THE BIOLOGICAL, IMMUNOLOGICAL, AND PHYSICOCHEMICAL CHARACTERIZATION OF A TRANSMISSIBLE AGENT CAPABLE OF INDUCING DNA AND THYMINE DEGRADATION IN CULTURED HUMAN CELLS*

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The isolation of an unidentified transmissible cytopathic agent, possibly from the acute phase blood of an infectious hepatitis patient, has been reported (1). This agent possesses an unique property of inducing active DNA and thymine catabolism in infected "liver" cells (2). Until recently, characterization of the agent was greatly hampered by its existence in cell-associated form (1). Our recent success in obtaining it in cell-free form has greatly facilitated the characterization of this transmissible agent. This report describes its biological, immunological, and physicochemical properties. Since some of the properties are sufficiently unique, the possibility of a new class of transmissible agent should be considered. The release of a lipogenic toxin and the mechanism of DNA and thymine degradation are described in the accompanying reports (3, 4). A study of the cytopathology of this agent using the electron microscope is in progress.[1]

Materials and Methods

Nutrient Media.—Unless specified otherwise, the medium consisted of 5 per cent inactivated horse serum in Eagle's basal medium (5) as modified in our laboratory for the cultivation of primary human amnion cells (6). Holmes' chemically defined medium (7) was simplified through the elimination of the coenzymes and the nucleotides. This simplification did not detectably affect its ability to sustain the growth of an adapted strain of human "liver" cells. This simplified Holmes' medium consisted of Eagle's basal medium, as modified for the primary amnion cells, and the following additional compounds (in mg per liter): L-cysteine HCl 75, reduced glutathione 15, ascorbic acid 17, alpha-amino-*n*-butyric acid 2.5, L-ornithine HCl 3.5, L-taurine 2, L-homocystine 1, L-ergothione·HCl·H_2O 1, D-glucosamine HCl 3.2, glucurono-lactone 1, Na glucuronate 1, glucose-1-phosphate 1, citric acid 1, Na acetate·3 H_2O 83, L-hydroxyproline 20, *p*-aminobenzoic acid 0.05, alpha-tocopherol phosphate 0.01, biotin 1, thiotic acid 1.5, adenosine-5-phosphate 0.02, D-ribose 0.23, D-2-deoxyribose 0.2, vitamin B_{12} 2.5,

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cholesterol 0.2, menadione 0.01, calciferol 0.1, vitamin A 0.1, beta estradiol 0.1, and tween 80 5.

Cell Cultures.—All cell cultures were grown on glass as monolayers. The tubes were revolved at 12 revolutions per hour in a 36°C incubator. Unless specified otherwise, an established strain of epithelial-like cell derived from an explant of human liver was used exclusively (8). This culture has been propagated in our laboratory since its original isolation without any animal passage.

Propagation of this Transmissible Agent.—The history of this agent has already been described (1). Infected "liver" cultures which showed complete cell destruction were found to retain infectivity for at least 10 months when stored at 0-4°C; these cultures served as convenient sources of this agent. To prepare the cell-free form, "liver" cultures nourished in the simplified Holmes' medium were used. After infected cultures showed complete degeneration, they were kept at 36°C for 3 additional days. The media were pooled and centrifuged at 2000 RPM for 15 minutes in a horizontal International centrifuge. The upper ¾ of this supernatant fluid was collected. Since this fluid was low in infectivity (containing about 10 infectious doses per ml) and contained interferon-like substance as well as a lipogenic toxin (3), it was further centrifuged at 14,000 RPM for 1 hour in a Servall SS-1 angle head centrifuge. The sediment, which contained the infectivity, was resuspended in $\frac{1}{10}$ its original volume of simplified Holmes' medium. This concentrated preparation was kept at 0–4°C and may be used for several months. The supernatant fluid which was not infectious, contained the lipogenic toxin to be elaborated upon in the accompanying report (3). In 3 recent experiments, the cell-free form was also obtained from infected "liver," primary amnion, and chick embryonic tissue cultures which were nourished in Eagle's basal medium containing 5 per cent horse serum. The results reported in this manuscript are based on study with this agent, which has undergone numerous passages in the "liver" culture since its original isolation in 1954.

Virus Titration.—The standard tube dilution technic was used. Serial two-, four-, or tenfold dilutions of the preparation were made; and 2 to 4 liver cultures were used for each dilution. The appearance of cytopathic changes was recorded. Cultures showing no cytopathic changes after 3 weeks were discarded as negative.

Neutralization.—The standard tube neutralization test was used. 0.25 ml of a preparation of this agent was mixed with 0.25 ml of serum. The mixture was stored at 0–4°C for 16 to 20 hours. 0.4 ml of this mixture was then inoculated in a "liver" culture. This amount of inoculum contained approximately 10 infectious doses. 3 control cultures receiving the same amount of this agent were included in each test. 3 days after the appearance of advanced cytopathic changes in these 3 control cultures, the test was read. Those showing no cytopathic changes were considered positive for antibody or inhibitor. All serums were tested undiluted and at dilution of ¼, $\frac{1}{16}$, and occasionally $\frac{1}{64}$. When the quantity was insufficient, the specimen was tested at ¼, $\frac{1}{16}$, and $\frac{1}{64}$. In the later portion of this study, serums were frequently surveyed for heat-stable antibody without dilution. For the detection of heat-stable antibody, serums were routinely heated at 56°C for 30 minutes prior to testing. In the titration of heat-labile inhibitor, this preheating was omitted.

Other Viruses.—Stock preparations of adeno 3 and polio 1 viruses were propagated in cultures of HeLa cells (9); Coxsackie B1 and herpes simplex in "liver" cells, and vaccinia virus, on chorioallantoic membranes of chick embryos.

Enzymatic Digestion.—All enzymatic digestions (other than those involving the use of lipolytic enzymes) were carried out by diluting the enzymes in Eagle's basal medium (pH 7.4–7.8), mixing equal portions of diluted enzymes and a preparation of this agent, incubating the mixture at 37°C for 20 hours, and then testing for infectivity in "liver" cultures. The final concentrations of the enzymes varied from 50 to 200 μg per ml. In experiments using trypsin, about 500 μg of crystalline soybean trypsin inhibitor were added prior to the transfer of trypsin-containing materials to "liver" cultures. Due to non-specific degeneration caused by

papain, a blind passage of the cell sediment was routinely made 2 days following the introduction of materials containing papain.

EXPERIMENTAL RESULTS

1. Cytopathology

5 to 7 days following inoculation with 10 infectious doses, the "liver" culture showed characteristic degenerative changes. These changes progressed rapidly involving all cells within 2 to 3 days. These cellular changes consisted of granularity, crenation, and loss of structural distinction between nucleus and cytoplasm (see Fig. 1). In preparations stained with hemotoxylin-eosin, intranuclear changes were characteristic. The nuclear changes consisted of condensation of basophilic materials along the nuclear membrane and the formation of basophilic globules of varying number and sizes (see Fig. 2 *A*). With further changes, the involved nucleus apparently collapsed and appeared as a small basophilic mass without structural detail (Fig. 2 *B*). In culture showing advanced degeneration, many cells consisted of cytoplasmic masses in which were situated a basophilic globule and a basophilic ring surrounding a basophilic dot (Fig. 2 *C*). In the end stage, the basophilic globule was no longer visible and the cytoplasm became more vacuolated (Fig. 2 *D*). Further disintegration of such shrunken cells occurred at a very slow pace; 1 month (at 37°C) after complete degeneration, numerous shrunken cells still remained. Feulgen staining showed that the abnormal basophilic masses and globules, illustrated in Fig. 2, were Feulgen-positive (DNA). The basophilic ring was also Feulgen-positive; the central basophilic dot was Feulgen-negative (nucleolus?), however. The condensation of basophilic materials along the nuclear membrane was also seen occasionally in presumably uninfected "liver" and primary amnion cultures.

In our earlier study with the cell-associated forms (1), it was described that the infecting process may at times show 3 other features: *1*, marked prolongation of the incubation period; *2*, establishment of a "chronic" infection, and, *3*, reappearance of colonies of healthy cells in apparently completely degenerated cultures. Such variations in the infecting process have not been encountered in "liver" cultures infected with the cell-free form.

2. Susceptibility of different types of cells in vitro

The reaction of primary human amnion cells was distinctly different from that of the "liver" cells. Seven lots of amnion cells each harvested from a single placenta had been studied thus far. Six of the seven failed to show characteristic progressive degeneration after inoculation with 3 to 300 infectious doses. Transient cytoplasmic granularity and non-progressive cell destruction were noted at irregular intervals. Infectious materials were being released from such amnion cultures (some for over 3 months) as demonstrated by the transfer of

uncentrifuged used media to "liver" cultures; the recipient "liver" cultures invariably showed characteristic cytopathic changes. An exception was noted, however. One lot of primary amnion cells was completely destroyed about 15 days following infection with about 300 infectious doses. Four lots of primary human embryonic skin and muscles from 3 to 4-month-old embryos, one lot of primary monkey-kidney cells, three lots of primary embryonic chick tissue, the HeLa (9), conjunctival (8), FL (10), KB (11), mouse fibroblast strain L (12), and sarcoma 180 II (13) cells were completely destroyed 4 to 10 days after infection with about 10 to 100 infectious doses. Two lots of mouse embryo cultures were also destroyed in about 14 days. Of some interest was an observation that the ribose variants derived from the HeLa and the conjunctival cells (14) were much less susceptible than the parent cultures; the ribose variants also multiplied at slower rates as compared to the parent cultures.

3. The production of interferons

The appearance of "chronic" infections in primary amnion cultures suggested that interferon-like substances may be released by the infected cells. The fluid phase of infected liver and primary amnion cultures, after removal of infectious particles by centrifugation at 14,000 RPM (SS1 Servall), were tested at final concentration of 30 per cent by a method currently used (15). The appearance of cytopathic changes in the liver or primary amnion infected with about 30 ID of Coxsackie B1 virus was significantly and regularly delayed. Such delay was not observed, however, in liver cultures infected with poliovirus type 1 or with this agent (30 ID used). It seemed reasonable to conclude that interferon-like activity was released from the infected cells. There was no evidence, however, that the interferon released was responsible for the "chronic" infection observed in the primary amnion cultures.

4. Stability of its infectious property

Thermostability.—At 0–4°C, this agent retained its infectivity for more than 6 months. At 37°C, a detectable reduction of infectivity (as judged by the prolongation of the interval between infection and degeneration) was noted on the 14th and 35th day, and a complete loss on the 70th day. At 56°C for 30 minutes, 5 out of 7 pools retained partially their infectivity. At 60°C for 30 minutes, infectivity was detectable in 2 of 3 pools. At below—60°C, 1 pool was found infectious after 1 month but non-infectious after 6 months; a 2nd pool, with about 10^8 infectious doses per ml before freezing, lost its infectivity after 1 month. After 5 cycles of freeze-thawing by alternate immersion in dry ice–alcohol and 37°C water baths, 3 of 5 preparations (infectious at dilution of 1/64 or more) became non-infectious when tested undiluted, while two were infectious at dilution of 1/4 but not at 1/8.

Inactivation by Ether and Chloroform.—Four lots of this agent were extracted once with equal portions of ether; infectivity was no longer demonstrable in the aqueous phase. Similarly, extraction with chloroform inactivated this agent.

Inactivation by Bile Salts.—When incubated with equal volume of 1 per cent sodium deoxycholate at 37°C for 2 hours, infectivity was partially reduced.

Insusceptibility to Various Proteases, Nucleases, and Carbohydrases.—The following enzymes had been tested and found ineffective in inactivating this agent: crystalline DNAse, crystalline RNAse, crystalline trypsin, crystalline papain, crude trypsin, crude pancreatin, crystalline eggwhite lysozyme, and bovine testicular hyaluronidase. In 1 experiment higher concentrations of DNAse, RNAse, or trypsin (0.5 to 1 mg per ml) were used; pH were adjusted to 6–7 for DNAse, 4.5–5 for RNAse, and 7.6–8.2 for trypsin; and digestion period was prolonged to 6 days at 36°C. Fresh enzymes were also added on the 3rd and 5th days. Again, no significant reduction in infectivity was detected.

Insusceptibility to Pancreatic Lipase and Lecithinase.—Examination of the effect of lipolytic enzymes on the infectivity of this agent was complicated by the necessity of introducing bile salts or ether into the reacting systems to bring about the optimal interactions of the enzymes and the lipids (16, 17). Both bile salts and ether have already been shown to destroy its infectivity. The effect of these 2 lipolytic enzymes were therefore tested without bile salts or ether in the reacting systems as follows:

1. 0.1 ml of virus preparation was mixed with 0.05 ml of pancreatic lipase (2 per cent suspension in 0.5 M tris buffered at pH 8), 0.05 ml 45 per cent $CaCl_5$, and 0.3 ml 0.5 M tris (pH 8). After 1 hour at 37°C, infectivity was titrated in "liver cultures."

2. 0.1 ml of virus was mixed with 0.1 ml of snake venom (*Crotalus adamanteus* venom, kindly furnished by the laboratory of Dr. E. P. Kennedy) and 0.3 ml Eagle basal medium. This amount of snake venom was equivalent to about 0.5 mg of dry venom. Infectivity was titered after 1 hour at 37°C. Since snake venom at this concentration was also destructive to the "liver" cells, passages were routinely made for culture destroyed with 24 hours after receiving the inoculi.

Both lipolytic enzymes fail to reduce the infectivity of this agent.

Slow Inactivation by Formalin.—Results of the inactivation of this agent by 0.025 per cent formalin are shown in Table I; the rate of inactivation is very much slower than that of vaccinia and herpes viruses, or polio and vesicular stomatitis viruses.

5. Filtration and centrifugation

Membrane filters (Millipore) retained the infectivity in most instances; 5 filtrates through filters with 5μ pore size (which allowed passage of *Staphylococcus aureus*) and 7 of 9 through 0.35 micron filter were found non-infectious.

2 filtrates (0.35 micron) were infectious; technical errors, however, could not be positively excluded. In a single trial, infectivity was retained by ultrafine glass filter.

Infectivity was not sedimented by centrifugation at 2000 RPM for 15 minutes (International horizontal model) but was regularly sedimented at 14,000 RPM for 1 hour (Servall SS-1 superspeed angle centrifuge). Only 1 of 12 supernatant fluids was found infectious.

TABLE I

The Inactivation of This Unidentified Agent, the Vaccinia and Herpes Simplex Viruses by Formalin, ‡*

Time	The agent		Infectivity§ vaccinia	Herpes
	A	B		
hrs.				
0	1280	2560	10^6	10^5
24	<80	80	<10	<10
48	80	640?	<10	<10
96	20	20	<10	<10

* Formalin used at final concentration of 1/4000; pH = 7.2–7.6 and temperature of incubation, 37°C.

‡ See Symposium on Inactivation of Viruses (*New York Acad. Sc.*, 1960, **83**, 513), for data on other viruses.

§ Expressed as infectious doses per ml (tested in liver cultures).

6. Suppression of its synthesis by 5-fluorodeoxyuridine

Since the type of nucleic acid present in a virus may be of value in its identification, experiments were designed to determine whether this is a DNA- or RNA-containing agent. Two preliminary attempts were made in extracting infectious nucleic acid from 10^7 specifically involved shrunken "liver" cells by the phenol method (18, 19). Infectious nucleic acid was not obtained.

Based on the well established observations that 5-fluorodeoxyuridine (FUDR) suppressed the formation of DNA- but not RNA-containing viruses (20-24), the effect of FUDR on the propagation of this transmissible agent was studied.

Three replicate cultures of primary human amnion cells were nourished for 7 days with a nutrient medium containing 1 μg FUDR per ml. Previous study showed that at this concentration of FUDR the primary amnion did not degenerate or multiply significantly for at least 14 days (6). The media of the 3 cultures were then changed to that containing no FUDR, 1 μg FUDR plus 50 μg thymidylate, and 1 μg FUDR, and were infected with 10 infectious doses of this transmissible agent. Nutrients were renewed every 3 or 4 days with

their respective media. The used media together with any detached cells were transferred to liver cultures which were then observed for evidence of characteristic degeneration.

The results are shown in Table II; FUDR suppressed partially or completely the formation of this agent. Thymidylate, however, fails to effect regularly a complete reversal of this suppressive action of FUDR. Since this latter finding is contrary to what has been reported for 2 other DNA-containing viruses

TABLE II

Effect of FUDR on the Formation of this Transmissible Agent in Primary Human Amnion Cell Culture

Expt.	Nutrient media*		Infectivity in media on day after infection		
	FUDR	Thymidylate	3	7	14
1	0	0		+ (5)‡	+ (2)
	1	0		−	−
	1	50		−	−
2	0	0	+ (4)	+ (3)	+ (3)
	1	0	+ (8)	+ (7)	+ (7)
	1	50	+ (8)	+ (7)	−
3	0	0	+ (7)	+ (3)	
	1	0	+ (13)	−	−
	1	50	+ (10)	+ (3)	+ (6)
4	0	0	+ (7)	+ (3)	+ (2)
	1	0	−	−	−
	1	50	+ (14)	+ (10)	+ (11)

* Regular medium containing FUDR or thymidylate in μg per ml.

‡ Signifies the day of onset of characteristic degeneration in liver cultures.

(20, 24), the possibility that the cell system used in these experiments may contribute to this difference was explored. Earlier work (6) indicated that the relatively dormant primary amnion cells differed considerably in their response to FUDR from the rapidly growing transformed cells used by other investigators. The propagation of 3 DNA-containing viruses (vaccinia, adeno 3, and herpes simplex) and of 2 RNA-containing viruses (polio 1 and Coxsackie B1) was investigated under experimental conditions similar to those described for this transmissible agent. The results of 1 single experiment are presented in Table III; FUDR exerts no detectable effect on the propagation of the RNA-containing viruses—it suppresses partially the multiplication of the DNA-containing viruses, and this suppressive effect is reversed by thymidylate. These findings are in complete agreement with those reported by

others (20-24). Based on these studies, it seemed reasonable to conclude that this transmissible agent contained DNA. We are unable, however, to account for the failure of thymidylate to reverse regularly and completely the suppressive action of FUDR.

TABLE III

Effect of FUDR, with or without Thymidylate, on the Formation of Several DNA- and RNA-Containing Viruses in Primary Human Amnion Cultures

Virus*	Nutrient media‡	Degree of degeneration§ on				
		day, 2	3	4	5	Titer‖
Polio 1	Control	++	+++			6.7
	FUDR	++	+++			6.7
	FUDR, thymidylate	++	+++			6.3
Coxsackie B1	Control		0	+	+++	6.7
	FUDR		0	+	+++	6.7
	FUDR, thymidylate		0	+	+++	7.0
Vaccinia	Control	0	+	++	+++	4.5
	FUDR	0	0	+	+	2.7
	FUDR, thymidylate	0	+	++	+++	4.5
Herpes simplex	Control		+++			4.5
	FUDR		+			3.0
	FUDR, thymidylate		+++			4.3
		day, 5	7	10	15	
Adeno 3	Control	0	+	+	+++	5.5
	FUDR	0	0	0	+	3.5
	FUDR, thymidylate	0	+	+	++	5.7

* About 30 to 300 LD_{50} were used.

‡ FUDR and thymidylate at 1 and 50 μg per ml medium, respectively.

§ 0, +, ++, and +++ represented no, early, advanced, and complete degeneration, respectively.

‖ Virus titer determined at the time when the control culture (without FUDR) showed complete degeneration; expressed as the negative logarithm of the high dilution which gave rise to cytopathic changes in 50 per cent of the inoculated cultures.

The inability of thymidylate to reverse completely the suppressive action of FUDR on DNA or viral synthesis has been reported (24, 25); reversals were accomplished, however, by further addition of uridine or uracil. The appearance of characteristic cytopathic changes and the formation of infectious materials were studied with liver and conjunctival cells treated with FUDR, FUDR plus thymidine, FUDR plus thymidine and uracil, and FUDR plus uracil. Results are shown in Table IV. Thymidine with or without uracil reverses the

cytotoxic action of FUDR; the suppressive action on the formation of this agent was not affected, however. The possibility that FUDR may affect cellular metabolism by another mechanism as yet unknown should be considered.

7. Continued release of infectivity from specifically involved shrunken cells

Infectious materials were found to be released continually from specifically involved shrunken cells for many weeks as demonstrated by the following

TABLE IV

Failure of Thymidine and Uracil to Reverse the Suppressive Effect of FUDR on the Formation of this Agent in Liver and Conjunctival Cells

Cell	Media*	Multiplication index‡	Viral degeneration§
Liver	Control	1728/225	6th day
	FUDR	218/225	Neg. after 14 days
	FUDR, U	126/225	Neg. after 14 days
	FUDR, T	1076/225	Neg. after 14 days
	FUDR, T, U	1202/225	Neg. after 14 days
Conjunctival	Control	1394/59	7th day
	FUDR	32/59	Neg. after 14 days
	FUDR, U	8/59	Neg. after 14 days
	FUDR, T	772/59	Neg. after 14 days
	FUDR, T, U	552/59	Neg. after 14 days

* Regular medium to which FUDR, U (uracil), T (thymidine) were added to concentrations of 1, 50, and 50 μg per ml, respectively.

‡ Multiplication of cells in uninfected culture; denominator = No. of cell × 10^3 at 0 day and numerator, at 7th day.

§ Extensive non-specific degenerations were noted in cultures treated with FUDR or FUDR plus uracil; these degenerative changes were not transmissible.

experiment. A suspension of specifically involved shrunken cells (about 5 to 10 million cells) was washed 3 times with 10 ml each of Eagle's basal medium by centrifugation at 2000 RPM for 15 minutes; the washed sediment resuspended in 2 ml of modified Holmes' medium was kept at 36°C for 7 to 34 days; the suspension was then centrifuged at 2000 RPM for 15 minutes, and the supernatant fluid tested for infectivity. This procedure was repeated at irregular intervals. In 1 experiment, infectivity was demonstrated on the 20th, 40th, 54th, and 57th day. In a 2nd experiment, the infectious titers were 10^3, 10^1, 10^2, and $< 10^1$ infectious units per ml on the 7th, 14th, 48th, and 81st days, respectively.

8. Failure to demonstrate neutralizing antibody

Heat Stable Serum Antibody.—A large variety of serums was tested for neutralizing antibody against about 10 infectious doses of this transmissible agent. No neutralizing antibody has so far been detected in serums preheated at 56°C

for 30 minutes. Serums that have been tested thus far are classified in the following groups:

1. Twenty-three convalescent infectious hepatitis sera collected 2 to 3 months after onset of jaundice from 3 different outbreaks in Massachusetts in 1954, 1955, and 1960.

2. Three serums collected about 2 months after onset of jaundice from individuals given the serum hepatitis virus (kindly furnished by Dr. Roderick Murray).

3. Two serums from patients with jaundice due to leptospirosis (diagnosis confirmed by the agglutination-lysis tests).

4. Three serums from elderly patients with severe obstructive jaundice.

5. Seventeen convalescent serums collected from patients recovered from the following illness: 3 rubella, 2 rubeola, 1 varicella, 2 ECHO-16 exanthema, 1 mumps, 1 herpetic gingivitis, 1 poliomyelitis, 1 influenza, 1 adeno-3, 2 Rocky Mountain spotted fever, and 2 rickettsialpox.

6. Human gamma globulin prepared from approximately 1000 units of outdate blood collected in Massachusetts (kindly furnished by Dr. Geoffrey Edsall of the Institute of Laboratories, Commonwealth of Massachusetts). This lot of gamma globulin was tested at the concentration of 4 per cent (antibody against 100 ID_{50} of poliovirus type 1 was demonstrable at 0.2 per cent).

7. Ninety-nine serums from healthy adults who were students or employees in the Harvard Medical Area.

8. Serums of 3 laboratory personnel working with this agent for 1 or more years (tested about every 6 months).

9. Seven specimens of human placental cord serums.

10. Serums from healthy adults (with or without clinical trachoma) residing in the following localities: 20 from Ethiopia, 10 from Hong Kong, 10 from Portugal, and 50 from Yugoslavia.

11. Forty-five white Swiss mouse, 19 white rat, 35 cotton rat, 6 rooster, 7 rabbit, 15 guinea pig, 8 horse, and 2 hamster serums (these were tested individually or in pools of 4 to 10 serums).

12. Hyperimmune serums against parainfluenza virus, types 1, 2, and 3.

13. One chimpanzee serum with high neutralizing titer against the D.A. virus, kindly furnished by Dr. Hsiung (26).

14. Two pools of monkey serum and 2 pools of chick plasma.

Failure to Produce Antibody in Rabbits and Mice.—Attempts to produce antibody have been unsuccessful thus far. The following immunizing procedures were used:

1. Three rabbits were given weekly intravenous injections of about 2×10^6 specifically involved shrunken "liver" cells for 3 successive weeks and were bled on the 5th and 10th days after the last injection.

2. Two rabbits were given an intramuscular injection of about 5×10^6

specifically involved shrunken "liver" cells suspended in Freund's incomplete adjuvant, and were bled 3 months later.

3. One white Swiss mouse was given intravenously 10 to 30 infectious doses at each injection for a total of 11 injections; daily for 3 injections, every 3rd day for 3 more injections, every 5th day for another 3 injections, and 2 more injections at weekly intervals. Another was similarly treated but by the intraperitoneal route. Serums collected 7 days after the last injection failed to neutralize this transmissible agent at 1/4 dilution.

4. Eight white Swiss mice were given subcutaneously 2×10^6 sarcoma 180 II cells (13) mixed with equal number of specifically involved shrunken "liver" cells. 2 months later, they were bled. No neutralizing antibody could be demonstrated when tested undiluted. At the time of bleeding, 3 mice developed large subcutaneous tumors and 5 were without tumors. From 2 of these tumors, this transmissible agent was readily recovered following explanation of the tumors *in vitro*.

9. The existence of an ubiquitous heat-labile inhibitor in serums

When the neutralization tests were performed without the preheating of test serums, many were found to contain inhibitor against this transmissible agent. Subsequent study showed that this inhibitor was present in almost all fresh serums or in those kept frozen at below −20°C within 48 hours after collection. The activity of this inhibitor may be manifested by a delay in the onset of cytopathic changes or complete inactivation of this transmissible agent. A total of 76 human and 81 animal serums have been tested. These serums were collected by the Staff of this Department and were known to have been stored at below −20°C within 48 hours after collection. The frequency with which they had been thawed and refrozen before testing was not known, however. The results are summarized in Table V: this inhibitor is present in at least 96 per cent of all the serums tested, in human as well as in animal serums, in human placental cord as well as human adult serum, and in human serums collected from Hong Kong, Ethiopia, as well as those in Boston. The inhibitor titers in the hepatitis serums are not any higher than that of the general population. The placental cord and the adult serum have comparable inhibitor titers. It seems reasonable to conclude that this heat-labile inhibitor is a normal serum component.

Thermostability of this Inhibitor.—The stability of this serum inhibitor was studied by determining the neutralizing titers of 2 pools of human serums kept at 37°C and at 0–4°C for varying durations. The results are summarized in Table VI; significant decreases in the inhibitor activity are noted after 4 days at 36°C and 4 weeks at 0–4°C. Heating at 56°C for 30 minutes completely eliminates its activity. Repeated freeze-thawing affects the activity of one pool and not the other.

Loss of Activity Following Cold Ethenol Fractionation and Ether Extraction.—Two pools of human serums with initial inhibitor titers of 1/4 and 1/16 were fractionated into fraction II + III and II + III supernate by the cold ethanol method (27). Inhibitor activity could not be demonstrated in both fractions tested singly or in combination. Two extractions with equal portion of ether

TABLE V

Titers of Heat-Labile Inhibitor in Various Serums

Serum	Duration of storage	No. tested	No. with titer of			
			None	1	4	16
Human						
a. Boston						
i. Medical students	5 yrs.	9		2	7	
ii. Blood donors	2 yrs.	2 pools of 5		1	1	
iii. Lab personnel	1 yr.	12			10	2
iv. Investigators	1 yr.	3			1	2
	fresh	3			2	1
v. Placental cord	1 wk.	7	1?*		5	1
vi. Hepatitis	<6 mos.	12		2	10	
b. Hong Kong	3 yrs.	10	2	8		
c. Ethiopia	2 yrs.	10		2	6	2
Horse	1 wk.	3	1	1	1	
Guinea pig	1 wk.	pool of 15			1	
Hamster	3 yrs. ?	2		2		
Cotton rat	3 yrs. ?	7 pools of 5		7‡		
White rat	3 yrs. ?	4 pools of 5		4‡		
White Swiss mouse	fresh	2	2*			
Rooster	3 yrs. ?	1 pool of 4			1	

* Negative at 1/4; quantity insufficient for testing without dilution.

‡ Negative at 1/4; all pools combined and found positive when tested without dilution.

for 1 minute each resulted in complete loss of inhibitor activity of both pools of serums.

Presumed Non-Identity of this Inhibitor and the Properdin System.—Heat-labile component of normal serum capable of neutralizing certain viruses had been reported (28–30, and their references). Subsequent study suggested that this normal labile inhibitor against the myxoviruses was identical to the properdin system (31). Experiments were designed to determine if our inhibitor was similar to the properdin system. The following results indicate their non-identity:

1. Neutralization at 0–4°C. The properdin system was said to be inactive at below 10°C, and the optimal temperature for its action, about 37°C (31). The inhibitor described by us was at least as active at 0–4°C as at 37°C. A pool of guinea pig serums and 1 of human serums were titrated for inhibitor against this transmissible agent. The interaction of the diluted serum and the agent was allowed to take place for 1 hour at 0°C or at 37°C before inoculation. Titers of inhibitor were similar whether the interaction occurred at 0 or at 37°C.

2. Neutralization in the presence of sodium citrate. Magnesium and/or calcium were demonstrated essential in the neutralization of the myxovirus by

TABLE VI

Neutralizing Titers of 2 Pools of Human Serum Stored at Different Temperatures for Varying Intervals*

Treatment	Titer of inhibitor	
	Pool A	Pool B
None	4‡	16
56°C, 30 min.	None§	None
Freeze thaws ×10	4	1
36°C, 1 day	4	1
36°C, 2 days	1	1
36°C, 4 days	None	None
0–4°C, 1 wk.	4	4
0–4°C, 2 wks.	4	16
0–4°C, 4 wks.	1	1

* Both pools have been kept frozen at below −20°C for about 2 years.

‡ Reciprocal of serum dilution which delayed the onset of degeneration for 3 or more days.

§ None detectable when serums were tested undiluted.

the properdin system (28–31). When 1 or 2.5 per cent sodium citrate was used in the diluent, the neutralizing activity against the myxovirus was inhibited (28, 29). The presence of 1 per cent sodium citrate in the diluent did not detectably reduce the inhibitor titers of 2 serums tested. Their titers were 1/8 and 1/2 when tested without sodium citrate, and 1/8 and 1/4 in the presence of 1 per cent sodium citrate.

3. Failure to restore the activity of fraction II + III. Properdin was reported to be present in Cohn's fraction III or II + III (32, 33). If the inhibitor against this transmissible agent were the properdin system, one should be able to restore the inhibitory activity of fraction II + III by the addition of guinea pig complement and magnesium ions. Concentrated fraction II + III was mixed with guinea pig serum, modified Holmes' medium, and the transmissible agent. The proportion of each component was such that the final concentrations

of fraction II + III were equivalent to undiluted serum and that of guinea pig serum at 1/10 dilution. After 1 hour at 37°C, "liver" cultures were inoculated with this mixture. Degeneration appeared at about the same time as the control containing similar quantities of guinea pig complement and the transmissible agent. Two human serum fraction II + III, kept frozen at −20°C for 2 years, have been so tested. The original unfractionated serums, also kept frozen at −20°C for about 2 years, were found to inhibit this transmissible agent at 1/4 and 1/16 dilution. The guinea pig serum, used in this study, hemolyzed sensitized sheep erythrocyte at 1/320 and inhibited this transmissible agent at 1/4 dilution. Since a combination of fraction II + III containing most of the properdin, guinea pig complement, and magnesium ions (which constituted the properdin system) failed to inactivate this transmissible agent, this may be considered additional evidence of the non-identity of these two heat-labile inhibitors.

Reactivation of the Agent Treated with Serum Inhibitor.—To elucidate the mechanism of this inactivating process, attempts were made to reactivate this agent previously inactivated by the serum inhibitor. We have found that reactivation may be readily accomplished through simple solution as illustrated by the following experiment:

0.2 ml of a preparation of this agent (± 100 infectious doses) was mixed with 0.4 ml of serum. The mixture was kept at 0–4°C for 24 hours and then divided into 2 equal portions. 1 of the portions was kept at 0–4°C for another 48 hours and then inoculated into a "liver" culture. The other portion was diluted to 10 ml with modified Holmes' medium, kept at 0–4°C for 24 hours, and centrifuged at 14,000 RPM for 1 hour in a Servall SS-1 angle head centrifuge. The upper 9 ml was discarded. The lower 1 ml was diluted to 10 ml with modified Holmes' medium, kept at 0–4°C for 24 hours, and centrifuged at 14,000 RPM again for 1 hour. The lower 1 ml together with whatever sediment that may be present was transferred to a "liver" culture. The cultures were then observed for the appearance of characteristic degeneration.

The results are shown in Table VII which is self-explanatory. These results suggest that the inactivation of this transmissible agent by the heat-labile serum inhibitor is due to some non-specific complexing of the agent and inhibitor rather than due to enzymatic degradation. The dissociation of the agent-inhibitor complex in the presence of magnesium ion is also contrary to that reported for the dissociation of myxovirus-properdin complex (22, 25).

10. Failure to reisolate a similar agent

Our failure to reisolate a similar agent from the original frozen blood specimens was reported previously (1). It was suggested then that (since this transmissible agent was unstable to freeze-thawing) isolation attempts should be made with fresh hepatitis blood. To date, we have inoculated "liver" cultures

with over 35 specimens of fresh blood collected from infectious hepatitis patients during the 1st or 2nd week of jaundice. These cultures were observed for 3 to 5 weeks with no evidence of progressive degeneration. 1 blind passage was routinely made and the culture was observed for another 3 to 5 weeks; again, no progressive degeneration appeared. In several instances, 3 or 4 blind passages were made before the cultures were discarded as negative.

It is conceivable that our failure to reisolate this transmissible agent from fresh hepatitis bloods may be due to its complexing with the ubiquitous serum inhibitor. Since this complexing of agent and inhibitor may be readily dissociated by simple dilution (see section on reactivation), seventeen frozen hepatitis bloods were subjected to the dilution process prior to inoculation of the

TABLE VII

Reactivation of Infectivity of Serum Inactivated Agent by Simple Dilution

Exp.	Infectious agent mixed with	CPE in culture-receiving mixture treated by	
		Dilution	No treatment
1	Holmes' medium	5*	5
	Guinea pig serum pool	5	17
	Normal human serum pool	5	Neg.
2	Holmes' medium	5	5
	Hepatitis serum A	5	11
	Hepatitis serum B	5	Neg.

* Number of days after inoculation when characteristic cytopathic changes appeared; negative indicated absence of degeneration after 21 days.

"liver" and the primary human amnion cultures. Again, no transmissible cytopathic agent could be demonstrated in cultures observed for at least 2 months after receiving such materials. Owing to shortage of available clinical materials, we have not yet tested any fresh hepatitis bloods which are subjected to this dilution process before inoculation into cell cultures.

11. Other miscellaneous observations

Similar to the cell-associated forms (1), 3 to 10 infectious doses of the cell-free form, given intracerebrally or intraperitoneally, failed to produce any overt disease in white Swiss mice (1 to 3-day-old) during observation periods of 3 weeks. About 100 infectious doses given by the amniotic route to 7-day-old chick embryo failed to increase significantly the embryonic mortality. Preparations containing about 10^3 infectious doses per ml failed to hemagglutinate human group O or embryonic chick erythrocytes at 0–4°C. Partially degenerated "liver" or primary human amnion cultures (known to be releasing infec-

tious materials) did not hemadsorb human group O or embryonic chick erythrocytes. No visible growth can be detected after cultivation on beef infusion agar containing 10 per cent fresh horse blood or serum-agar medium for pleuropneumonia-like organisms (34); these cultures were kept for 7 days at 36°C in an atmosphere of 5 per cent CO_2 in air.

DISCUSSION

Data presented suggest that this transmissible agent is a virus consisting of DNA protected by a surface coat rich in lipids. Since many of its unusual characteristics can best be explained by a hypothesis that its outmost surface consists chiefly of lipids, we would like to propose that this agent be referred to tentatively as a lipovirus.

The suppressive action of 5-fluorodeoxyuridine on the formation of this virus in primary human amnion, liver, and conjunctival cells indicates that DNA is an essential component. This line of reasoning is somewhat weakened by our failure to obtain regular and complete reversal of this suppressive action of FUDR by thymidine or thymidylate. There has been, as yet, no reported exception to the finding that FUDR suppresses the synthesis of DNA but not RNA viruses in mammalian cells. The failure of thymidine and/or uracil to reverse the suppression of the formation of this virus by FUDR may be considered a characteristic so far unique to this lipovirus.

Our contention that its surface coat consists chiefly of lipids is based on the following findings: *1.* the complete inactivation by ether and chloroform, and partial reduction of infectivity by sodium deoxycholate; *2.* the slow inactivation by 0.025 per cent formalin; *3.* the reversible complexing with an ubiquitous serum inhibitor, presumably lipoprotein-like; *4.* the retention of infectivity by membrane filter (Millipore) with pore size of 5 microns; *5.* the inactivation by freeze-thawing in contrast to its marked stability at 0–4°C; *6.* the failure of DNAse, RNAse, trypsin, papain, lysozyme, hyaluronidase, lecithinase, and pancreatic lipase to reduce significantly its infectivity, and *7.* its apparent immunological non-reactiveness.

The failure of lecithinase and lipase to inactivate this lipovirus appears paradoxical. This, however, may be explained on the basis of high substract specificity and our inability to provide optimal conditions for lipolysis.

The statement that the lipovirus is apparently non-reactive immunologically is based on the following two observations: *1.* our failure to demonstrate heat-stable neutralizing antibody in a large variety of human and animal serums, and in human gamma globulin; and *2.* our inability to elicit an immunological response in rabbits and mice.

It should be emphasized that the failure to elicit antibody response by the methods used may also be due to our inability to give sufficient quantity of antigenic materials. We have tried to overcome this difficulty by creating sar-

comas in the white Swiss mice with sarcoma 180 cells of Foley (13), infecting the sarcoma 180 cells with this agent, demonstrating the presence of this agent in the sarcoma 2 months after infection, and then testing for antibody in the serums of these mice. None were demonstrated. While it is reasonable to conclude that limited multiplication of this agent had taken place in the sarcoma-bearing mice, the possibility that the agent existed in latent forms cannot be excluded. Inability to obtain satisfactory immune response in the laboratory animals has been reported for the cytomegalovirus (35). Antibody to the cytomegalovirus, however, can be readily demonstrated in humans (35). The possibility that our serological survey is not sufficiently exhaustive or method not sufficiently sensitive to detect a very low incidence of antibody should also be mentioned.

The susceptibility of sarcoma 180 cells *in vitro* (completely destroyed in 2 to 3 days by the dose of specifically involved shrunken cells used) in contrast to the markedly reduced susceptibility when transplanted *in vivo* (cells continued to divide forming large sarcoma in 2 months) is an observation of some interest. Similarly, explants of chick embryonic tissues were completely destroyed while chick embryos survived infection *via* the yolk sac or amniotic routes. Differences in the *in vitro* and *in vivo* susceptibility of tissues to poliovirus and myxoma viruses have been described (36–38). Little is known, however, about the basic mechanism of the reduced *in vivo* susceptibility of certain tissues to these viruses.

The nature of the ubiquitous inhibitor against this transmissible agent deserves discussion. It is presumably a normal component of most mammalian serum since it is present in at least 96 per cent of the 157 serums tested, and since comparable quantity (1/1 to 1/16) was found in human placental cord serums as in serums collected from the adult populations. The evidence presented appeared sufficient to conclude that this inhibitor is not the properdin system. Its instability to heating at 56°C, to storage at 37°C, or 0–4°C, to repeated freeze-thawing, to ether extraction, and to cold ethenol fractionation suggests the possibility that this inhibitor is a lipoprotein. The mechanism of this reversible complexing of an agent rich in surface lipids and a lipoprotein may be similar to that described by Younger and Noll (39). The role of such an inhibitor in the defense mechanism of an organism against infection by this transmissible agent is not known at the present time. It is not difficult, however, to visualize its role in limiting hematogeneous dissemination of infectious processes. It is possible that a transient or prolonged deficiency in this lipoprotein inhibitor may impair the barrier against infection by this agent. We are not certain whether the failure to detect this inhibitor in a few of the 157 serums tested was representative of such a deficiency or the result of excessive freeze-thawing which many of these serums may have been subjected to.

The origin of this transmissible agent remains obscure. Although it presum-

ably originated from blood taken from an infectious hepatitis patient during the acute phase, we have not been able to reisolate a similar agent (or any transmissible cytopathic agent) from the bloods of 43 infectious hepatitis patients collected in 1954, 1955, and 1960. The possibilities that this agent is indigenous in the "liver" culture or that it had been introduced inadvertently from an extraneous source should be considered. We have not been able, however, to induce the appearance of a similar agent from uninfected culture, or to define the extraneous source. The data presented appear sufficient for us to conclude that this is a new transmissible agent not described previously. The characteristic cytopathology and the unique property of thymine degradation (4) rule out the possibility that the lipovirus is a common virus which has lost its ability to synthesize immunologically reactive coat.

The existence of a viral agent with immunologically non-reactive coat may provide attractive explanations for some of the immunological anomalies in infectious diseases. The inactivation of an immunologically non-reactive virus by an ubiquitous serum component may provide basis for hypothesizing the existence of a new type of defense mechanism. We feel, however, that our present findings are inadequate in establishing any clinical role of this agent and that further study is required to establish conclusively its immunologic non-reactiveness. The possible existence of infectious nucleic acids wrapped in lipid coats has been discussed in a recent article by Herriott (40).

SUMMARY

Experiments designed to characterize an unidentified transmissible agent brought forth the following findings:

The cytopathology consisted of the formation of intranuclear globules, collapse of the involved nuclei, and the extrusion of nuclear materials.

The relatively dormant primary human amnion cells were less susceptible than the rapidly growing cell lines. Similarly, the slowly multiplying ribose variants were less susceptible than their corresponding parent cell lines.

Interferon-like activity was released from infected cells.

Infectivity was readily demonstrated following storage at 0–4°C for at least 8 months or at 37°C for at least 2 weeks. Freeze-thawing, however, markedly reduced or completely destroyed its infectivity.

Infectivity was destroyed completely by ether and chloroform; partially by desoxycholate, and not affected by trypsin, papain, RNAse, DNAse, hyaluronidase, lysozyme, lecithinase, or pancreatic lipase.

The rate of inactivation by 0.025 per cent formalin was much slower than that of vaccinia and herpes viruses.

Its synthesis was suppressed by 5-fluorodeoxyuridine. This suppression was not reversed by thymidine and/or uracil.

Heat-stable neutralizing antibody could not be demonstrated in 379 human

and animal serums, in human gamma globulins, or in serums from animals "immunized" with this agent.

Heat-labile inhibitors (lipoprotein-like) capable of inhibiting the infectivity of this agent were demonstrated in 154 of the 157 serums tested.

Experimental evidence indicated the non-identity of this ubiquitous inhibitor and the properdin system.

The non-infectious complex between this agent and the ubiquitous serum inhibitor may be dissociated (hence, become infectious) by simple dilution.

Repeated attempts to reisolate a similar agent have not been successful.

We have hypothesized that this agent is a virus consisting of DNA wrapped in a surface coat rich in lipid, and suggest that this virus be referred to tentatively as a lipovirus.

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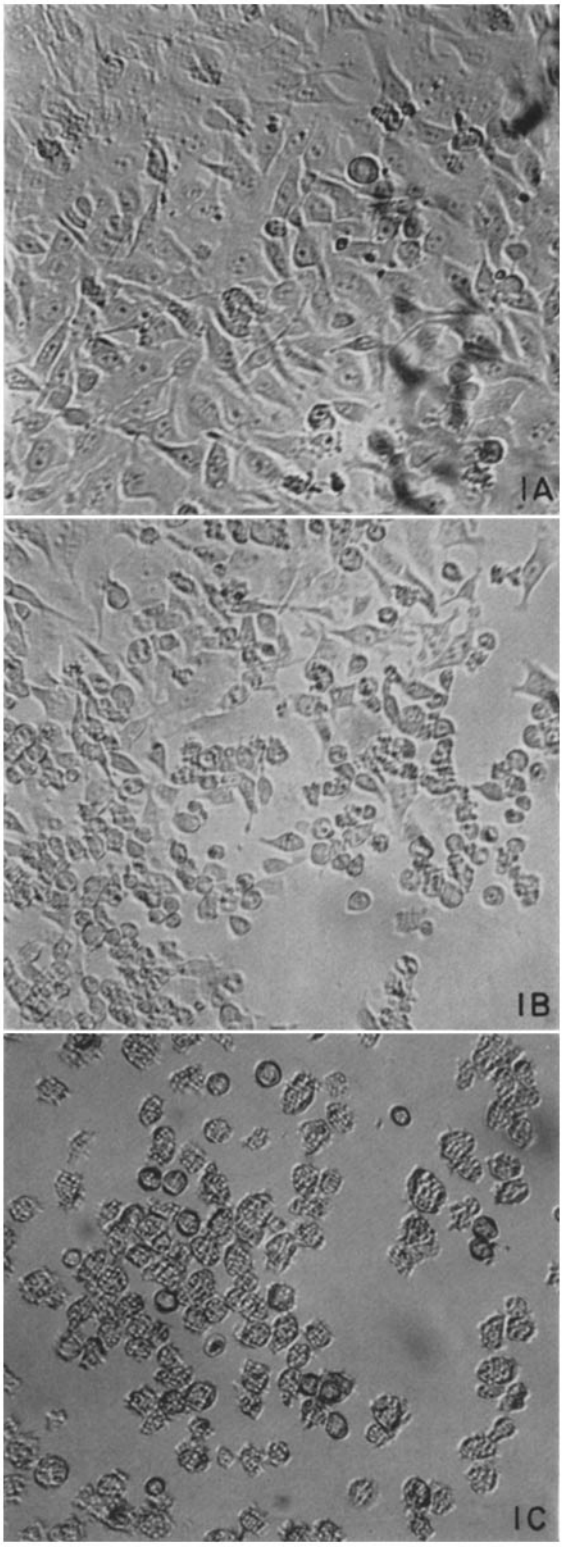
EXPLANATION OF PLATES

PLATE 102

FIG. 1. Cytopathology in unstained liver cultures, × 100.
(*A*) uninfected control, (*B*) partial degeneration, and (*C*) complete degeneration.

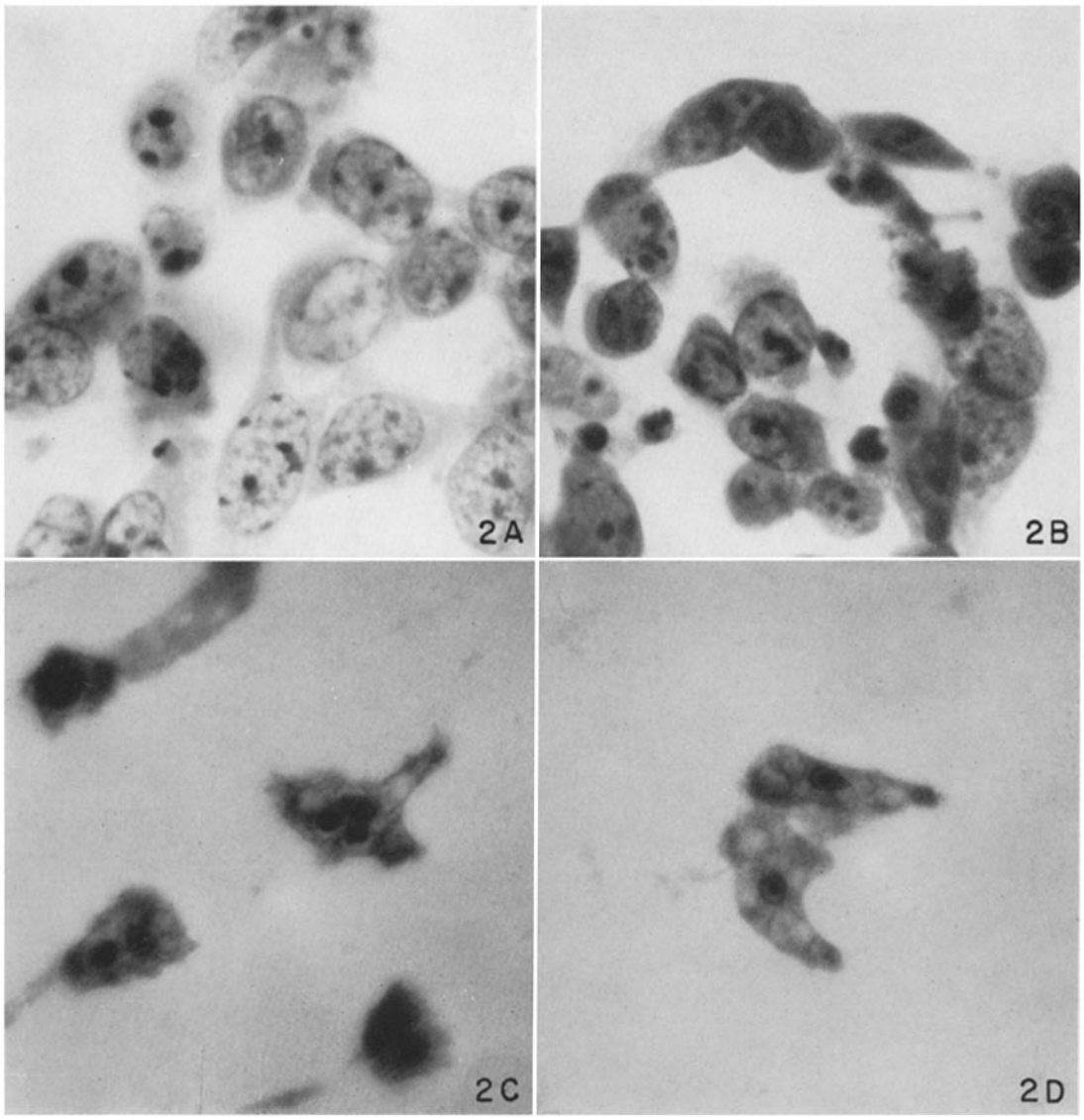


(Chang and Humes: Cultured human cells)

PLATE 103

FIG. 2. Cytopathology in liver cultures stained with hematoxylin-eosin and magnified × 400.

(*A*) basophilic globules in nuclei, (*B*) "collapse" of involved nuclei, (*C*) partial extrusion of nuclear materials, and (*D*) end stage consisting of a "shrunken" cell. See text also.



(Chang and Humes: Cultured human cells)