other workers (*2, 6*). Data such as total migration, movement toward the anode or cathode or both, and the presence or absence of specific fractions have been used as taxonomic characteristics. With this key, it should be possible to assign an amphibian or reptile to its proper order on the basis of the plasma pattern of the specimen. The key is based on patterns from 87 species distributed among the families as follows (*7*).

(Caudata) Proteidae, 1; Ambystomidae, 3; Salamandridae, 3; Amphiumidae, 1; Plethodontidae, 6. (Salientia) Bufonidae, 3; Hylidae, 5; Microhylidae, 1; Ranidae, 5. (Chelonia) Chelydridae, 1; Kinosternidae, 2; Emydidae, 5; Trionychidae, 1. (Crocodilia) Alligatoridae, 2; Crocodylidae, 1. (Sauria) Iguanidae, 4; Anguidae, 3; Xantusidae, 1; Teidae, 1; Scincidae, 2. (Serpentes) Colubridae, 30; Crotalidae, 7.

Since there are many major gaps in our preliminary survey of the Amphibia and Reptilia, it is very likely that this key will have to be altered as more data are accumulated. Thus far, however, these studies suggest that paper electrophoretic analysis is useful in revealing characteristics of plasma proteins that may serve as another means of illustrating basic similarities and differences between taxonomic groups.

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22 May 1956

Tissue-Culture Cultivation of Cytopathogenic Agents from Patients with Clinical Hepatitis

The susceptibility of a number of established cell lines of human and animal origin to a variety of selected strains of viruses, as well as to clinical specimens, from various diseases of unproved etiology is being investigated in our laboratories. During the course of these spectrum studies, an icteric serum specimen (MR_1), obtained from a patient presumed to have infectious hepatitis (*1*), apparently induced a destructive effect on an established line of spindle cells from human embryonic testicle (line PD 39T). Subsequently, confirming this original observation, a more striking cytopathogenic effect was observed on the Detroit-6 strain of human epithelial-like cells (*2*) in cultures containing this serum.

Table 1. Isolation of agents in Detroit-6 strain of cells from patients with hepatitis.

Designation	Test material	Data pertaining to cellular cultures			Calculated total dilution of original specimen
		No. of serial passages	Time cytopathogenicity observed (range in days)	Infectivity titer attained	
MR_1	Acute serum	6	6–9	$10^{-4.0}$	$10^{-9.2}$
Sal.	Acute serum	4	6–9	$10^{-1.5}$	$10^{-4.6}$
G. Pas.	Acute serum	6	4–7	$10^{-1.5}$	$10^{-6.0}$
G. Pas.	"Convalescent" serum	1	6–9		$10^{-2.0}$
D. Keb.	Acute serum	5	5–8	$10^{-1.0}$	$10^{-5.2}$
D. Keb.	"Convalescent" serum	1	6–9		$10^{-2.0}$
H. Pom.	Acute serum	7	5–8	$10^{-1.0}$	$10^{-5.3}$
H. Pom.	"Convalescent" serum	1	6–9		$10^{-2.0}$
New	Stool	5	6–9	$10^{-3.0}$	$10^{-5.0}$
Gar.	Stool	6	5–8	$10^{-1.5}$	$10^{-6.0}$
USA	Stool	2	6–9	$10^{-1.5}$	$10^{-3.2}$

A number of spindle and epithelial cell lines of human embryonic origin (skin, lung, liver, adrenal, spleen, uterus, fallopian tube, trachea) (*3*), as well as monkey kidney tissue, have not shown any degenerative change when they were inoculated with MR_1. In further studies with other cell lines, the original serum and fluids removed from the fifth passage in Detroit-6 cells have exhibited a similar cytopathogenic effect toward cultures of human amnion cells (*4*). In addition, after five passages in the Detroit-6 strains of cells, the harvested fluids produced a cytopathogenic effect on cultures of HeLa cells (*5*).

However, transfers beyond three successive serial passages have not been made in either amnion or HeLa cultures, and evidence for actual multiplication is as yet inconclusive. Syverton (*6*), while attempting to establish serum and infectious hepatitis virus in HeLa cell cultures, observed degenerative changes that were not transferable beyond three passages. Further work on use of the HeLa strain and amnion cells for primary isolation and maintenance of similar agents is in progress, but it appears to date that their susceptibility is of a lower order than that of Detroit-6 cells. The testicular line of spindle cells (PD 39T) used originally was lost in passage. Consequently, the studies reported here have been mainly with the Detroit-6 strain, in which the MR_1 agent has been passed serially without difficulty.

Following the repeated confirmation of the observation with MR_1, a number of similar transmissible agents, presumably viral in nature, have been isolated from tubes inoculated with serum and stools of patients (*7, 8*) diagnosed as having clinical hepatitis. The specimens had been stored for variable periods at −70°C until tested. Young cultures of Detroit-6 cells have been used throughout the study, and the cells are washed three times with a maintenance solution consisting of mixture No. 199 (*9*) containing 10-percent horse serum to remove the human serum contained in the growth medium. The primary inoculum has been either 0.5 ml of 1/5 serum dilution or a 10^{-2} dilution of centrifuged supernate in the case of stool specimens.

Following 4 to 14 days of incubation at 36° to 37°C, the cell monolayer becomes disrupted and extended. Groups of small dark granular cells, irregular in size and shape, then aggregate in clumps throughout the culture. No inclusions have been noted, and areas of apparently normal cells are observed that indicate that all cells are not affected. Subcultures to younger cells are usually prepared at intervals ranging from 3 to 10 days, and characteristic cellular changes have been noted after each transfer.

Figure 1 shows the appearance of a control culture as observed in a fresh preparation (upper left) and stained with azure-eosin (upper right). The cytopathogenic changes produced by one of the agents (G. Pas.) is illustrated from a fresh preparation (lower left) and stained with azure-eosin (lower right). These cultures were incubated for 3 days and then were inoculated with virus, and the photographs (*10*) were taken following 8 days of incubation. Controls have consisted in simultaneous passage of serum from individuals with

no history of hepatitis as well as tissue-culture fluids obtained from uninoculated Detroit-6 cells. No antibiotics have been employed, and sterility tests on a number of media indicate that the tissue-culture preparations are free of bacterial contamination.

To date, experimentally transmissible agents exhibiting essentially similar cytopathogenic effect on the Detroit-6 strain of cells have been isolated from eight serums and three stools. Passage fluids capable of producing characteristic cytopathogenic changes have not shown evidence of any pathological effects in common laboratory animals or in embryonated eggs.

A summary of the data on the successful isolations from serum and stool specimens is contained in Table 1. Stool suspensions, as well as certain serums, may exhibit "toxicity" for cells in culture, but transmissible degeneration of this type seems improbable when one considers the total dilution of the original inoculum producing the specific cellular changes among the final subcultures. In addition, there has been a slight progressive rise in titer with each successive transfer as well as a decrease in incubation period required for cytopathogenicity. One serum (Sal) also produced degenerative change when it was inoculated into human amnion cells which could be passed to Detroit-6 but could not be transferred on passage to either amnion or HeLa cultures. This confirmed similar results with the MR_1 agent.

Seven serums obtained from laboratory personnel have been tested for ability to neutralize the cytopathogenicity of several of these agents. A serum obtained from the only individual (FT) known to have had hepatitis, 7 years previously, was capable of neutralizing the agents tested (D. Keb., Gar., G. Pas., MR_1). The other serums were negative, except that of one of the authors (RK_2), which showed a limited capacity to neutralize two strains (D. Keb. and Gar.). This serum was obtained following a mild illness that developed during the course of the work and had been diagnosed on the basis of laboratory and clinical evidence as probable infectious mononucleosis. An earlier serum from the same individual (RK_1) was without neutralizing activity. It has been reported that certain preparations of immune globulin are useful in clinical hepatitis (*11*). Therefore, two samples of commercial gamma globulin from different manufacturers were tested for their ability to neutralize representative agents of this group of isolates. One of the preparations at a 1/320 dilution neutralized the cytopathogenic effect; the other preparation was without effect at a 1/50 dilution.

Serums were obtained during convalescence (7) from three of the patients

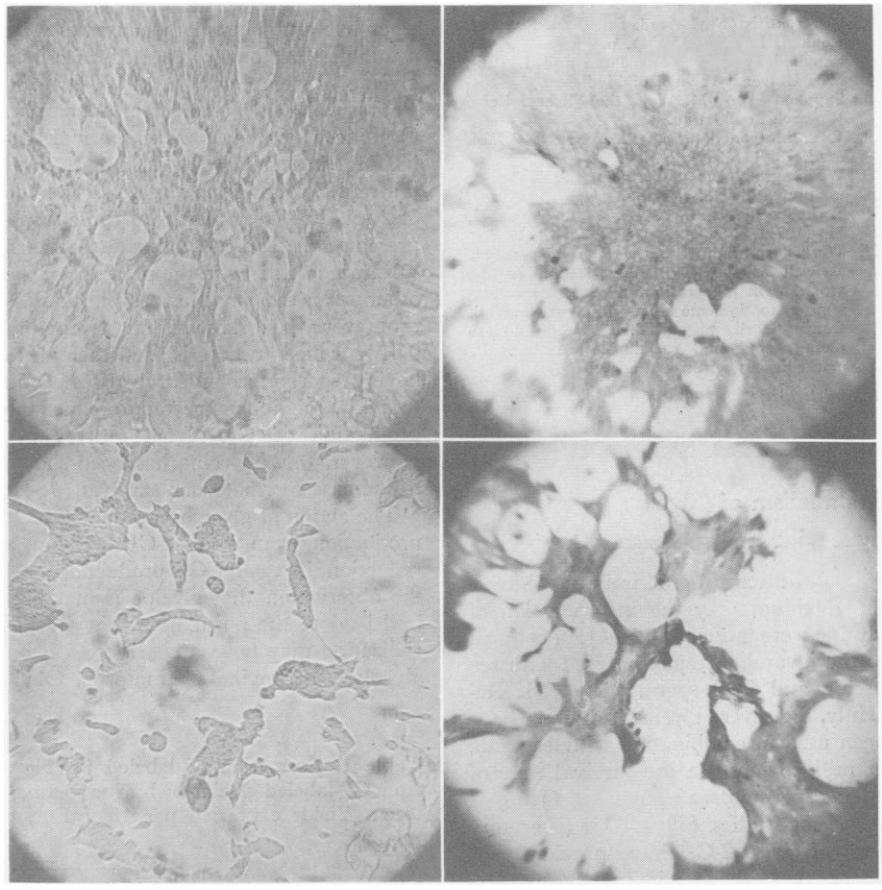

Fig. 1. Control cultures of Detroit-6 cells and changes produced by G. Pas. after 8 days of incubation. (Top) Control cultures unstained (left) and stained with azure-eosin (right). (Bottom) CPE produced by G. Pas. unstained (left) and stained with azure-eosin (right).

(D. Keb., G. Pas., H. Pom.) 2 to 3 months after acute illness. During an attempt to run neutralization tests against the homologous agent isolated in studies on the acute phase material, a typical cytopathogenic effect was observed in the serum controls on Detroit-6 cells. Therefore, even 3 months after onset and during convalescence, it appears that serums may reman infectious. The agents isolated from these "convalescent" serums appear in every way to be similar to the original isolates. This finding is not surprising when one considers the protracted clinical course of hepatitis. We are endeavoring to obtain later serum specimens from these patients and hope to be able to obtain serial stool and serum specimens from additional cases to substantiate further this finding.

On the basis of preliminary evidence, these agents appear to be filterable and relatively heat-stable. Material harvested from the fourth passage of Gar. was not inactivated by heating at 60°C for 30 minutes. Filterability through an 03 Selas has been established for the sixth passage of the G. Pas. agent. Similar studies on the other agents are in progress.

This unique group of similar viruslike cytopathogenic agents appear to be closely associated with cases of human hepatitis. However, it cannot be firmly ruled out to date that this group of agents is not originating from some of the components of the particular tissue-culture system. Final evaluation of their possible etiological significance in disease must await extensive clinical and serological studies as well as additional laboratory confirmation under carefully controlled conditions (*12*).

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22 March 1956

Metabolism of Glycine by Bovine Spermatozoa

Early attempts to improve bovine semen diluents by the incorporation of glycine were unsuccessful (*1*), in contrast to the favorable response noted with sea urchin and fowl spermatozoa (*2*). Recently, however, glycine solutions have been used in combination with egg yolk or milk to increase the survival time of bovine spermatozoa at 5°C (*3, 4*).

Recent studies (*5*) in this laboratory with glycine-1,2-C^{14} have provided evidence that glycine is metabolized by freshly collected bovine spermatozoa. Previously, a metabolic role for glycine had received little consideration, since it apparently was not utilized (*2, 6*).

In a preliminary trial, 1.7 ml of whole semen containing 2×10^9 spermatozoa was incubated in a diluent of heated skimmilk and 0.5*M* aqueous glycine (1/1) (*4*) containing 4 μc of glycine-C^{14}. Respiratory CO_2, collected in alkali during 3 hours of incubation at 37°C, was converted to barium carbonate and yielded a total of 11,700 count/min.

Subsequent trials involved twice-washed spermatozoa suspended in Ringer-phosphate buffer containing 0.001*M* adenosine triphosphate. In experiment 1, 0.01*M* sodium formate was added as a metabolic trap (*7*); 0.01*M* glyoxylic acid was used similarly in experiment 2. Formate was recovered by steam distillation and was oxidized with mercuric oxide. Respiratory CO_2 and formate were radioassayed as solid barium carbonate. Glyoxylate was recovered as the 2,4-dinitrophenylhydrazone and was radioassayed as such. Results of these two experiments are presented in Table 1. Obviously, spermatozoa are capable of metabolizing glycine. The products recovered indicate that the pathways involved may be similar to those observed in other mammalian tissue (*7*).

Further evidence of the nature of glycine metabolism was sought by determining the glycine and glucose oxidation when one and when both substrates were available. Uniformly labeled glucose or glycine was employed in 0.01*M* concentration with 10^6 count/min, per flask. Osmotic pressure was within normal limits for these cells (*8*). Each flask contained 10^9 spermatozoa in a final volume of 2 ml. The mean results of three trials, presented in Table 2, indicate that each substrate is utilized in the presence of the other under the conditions employed. Recent work has shown that glycine at 0.01*M* concentration is detrimental to bovine spermatozoan survival (*4*) and therefore might be expected to reduce the production of CO_2 from glucose-C^{14}. However, 0.1*M* glycine has been shown to increase spermatozoan survival (*3, 4*).

The action of glycine in reducing CO_2 production from glucose-C^{14} without a reduction in total CO_2 production and the observation that glycine is metabolized lead to the postulation that glycine utilization may have a sparing effect on glycolysis in bovine spermatozoa. Obviously such an effect may not account for all of the benefit derived from glycine.

These studies are being extended to obtain additional information regarding the metabolism of glycine by bovine spermatozoa, particularly with regard to the nature of other possible intermediates and end-products.

Table 1. Formation of labeled products from C^{14}-labeled glycine by bovine spermatozoa. Yields expressed per 10^9 cells for 2 hours.

Expt.	Product			
	CO_2		Formate	Glyoxylate
	μ mole	(count /min)	(count /min)	(count /min)
No. 1 4 μcurie glycine + formate	13	4240	2300	
No. 2 2 μcurie glycine	28	2680		
2 μcurie glycine + glyoxylate	10	1700		1040

Table 2. Oxidation of glucose and glycine in suspensions of bovine spermatozoa in Ringer-phosphate buffer, *p*H 7.1. Results expressed per 10^9 cells for 2 hours.

Substrate	Respiratory CO_2	
	count/min	μmoles
Glycine-C^{14}	5295	32
Glycine-C^{14} + unlabeled glucose (0.01*M*)	4085	31
Glucose-C^{14}	7305	33
Glucose-C^{14} + unlabeled glycine (0.01*M*)	6935	30
Glucose-C^{14} + unlabeled glycine (0.1*M*)	6150	34

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14 May 1956

Maternal and Sexual Behavior Induced by Intracranial Chemical Stimulation

A technique permitting chemical or electric stimulation, or both, of restricted brain areas in unanesthetized rats, and electroencephalographic (EEG) recording from these areas, has been developed and found to be of value (*1*).

Implants are prepared as follows. Two Tygon-insulated copper or silver wires (0.1 mm in diameter) are baked along the outside of No. 22 hypodermic tubing extending from 2 to 9 mm below the base of a plastic holder (*2*). The wires lead from contact points on the holder and terminate at opposite sides of the end of the shaft as a bipolar stimulating-and-recording electrode. The implant shaft is permanently inserted in the brain while the anesthetized rat is held in a stereotaxic instrument. Four holes in the base of the holder permit rigid attachment to the skull with jeweler's screws.

Two or more days later, rats are placed in 3- by 3- by 2.5-ft boxes for stimulation testing. A small clip connects the implant to light overhead leads from a 0- to 12-v, 60 cy/sec stimulator, or to an EEG machine. The clip also contains a