract 0.025% malt extract 0.025%, methyl-silicon oil 0.02%, trace element solution 1.0% (the composition of the latter was $ZnSO_47H_2O$ 0.1 g, H_3BO_3 0.1 g, $MnCl_2$ 0.1 g, $FeCl_36H_2O$ 0.05 g, KJ 0.01 g, $CuSO_45H_2$ 0.01 g, dissolved in 1000 ml of distilled water).

After 48 hours of inoculum fermentation, four types of culture medium, marked "A", "B", "C" and "D", were inoculated with microorganism cultures in quantities of 10% for the volume of the fermentation medium; the composition of the four substrata was the following:

Culture medium "A": saccharose 3.0%, CSL 2.0%, $Ca(NO_3)_2 \cdot 4H_2O$ 0.75%, KH_2PO_4 0.25%, citric acid 0.075%, KCl 0.05%, $MgSO_4 \cdot H_2O$ 0.0125%, $FeCl_3 \cdot 6H_2O$ 0.001%, methyl-silicon-oil 0.02%.

Culture medium "B": $NaH_2PO_4 \cdot 1H_2O$ 0.1%, $NaNO_3$ 0.19%, NH_4NO_3 0.03%, $Na_2HPO_4 \cdot 12H_2O$ 0.07%, $MgSO_47H_2O$ 0.015%, KH_2PO_4 0.01%, K_2HPO_4 0.0075%, yeast extract 0.025%, malt extract 0.025%, methyl-silicon-oil 0.02%, CMC 0.5%.

Culture medium "C": same as substratum "B", but cellulose powder 0.5% instead of CMC.

Culture medium "D": same as „B" plus cellulose powder 0.5%.

The pH of all substrata was set to 4.5.

In the course of fermentation the pH of the culture medium was regulated with phosphoric acid every 24 hours, for keeping it at 4.5 pH; fermentation lasted 9 days in every case for studying the intensity trends in the periods of cellulase enzyme formation and decomposition. The activity values apply to 1 ml of culture filtrate; in case of cellulose powder tests they are expressed in micrograms of glucose on the basis of reducing-sugar value determined after 48 hours of incubation; in case of carboxymethylcellulose tests they are expressed in units of CMC viscosity decrease, where

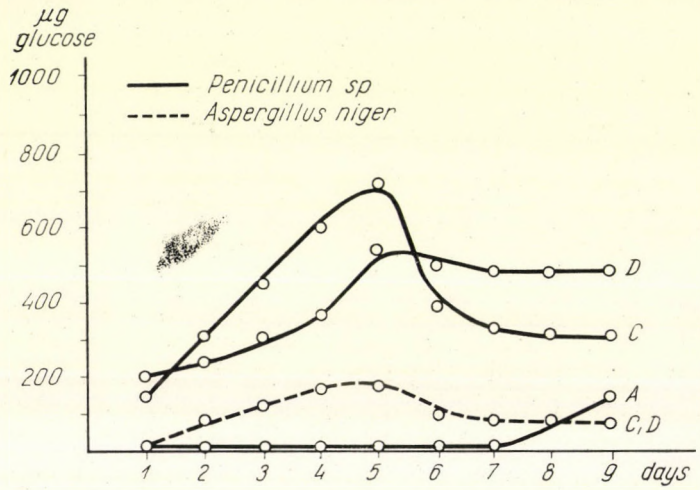


FIG. 9. C_1 activity of 1 ml culture filtrate of "A", "C" and "D" during fermentation

the unit is 1 microgram of the Cellulase 36 preparation and 150 units are the equivalent of 0.25 degree of degradation. Figure 9 shows the activity according to the cellulose powder assay, as a function of fermentation days. Figure 10 shows the activity values according to the carboxymethylcellulose assay, as a function of fermentation days.

The *Aspergillus niger* strain yielded no activity relating to the C_1 system, neither in culture medium "A", nor in "B"; only in culture medium "C" and "D" did such activity appear to a lesser extent, beginning from the second day, and reaching the maximum values by the fourth day. C_x yielded high values in culture medium "A"; the activity curve showed the maximum on the sixth day of fermentation; following this it declined and dropped to nearly 25% by the ninth day. It is an interesting finding that in culture media "C" and "D" which contained also cellulose powder, the C_x enzyme system was totally suppressed to the advantage of the C_1

system, while in medium "B" the presence of CMC inhibited the development of the activity of both enzyme systems. Consequently the *Aspergillus* strain studied here contained the C_1 factor of the cellulase enzyme as a constitutive enzyme system, which factor appeared as an induced enzyme under the effect of cellulose, but would not be induced with CMC; on the other hand, the enzyme activity, already induced through cellulose, is preserved even in the presence of CMC. It also contains as a constitutive enzyme system the C_x factor of the cellulase enzyme, which, however,

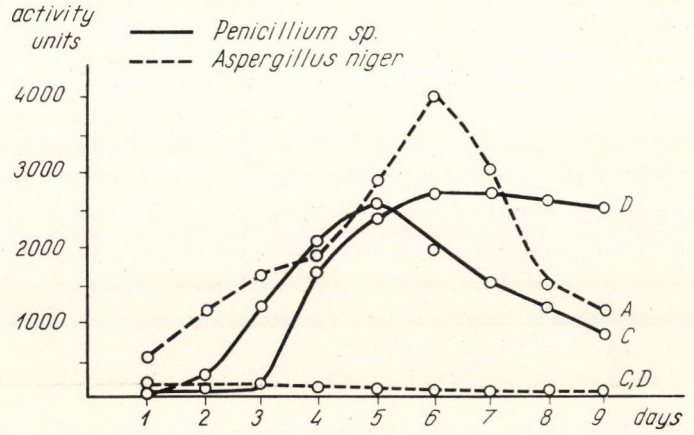


FIG. 10. Viscosimetric activity of 1 ml culture filtrate of "A", "C" and "D" during fermentation

is not induced by CMC; moreover, both CMC and cellulose exert an inhibitory effect on it.

In the fermentation of the *Penicillium* species no C_1 is formed in medium "B"; an activity of lesser extend appeared in medium "A" only by the time the saccharose content of the substratum had decreased to a minimum.

On the other hand, the activity increased most intensively in media "C" and "D", and reached the maximum by the fourth and fifth day: at the end of fermentation only about 50% of this activity value remained in medium "C". In medium "D" the maximum of activity was at a lower level, but this did not substantially decrease even at the end of fermentation. But in case of this strain the activity relating to the C_x enzyme system did not appear at all in medium "A", in sharp contrast to the *Aspergillus* strain. The activity value is insignificant also in medium "B". A considerable C_x activity appeared in media "C" and "D", where the maxima appeared on the respective fifth and sixth day, nearly on an identical level. In the course of further fermentation, the activity in medium "C"

decreased to about 30% by the end of fermentation. This decrease of activity did not take place in medium "D", and stayed at the maximum up to the end of fermentation. It may be concluded, then, that the *Penicillium* sp. contains the C_1 and C_x factors of the enzyme system, and that these equally appear as induced enzymes under the effect of cellulose powder. CMC does not in itself exert such an effect; but it maintains the activity, if applied in combination with cellulose powder, in case of both enzyme factors, although the activity of the C_1 enzyme system is somewhat lower if CMC is also present.

Summary

Cellulase enzyme preparations commercially available were studied. For the C_x system of Cellulase 36 and Cellulase Serva, the optimum pH is 3.0 to 3.5. For the C_x system of Cellulase Typ. I and Meicelase P, the optimum pH is 4.5. The optimum for the C_1 system of all four enzyme preparations is pH 4.5. The C_x enzyme system of some cellulase preparations is less stable at storage than the C_1 system. A gradual decrease in activity appears even when storage is at 0 °C. The same was observed in case of the authors' own powdered enzyme preparation and non-purified fermentation broth concentrates.

In submersed fermentation the cellulase-production properties of one *Aspergillus niger* strain and one *Penicillium* strain were studied. Our Penicillium strain is able to produce the C_1 and C_x enzyme factors in adequate proportion, and this is induced by the presence of cellulose powder in the culture medium; CMC in itself has an inhibitory effect on both enzyme systems, but its presence when accompanying cellulose powder is favourable. In investigated circumstances the *Aspergillus* strain possesses the capacity of producing cellulase, mainly that rich in the C_x enzyme system; this is inhibited by the presence of cellulose derivatives, while the production of the C_1 enzyme system is induced by the cellulose powder content of the nutritive medium of fermentation.

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THE ACTION OF CELLULASE ENZYME ON THE UNICELLULAR ALGAE CHLORELLA PYRENOIDOSA AND SCENEDESMUS QUADRICAUDA

by

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Introduction

This paper deals with the decomposition by various cellulase preparations of the cellulose substance of *Chlorella pyrenoidosa* and *Scenedesmus quadricauda*, with the purpose of increasing the digestibility of the alga cells.

Owing to the considerable cellulose content of their cell walls, algae are difficult to digest, and their nutritious value is not satisfactory as a result. Studies conducted by Nordhcote, Goulding and Horne (1958) have shown that the cell walls of *Chlorella pyrenoidosa* contain 15.5% alpha cellulose and about 31% hemicellulose, in all 13.6% of the dry substance of the cells.

The digestibility of algae could be increased substantially by decomposing the cell walls. This question was studied by Nordhcote et al., Boriko, Klyushkina and Kondratiev (1964), Klyushkina and Fofanov (1966). Cellulase is composed of several enzyme factors (C_1 and C_x), which act partially on alpha cellulose, partially on hemicellulose.

In our previous studies (Ürmössy 1967) we have found in respect to some commercial cellulase preparations, and to one preparation of our own, that their optimum activity is displayed at 47 °C in a medium of 4.5 pH.

We have studied the effect of various enzyme preparations on unicellular algae, by means of determining (a) the reducing sugar, which forms from cellulose, and (b) the dissolved proteins.

Materials and Methods

Dried, non-viable *Chlorella pyrenoidosa* was obtained from the Biological Institute of Leningrad, *Scenedesmus quadricauda* from the Alga Experimental Station of Trebon (Czechoslovakia). 0.1–1.0–15% suspensions of algae were acted upon by 0.1–10.0 mg/ml cellulase preparations.

The cellulase preparations were the following: Cellulase 36[1], Takamine Cellulase[2], Meicelase P[3], Cellulase Typ I[4], and Cellulase pract[5].

The alga cells used as substrate were disintegrated by a rotary mixer in a 0.05 M citrate buffer of 4.5 pH, in the presence of 0.1% cetylpyridinium bromide and 0.01% methyl-silicon-oil until we obtained a homogeneous suspension. In 100 ml iodine-value flasks the enzyme preparations used for the experiments and corresponding in quantity to the buffer solutions of the above composition were completed to 40 ml with the buffer solution and to this were added 10 ml each of the prepared alga suspension. The reaction mixture was shaken respectively at 37 and 47 °C, incubated for 2, 4, 20 and 48 hours, then centrifuged at 8000 g for 15 min, after which the reducing-sugar value and the protein content were determined in the supernatant. As control we determined the reducing-sugar value and the dissolved-protein content of the mixture before incubation and of the solution which contained only algae and no enzyme.

Reducing sugar was determined by the orthotoluidine method developed by Bartelheimer et al. (1966) as modified by Novák (1967) and Ürmössy et al. (1967).

The quantitative determination of the protein content of the cellular substance that had entered the solution as a result of the digestion of the alga cells was carried out with Ditterbrandt's method (1948). For preparing the reagent, 9.0 g potassium sodium tartrate, 3.0 g $CuSO_4 \cdot 5\ H_2O$ and 5.0 g KJ were dissolved in 400 ml of 0.2 n NaOH; 0.2 n NaOH were then added to complete the solution to 1000 ml. 0.5 ml of the supernatant centrifuged from the alga suspension were completed with distilled water to 2.0 ml, to which 3.0 ml of the reagent were added; the solution was then incubated at 37 °C for 30 min. The extinction of the colour mixture formed in this way was measured with the Linson photometer at 540 nm against a blind solution, and expressed in mg value by comparing it to a calibration curve plotted for bovine serum albumen.

The decrease in the number of alga cells after enzyme action was determined by the usual method.

[1] Rohm and Haas Co., Philadelphia, USA
[2] N. W. Chemische Fabriek, Naarden, Holland
[3] Meiji Seika Co., Tokyo, Japan
[4] Sigma Chemical Co., St. Louis, USA
[5] Serva Lab. Heidelberg, Germany

Results

We studied the 1% suspension of *Chlorella pyrenoidosa* in the presence of 0.5, 1.5 and 2.5 mg/ml Cellulase 36 enzyme, after incubation at 37 °C for respective 0.5, 2, 4, 20 and 48 hours. We determined the reducing-sugar values released from the reaction mixture. The decrease in the number of intact alga cells compared to the starting time was determined after 48 hours (Figs 1–2). The decomposition of the cell walls increases with the concentration of the enzyme. In the control solution containing no enzyme the number of alga cells stayed practically unchanged during incubation at 37 °C in a 4.5 pH medium.

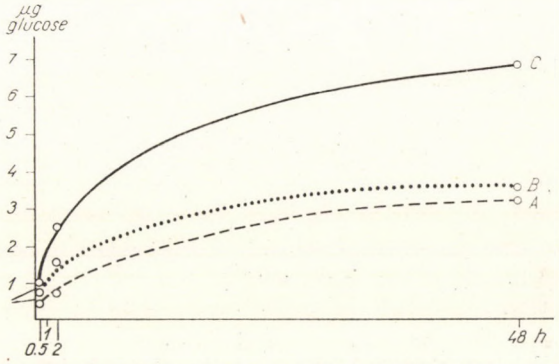

FIG. 1. Reducing sugar found in the suspension of *Chlorella pyrenoidosa* under the effect of treatment with the Cellulase 36 enzyme, in case of A = 0.5 mg/ml, B = 1,5 mg/ml, and C = 2.5 mg/ml enzyme concentrations.

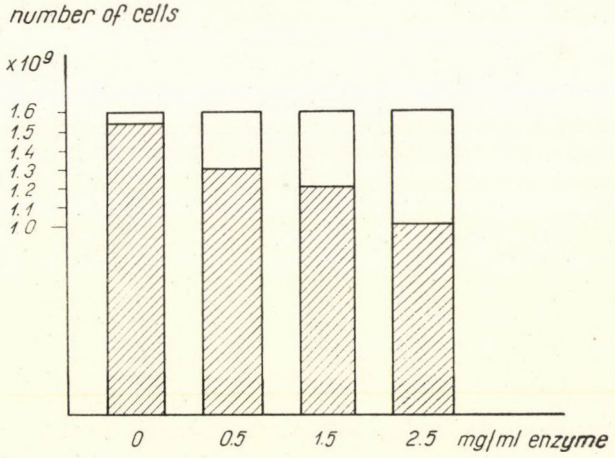

FIG. 2. Decrease of the cell count in the suspension of *Chlorella pyrenoidosa* after 48 hours of incubation, under the effect of treatment with different concentrations of the Cellulase 36 enzyme.

We studied the effect of 1 mg/ml Cellulase 36 and Cellulase Typ. I on respectively Chlorella and Scenedesmus cell suspensions of 1 mg/ml concentration at 37 °C. Table I shows the reducing-sugar values expressed in micrograms of glucose and converted to 1 ml of the suspension.

TABLE I

Reducing sugar values in μg/ml glucose

Alga sp.	Enzyme	Incubation time		
		2	4	24
		hours		
Chlorella	(Control)	8.0	9.0	12.0
Scenedesmus		8.0	9.0	12.0
Chlorella	Cellulase 36	14.0	16.0	50.0
Scenedesmus		17.0	20.0	41.0
Chlorella	Cellulase Typ. I	143.0	155.0	340.0
Scenedesmus		130.0	148.0	338.0

It appears, then, that the enzymes attacked *Chlorella* and *Scenedesmus* more or less with the same intensity, and that the Cellulase Typ. I that has proved more active in other experimental conditions (Ürmössy et al. 1967) was more active in the case of alga cells too.

Table II shows the effect of Cellulase Serva and Meicelase P on 1 mg/ml *Chlorella* at 37 °C.

TABLE II

Reducing sugar values in μg/ml glucose in *Chlorella pyrenoidosa* suspensions

Enzyme preparation	Enzyme concentration mg/ml	Incubation time				
		2	4	20	24	48
		hours				
Control Chlorella		8.0	10.0	13.0	15.0	20.0
Cellulase Serva	0.1	10.0	22.0	40.0	44.0	46.0
	0.2	20.0	34.0	50.0	46.0	62.0
	0.3	28.0	44.0	60.0	66.0	77.0
	0.4	36.0	58.0	72.0	78.0	92.0
	0.5	46.0	68.0	84.0	90.0	108.0
Meicelase P	0.1	96.0	124.0	174.0	180.0	179.0
	0.2	164.0	200.0	256.0	258.0	259.0
	0.3	234.0	276.0	336.0	339.0	342.0
	0.4	300.0	352.0	418.0	421.0	426.0
	0.5	372.0	428.0	500.0	515.0	512.0

As the concentration of the enzymes increases, the content in reducing sugar grows with both enzymes; Meicelase P is considerably more active than the Cellulase Serva preparation. The quantity of reducing sugar grows also in the control with no enzyme during incubation; this is a result of endogenous decomposition. Figure 3 illustrates the effect of Meicelase P as a function of enzyme concentration and incubation time. As the values

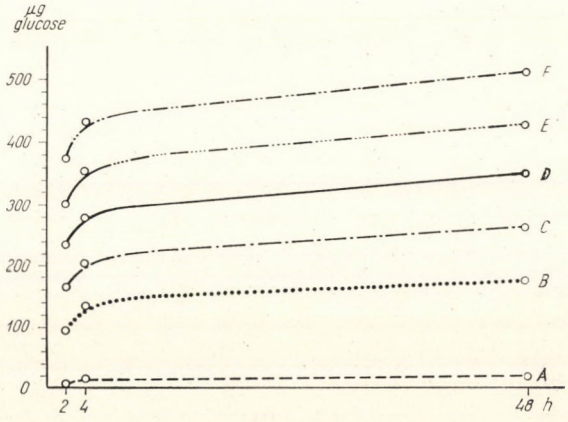

FIG. 3. The quantity of reducing sugar formed from the suspension of *Chlorella pyrenoidosa* under the action of various concentrations of the Meicelase P enzyme, in case of A = alga suspension with no enzyme, B = 0.1 mg/ml, C = 0.2 mg/ml, D = 0.3 mg/ml, E = 0.4 mg/ml, and F = 0.5 mg/ml enzyme concentrations.

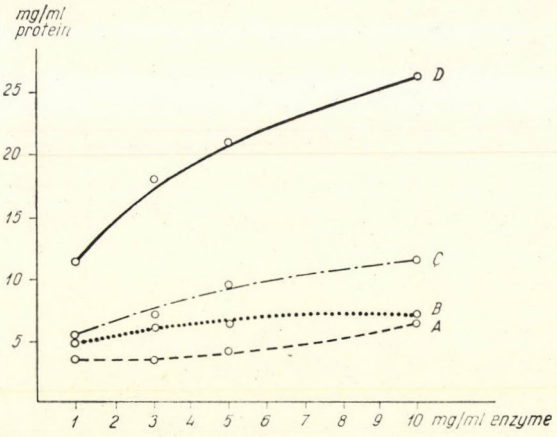

FIG. 4. Protein content determined in the 15% suspension of *Scenedesmus quadricauda* after 20 hours of incubation, in case of A = Cellulase 36, B = Cellulase (Serva), C = Meicelase P, and D = Takamine Cellulase enzymes. The protein content of the control alga suspension is 3.5 mg/ml.

after 20, 24 and 48 hours were identical, apart from minor differences, this indicates that the process of cellulolysis came practically to a standstill after about 20 hours.

We studied a suspension of *Scenedesmus* of 15% concentration incubated at 47 °C, under the action of 1 mg/ml, 3 mg/ml, 5 mg/ml and 10 mg/ml of Takamine Cellulase, Cellulase (Serva), Cellulase 36 and Meicelase P. The results are shown in Table III; owing to the concentration of the solutions, the values given are determined from a 0.01 ml suspension.

TABLE III

Reducing sugar values in μg /0.01 ml glucose after enzyme action on 15% *Scenedesmus* suspension

Enzyme preparation	Enzyme concentration mg/ml	Incubation time	
		4 hours	20 hours
Control without enzyme		33.7	41.9
Takamine	1	34.0	41.0
	3	35.0	41.8
	5	36.0	42.0
	10	37.0	42.0
Cellulase Serva	1	55.0	40.2
	3	64.0	42.0
	5	67.0	48.0
	10	74.0	52.0
Cellulase 36	1	54.0	47.0
	3	61.0	56.0
	5	66.0	66.0
	10	69.0	70.0
Meicelase P	1	50.0	50.0
	3	60.0	71.0
	5	77.0	88.0
	10	116.0	157.0

After 4 hours of incubation, digestion of smaller or larger extent in proportion to enzyme concentration was found in case of all enzyme preparations; but by the 20th hour the enzyme effect ceased altogether in case of

Takamine Cellulase, ceased practically with Serva Cellulase, and the activity of Cellulase 36 stayed on an identical level. Meicelase P proved to be the most efficient, as the reducing-sugar value increased even in the 20th hour.

Figure 4 shows the determination of the dissolved protein content of the suspension in the above experiment, after 20 hours of incubation. While regarded on the basis of reducing-sugar value the Takamine Cellulase seemed to have practically no effect on the alga cells, it proved to be the most active of the four tested cellulase preparations if judged on the basis of the protein content in the solution. The protein content of the enzyme added to the suspension was taken into account as correction for the measuring results; mg quantities of protein determined in this way from 1 ml of the experimental material are shown in the figure.

Discussion

We studied how *Chlorella pyrenoidosa* and *Scenedesmus quadricauda* cellulose can be degraded by means of cellulase preparations, and how this process of decomposition can be observed through the determination reducing sugars and dissolved protein. As a result of treating the alga suspension with cellulase enzymes, the released quantities of reducing sugar grow with enzyme concentration, and, to a certain limit, also with the period of treatment. We concluded that when the effect of cellulase enzymes on algae is investigated, the evaluation of enzyme activity, and of the extent of cell digestion, should be completed by the quantitative determination of protein that gets from the cells into the solution, as a practical judgement of the efficiency of cellulolytic enzymes seems to be possible only in this way. We consider *Chlorella pyrenoidosa* to be suitable as a substrate for studying enzyme effects, as this species is available in reproducible uniform quality, and has the advantage that by its application as a substrate in the methods of chemically determining enzyme activity the practical purpose, i.e. the study of the effects of enzymes on natural substances, can best be approached.

We have found that the unicellular algae *Chlorella pyrenoidosa* and *Scenedesmus quadricauda* can be digested enzymatically through the decomposition of the cell walls by means of commercial cellulase enzyme preparations, and that by such treatment their digestibility can probably be increased considerably.

Summary

1. 0.1–0.5 mg/ml concentrations of cellulase enzymes added to the 1% suspension of the cells of *Chlorella* and *Scenedesmus* result in great quantities of reducing sugar in the solution in case of pH 4.5 and at 37 °C.

2. The activity of the tested enzyme preparations proved not to be the same; the method of measuring the effect of these preparations on alga cells can be applied to a certain extent to the determination of enzyme activity in case of native cellulose.

3. The effect of 1–10 mg/ml cellulases on concentrated 15% alga suspensions is lower in respect of the formation of soluble reducing sugar, but leads to a great release of dissolved protein.

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