

Expression of Liver Phenotypes in Cultured Mouse Hepatoma Cells^{1, 2}

Gretchen J. Darlington,³ Hans Peter Bernhard,⁴ Richard A. Miller,⁵ and Frank H. Ruddle⁶

ABSTRACT—Mouse hepatoma cells were established *in vitro* as a permanently growing line designated Hepa. The mass population and a subclone were characterized for their karyotype and their retention of liver-specific properties. An examination of 17 hepatic traits revealed that the cell lines secreted several serum proteins. The activities of a number of liver-specific enzymes, however, appeared to be absent in these cells. The identification of differentiated properties of cultured hepatoma cells permits the use of these lines in a variety of studies such as cell hybridization, biochemical analysis of tissue-specific gene products, and the modulation of expression of genes governing differentiated phenotypes. This report presents the analysis of a broad spectrum of characteristics and thereby describes one of the most fully defined hepatoma cell lines of murine origin in the literature.—JNCI 64: 809-819, 1980.

Cultivation of somatic cells *in vitro* is an advantageous system for the study of organ-associated functions. When serially propagated outside the body, somatic cells obtained from various tissues often fail to express specific functions. However, a number of mammalian cell lines have now been described that retain characteristics of the tissue of origin. Cell lines from hepatomas include several derived from rat tumors (1) and the line reported by Szpirer and Szpirer (2) from the mouse.

In addition to the study of tissue-specific phenomena such as enzyme induction, differentiated cell lines have been utilized in the production of hybrid cells. The interaction of differentiated and undifferentiated genomes and the expression of various organ-associated traits in hybrid populations have been the subjects of several investigations (3).

It is our purpose to describe a cell line, isolated from a mouse hepatoma, that expresses liver characteristics. At the time of analysis, this cell line, Hepa, and a cloned subpopulation, Hepa-1, had TAT activity inducible by corticosteroids. They synthesized and secreted several serum proteins including albumin, ceruloplasmin, transferrin, and AFP. These cells had activity for esterase-2 and pseudocholinesterase, although other enzymes such as aldolase B, alcohol dehydrogenase, and XOx were not found. The chromosome complement was also found to differ from that of the normal mouse karyotype.

MATERIALS AND METHODS

Medium.—Dry powdered medium MAB 87/3 (4) was obtained from GIBCO, Grand Island, New York. Penicillin (50,000 U), streptomycin (50,000 µg), and FCS (100 ml) were added to 900 ml of medium. FCS was obtained from two sources: GIBCO and Flow Laboratories (Rockville, Md.).

Isolation of Hepa cells.—Male C57L/J mice carrying a subcutaneous hepatoma (BW7756) were obtained from The Jackson Laboratory, Bar Harbor, Maine. The serially transplantable BW7756 hepatoma has been termed a "minimal deviation tumor" (5). The tumor was excised from a mouse under sterile conditions when the cancer became a sphere approximately 2 cm in size. The tissue was minced into pieces 1-2 mm at the longest axis and then placed in a 25-ml Erlenmeyer flask containing 15 ml of a pancreatic enzyme solution [tradename, Viokase (GIBCO)] and a small magnetic stirring bar. The tissue chunks were further dispersed by agitation of the spinning bar for 20 minutes. The solution containing Viokase, mono-dispersed cells, and small tissue fragments was then transferred from the Erlenmeyer flask to a tube and centrifuged. Pelleted cells were resuspended in medium MAB 87/3, plated, and allowed to grow for 1 week. After that time a 0.5-ml aliquot containing approximately 10⁶ cells was inoculated ip into each of 2 C57L/J males. Tumors appeared in 2-3 weeks and were not encapsulated. When the tumors reached the appropriate size (2 cm in diameter), they were minced and cultured as above. The cells were passaged from animal to *in vitro* culture six times over a period of 6 months. This procedure for cell selection was described by Buonasissi et al. (6). After passage 6, the cells, designated Hepa, grew in culture with a generation time of about 30 hours. We generated colonies by plating 50 cells per 60-mm² Falcon culture petri dish. One colony, Hepa-1, was selected for further study.

Cell lines.—Other cell lines were used as controls and are referred to in various experiments. WI-38 is a human diploid cell line obtained from the American Type Culture Collection, Rockville, Maryland. RAG is derived from a BALB/c murine renal adenocarcinoma

ABBREVIATIONS USED: AChE = acetylcholinesterase. AFP = alpha-fetoprotein; AHh = aryl hydrocarbon hydroxylase; FCS = fetal calf serum; TAT = tyrosine aminotransferase; XOx = xanthine oxidase.

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² Animals were maintained under the guidelines set forth by Yale University, New Haven, Conn.

³ Department of Medicine, The New York Hospital-Cornell University Medical Center, 525 East 68th St., New York, N.Y. 10021.

⁴ Department of Cell Biology, Biocenter, University of Basel, Basel, Switzerland.

⁵ Sydney Farber Cancer Center, 44 Binney St., Boston, Mass. 02115.

⁶ Department of Biology, Yale University, New Haven, Conn. 06520.

(7). LM(TK⁻) is derived from the mouse L-cell and lacks thymidine kinase activity (8). KOP is a human diploid fibroblast line (9). H₄AzC₂, a subclone of a rat hepatoma line originally isolated by Richardson et al. (10), was supplied by Dr. Armen Tashjian, Harvard University, Cambridge, Massachusetts. Strain 501-1 is a mouse fibroblast line which, like LM(TK⁻), was derived from L-cells. Testing for mycoplasma is routinely done with the use of Hoechst 33258.

Histochemical analysis for intracellular glycogen.—Hepa cells were grown on microscope slides and stained with periodic acid-Schiff following the procedures outlined by Merchant et al. (11). Slides were examined under bright-field and phase illumination.

Karyotype studies.—The general methods for chromosome preparation from cultured cells were previously described (12).

Starch gel electrophoresis.—The general procedures for starch gel electrophoresis were described by Brewer (13). Cell samples were frozen prior to their use and stored as pellets. Shortly before electrophoresis the samples were thawed and homogenized in distilled water at a concentration of 50×10⁶ cells/ml unless noted otherwise.

XOX (EC 1.2.3.2).—Activity for XOX was observed by histochemical staining of starch gels according to the method of Yen and Glassman (14). The staining solution was modified by the omission of NAD from the reaction mixture. The stock gel buffer (Tris-ethylenedinitrilo tetra-acetic acid-borate buffer) has been described (15).

Alcohol dehydrogenase (EC 1.1.2.1).—Alcohol dehydrogenase isoenzymes were separated with the use of the electrophoretic conditions described above for XOX. The staining mixture contained 3 ml of 70% ethanol, 0.6 mM NAD, 0.1 mM phenazine methosulfate, 0.43 mM nitro blue tetrazolium, 0.012 N potassium cyanide, and 0.035 M Tris-HCl (pH 8.0).

Esterase-2.—The procedures for this assay have been previously reported (16).

Aldolase (EC 4.1.2.13).—Following the procedure of Omenn and Cohen (17), Hepa and Hepa-1 were examined for the presence of aldolase activity.

TAT activity (EC 2.6.1.5).—TAT activity was measured by the method of Granner et al. (18). Protein determinations were performed as described by Lowry et al. (19). Specific TAT activity was examined in Hepa-1 cells in the presence of varying amounts of dexamethasone (Sigma Chemical Co., St. Louis, Mo.) to determine the most effective dose for enzyme induction. Cells were plated in 25-cm² flasks at a density of 2.6×10⁵ per bottle. The cells were allowed to attach and grow for 72 hours. At that time flasks were divided into groups of three, and each group received medium containing one of the following concentrations of dexamethasone: 0, 10⁻⁵ M, 10⁻⁶ M, 10⁻⁷ M, 10⁻⁸ M, or 10⁻⁹ M. Twenty-six hours after exposure to the test medium, the cells were harvested and the contents of each bottle were assayed for specific TAT activity.

Induction of TAT over a 48-hour period.—To test

the effects of cell density on the inducibility of TAT by dexamethasone, Hepa-1 cells were assayed periodically for 48 hours. Replicate flasks were plated with 4×10⁵ cells in MAB 87/3 medium without dexamethasone. After 24 hours, 3 flasks were sampled for base-line TAT activity, and the remainder was fed dexamethasone (10⁻⁵ M) in fresh medium.

Antisera.—Monospecific antimouse AFP is a goat antiserum prepared against electrophoretically purified fetoproteins 1-3 derived from mouse amniotic fluid and was the gift of Dr. Ernest Zimmerman, University of Cincinnati, Cincinnati, Ohio. The antiserum gives a single precipitin on immunoelectrophoresis of amniotic fluid and does not react with adult mouse serum.

Commercially prepared rabbit antisera to mouse fibrogen, C3 (third component of complement), and transferrin were purchased from Cappel Laboratories, Inc. (Cochranville, Pa).

Antigens.—Amniotic fluid from 14½-day mouse embryos was also sent to us by Dr. Zimmerman. Supernatant, serum-free, tissue culture medium containing the secreted proteins of H₄AzC₂, KOP, 501-1, and Hepa-1 was collected in the following way. Confluent monolayers were washed twice with serum-free MAB 87/3, then overlaid with 10 ml of this medium. After 24-48 hours, the supernatant medium was concentrated in a Schleicher-Schuell membrane (Arthur H. Thomas Co., Philadelphia, Pa). These concentrates were frozen until use in polyacrylamide electrophoresis and immunologic procedures.

Secreted cell proteins were radiolabeled with [¹⁴C]leucine as follows. Serum-free medium minus leucine to which 10 μCi [¹⁴C]leucine/ml had been added was placed over plateau-phase cells and incubated at 37° C for 24 hours. The supernatant medium was collected, added to carrier protein, and tested by the immunoelectrophoresis method of Laurell (20). Autoradiography of the precipitin peaks permitted visualization of the radiolabeled cell products.

Acrylamide gel electrophoresis.—Electrophoretic analysis was done on Hepa-1-secreted proteins with the use of ORTEC (Ortec, Inc., Oak Ridge, Tenn.) slab acrylamide gels by the procedure of Tischfield et al. (21) with a 7.65% separating gel and a 4.5% spacer gel. The voltage levels were 280 V for 1 gel or 320 V if 2 gels were being run simultaneously.

The acrylamide slabs were removed from the gel mold and fixed for 30 minutes in 12% trichloroacetic acid if the gel was to be stained for protein. If the gel was to be stained for ceruloplasmin or cholinesterase (as described below), no prior fixation was done.

Serum proteins.—Protein bands were stained with Coomassie brilliant blue. Prior to electrophoretic analysis, some samples were pretreated with neuraminidase (Sigma Chemical Co.), as described by Gustine and Zimmerman (22). One volume of purified neuraminidase, 1.1 U/mg, 200 μg/ml in 35 mM sodium acetate (pH 5.0), was added to one volume of Hepa-1-secreted protein mixture or to mouse serum or amniotic fluid as described below. The samples were incubated at

37° C for 40 minutes, and the reaction was stopped by immersion of the samples in ice and addition of 0.5 volumes of 1.25 M Tris base.

Ceruloplasmin.—Ceruloplasmin was detected by the method of McCombs and Bowman (23) with the use of σ -dianisidine (Sigma Chemical Co.) in acetic acid. The orange bands of activity were visible within 1-2 hours.

Cholinesterase.—After electrophoresis, cholinesterase isoenzymes were identified by the direct coloring method of Karnovsky and Roots (24) with the use of acetylthiocholine iodine or butyrylthiocholine iodide (Sigma Chemical Co.) as substrates. The cholinesterase inhibitor eserine (Schwarz-Mann, Orangeburg, N.Y.) was used at 10^{-4} M, and the AChE inhibitor BW284C51 (a gift of Burroughs Wellcome, Inc., Beckenham, Kent, England) was used at 10^{-5} M; when inhibitors were used, the gel was incubated with inhibitor for 30 minutes before the substrate was included.

Double diffusion.—Double-diffusion analysis was performed on microscope slides coated with 1% agarose (Seakem, Rockland, Maine) in barbital buffer (pH 8.6) with an ionic strength of 0.025 M. Each well contained about 5 μ l of antigen or antiserum.

RESULTS

Cell Morphology and Growth Characteristics

Populations of Hepa-1 cells kept in logarithmic phase doubled every 24 hours. The generation time of Hepa was 30 hours.

The cell morphology of Hepa and Hepa-1 (fig. 1) was epithelioid. The nuclei contained many nucleoli, and the cytoplasm was granular, making the nuclear outlines very apparent.

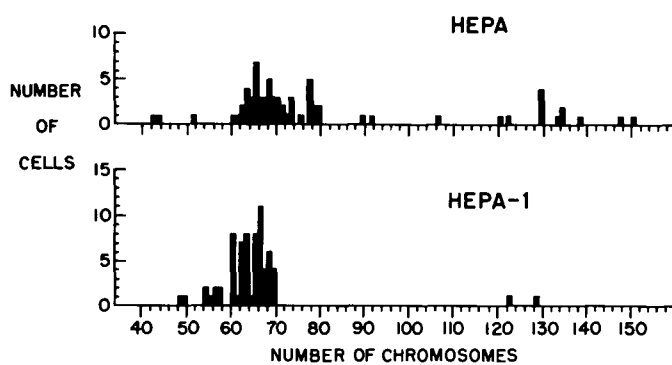
Histochemical staining of Hepa cells for stored glycogen was negative.

Karyotype Analysis

Table 1 compares the mean chromosome numbers of Hepa and Hepa-1. In general, populations of Hepa-1 had less variability in the total number of chromosomes per cell. The distribution in the Hepa-1 subpopulation was clustered around the mode of 67, whereas the numbers in the parental Hepa population ranged from 43 to 151 with a very weak mode of 66 (text-fig. 1). Two to four biarmed chromosomes appeared in both Hepa and Hepa-1 cells.

TABLE 1.—Comparison of the mean chromosome numbers of Hepa and Hepa-1

Cell line	No. of chromosomes				No. of cells counted
	Mean	Range	Mode	Biarmed chromosomes	
Hepa	81.0	43-151	66	2	69
Hepa-1	65.9	49-129	67	5	70



TEXT-FIGURE 1.—Histogram of chromosomes in Hepa and Hepa-1.

Secreted Proteins

Serum proteins.—Serum-free medium collected from cultures of Hepa-1 and analyzed by polyacrylamide gel electrophoresis contained at least 20 electrophoretically distinguishable protein components. In addition to albumin [cf. (25)], we have been able to identify AFP, ceruloplasmin, and transferrin among the secreted proteins. Our identifications were based on immunologic, electrophoretic, and biochemical techniques. Figure 2 shows the positions of AFP and transferrin secreted by Hepa-1 after polyacrylamide slab gel electrophoresis. Gustine and Zimmerman (22) showed that amniotic fluid from 14½-day mouse embryos contained three principal regions of protein: an albumin region moving just behind the dye front, a set of five AFP bands behind albumin, and a set of three transferrin bands that migrated least anodally. They also demonstrated that the faster members of each set were derived in vivo by the addition of sialic acid residues to the slower bands and that neuraminidase treatment of amniotic fluid prior to electrophoresis partially converted the fast-migrating bands into the slower species. Figure 2 shows the results of neuraminidase treatment of supernatant medium from Hepa-1 (channel 4). On the basis of mobility and response to removal by sialic acid residues, we were able to identify the bands indicated as AFP (fetoproteins 3, 4, and 5) and transferrin. No AFP-like protein was present in samples of adult mouse serum (channels 1 and 2) regardless of neuraminidase pretreatment. The predominant transferrin species of adult mouse serum migrated to the same electrophoretic position as did the fastest species seen in Hepa-1 and in amniotic fluid.

In addition, transferrin and AFP could each be identified by double-diffusion analysis in agarose. Figure 3A depicts a reaction of immunologic identity among antigens common to adult mouse serum, Hepa and Hepa-1 secretions, precipitated by a rabbit anti-mouse transferrin serum that gave a single band when tested by immunoelectrophoresis against adult mouse serum. FCS and an aliquot of concentrated medium were included as negative controls to demonstrate that the antiserum would not detect either FCS contamination or some artifact of our concentration technique.

Figure 3B shows a reaction of immunologic identity between antigens present in mouse amniotic fluid and in the supernatant medium from Hepa and Hepa-1 cells that were precipitated by antimouse AFP sera.

Albumin, AFP, and transferrin were synthesized and secreted by the Hepa and Hepa-1 cells. Figure 4 illustrates autoradiographs of Laurell immunoelectrophoresis plates on which was tested supernatant medium containing secreted proteins labeled with [14 C]leucine. Precipitin peaks contained labeled proteins for each serum protein when media from Hepa and Hepa-1 were used (wells 1 and 2). No peak was seen on the autoradiograph above well 3, which contained supernatant medium from RAG cells for albumin and AFP. However, RAG cells produced a small amount of transferrin.

Ceruloplasmin.—Ceruloplasmin was identified on the basis of its characteristic orange-staining reaction with σ -dianisidine. Figure 5 demonstrates that the stained protein in Hepa-1 supernatant was similar in mobility to a protein found in adult mouse serum. The slightly slower mobility of the material produced by Hepa-1 was reproducible from run to run. The lot of FCS used for cell growth did not contain histochemically detectable ceruloplasmin.

Attempts to demonstrate the presence of fibrinogen or of C3 in freshly concentrated supernatant from Hepa-1 cultures by immunoelectrophoresis were unsuccessful.

Cholinesterase.—Mammalian sera contained several isoenzymes of pseudocholinesterase (EC 3.1.1.8) capable of hydrolyzing acetylcholine or butyrylcholine in addition to other esters. Pseudocholinesterase (EC 3.1.1.8) was synthesized principally in the liver and was distinguishable from the AChE (EC 3.1.1.7) that is characteristic of cholinergic synapse by the resistance of pseudocholinesterase to the inhibitor BW284C51 and by its more rapid hydrolysis of butyrylcholine esters. We examined Hepa-1-conditioned medium for its ability to hydrolyze esters of thiocholine; the results are presented in figure 6.

Normal mouse serum, channels 4, 6, 8, and 10, had several bands of cholinesterase activity when acetylthiocholine was used as substrate. Of these, two bands (labeled with an asterisk in channel 4, fig. 6) appeared to be AChE inasmuch as both disappeared in the presence of the AChE inhibitor BW284C51 (channel 6) and failed to utilize butyrylcholine as substrate (channel 8). The remaining bands were classified as pseudocholinesterases; channel 10 shows that all were eserine-sensitive.

Hepa-1, channels 3, 5, 7, and 9, produced at least four electrophoretically distinguishable regions of cholinesterase activity with acetylthiocholine as substrate. One band (identified by an asterisk, fig. 6) could be classified as an AChE on the grounds that it was much less active either when butyrylthiocholine was provided as substrate (column 7) or when BW284C51 was included with an acetylthiocholine substrate (column 5). The other three bands appeared to be pseudocho-

linesterases. These three bands exhibited electrophoretic mobilities similar but not identical to those of pseudocholinesterases found in adult mouse serum (column 4). In addition, the more rapidly migrating bands of normal mouse serum pseudocholinesterase activity were not observed in the Hepa-1 supernatant.

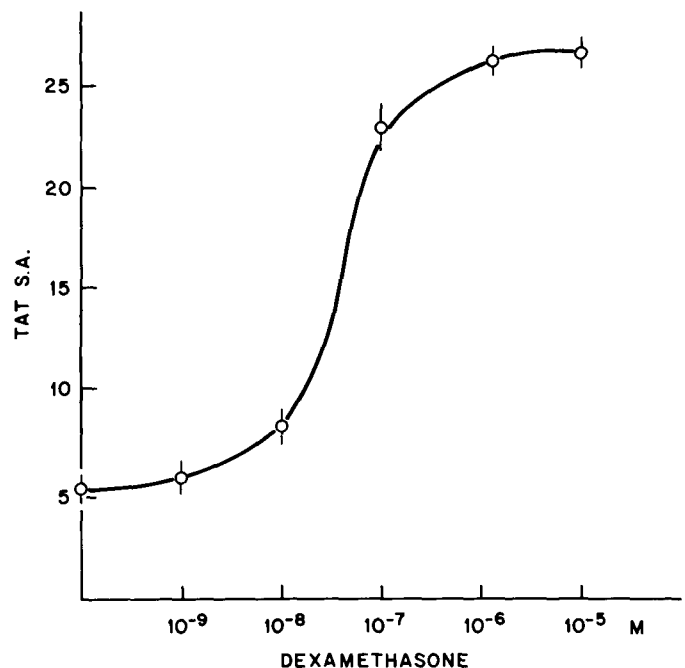
The cholinesterases of FCS are shown in column 10 of figure 6. Inasmuch as at least two of these bands did not occur in the Hepa-1 concentrated medium, we believe that there had been no significant contamination by FCS and thus that all four cholinesterase bands in the Hepa-1 supernatant had been synthesized by the cultured cells.

The Hepa-1 AChE migrated slightly less rapidly than did the corresponding band of serum AChE from normal mouse serum, and the Hepa pseudocholinesterase migrated slightly less rapidly than did the strongest serum pseudocholinesterase. These relationships were reproducible from experiment to experiment.

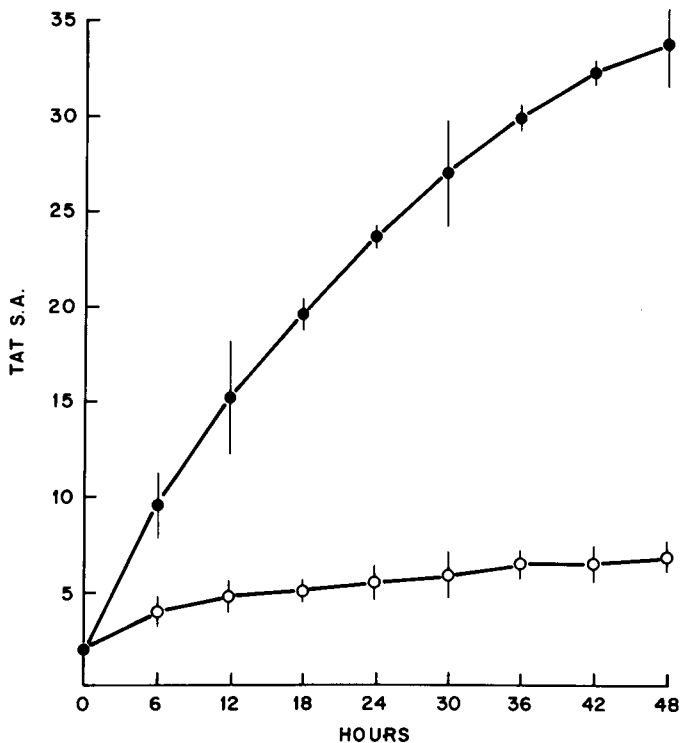
TAT Activity in Hepa-1 Cells

TAT activity was measured in the absence of dexamethasone during lag, logarithmic, and plateau phases of the growth of the culture. The greatest specific TAT activity in the absence of dexamethasone was observed immediately after attachment while the cells were still in the lag phase of growth. During logarithmic growth and plateau, the activity declined somewhat.

The induction of TAT by dexamethasone was examined shortly after attachment, i.e., in lag and early log phase rather than when the cells were approaching confluence. TAT activity in Hepa-1 cells during that



TEXT-FIGURE 2.—TAT activity in cells grown in dexamethasone at various concentrations. Specific activity (S.A.) is expressed as nmol α -ketoglutarate converted per milligram cell protein/min at 25° C.



TEXT-FIGURE 3.—Kinetics of TAT induction with or without dexamethasone. Hepa-1 cells in the presence of 10^{-5} M dexamethasone (●); Hepa-1 cells in the absence of dexamethasone (○).

period was increased by the addition of this synthetic corticosteroid fourfold to sixfold over the basal activity.

For the determination of the most effective concentration of inducer, Hepa-1 cells were grown in varying doses of dexamethasone as described in "Materials and Methods." Text-figure 2 shows that 10^{-6} dexamethasone and 10^{-5} M dexamethasone were equally effective in induction, both concentrations evoking a fourfold elevation of TAT activity above the control level. Dexamethasone at a 10^{-9} M concentration had little or no effect on enzyme activity, whereas at 10^{-8} and 10^{-7} M, TAT reached partially induced levels.

The increase in TAT activity for 48 hours following the addition of 10^{-5} M inducer to the medium is illustrated in text-figure 3. The cells in the control medium and in the medium containing dexamethasone both showed an increase in TAT activity at 6 hours. The enzyme activity of the cells growing in the presence of dexamethasone continued to rise to a peak of 30 nmol/mg cell protein/minute at 48 hours, at which time the experiment was terminated. In the absence of dexamethasone, the TAT activity leveled off after 6 hours and increased only slightly. The treated culture had a final increase of fivefold above the untreated cells.

AHH Activity

The activity of AHH was measured in Hepa-1 cells by Dr. Daniel Nebert (National Institute of Child

Health and Human Development, Bethesda, Md.) and co-workers and was presented in a separate report (26). AHH activity could be elevated in Hepa-1 by the addition of phenobarbital, aromatic hydrocarbons, and biogenic amines. Inducibility by the first compound appeared to be a liver-specific trait.

Starch Gel Electrophoresis

Activity for aldolase A (the form characteristic of muscle) was observed in Hepa, Hepa-1, and the BW7756 tumor. Mouse aldolase A migrated anodally, whereas the B-form of aldolase, seen in the liver, moved toward the cathode. Neither Hepa nor Hepa-1 had aldolase activity corresponding to the B-form of the mouse enzyme. An additional band, the C-form, was most prominent in brain. One heteropolymeric band of A- and C-subunits was observed in the cultured cells (data not shown).

Other enzyme activities tested by starch gel techniques were XOX and alcohol dehydrogenase. Neither of these enzymes was detected in Hepa or Hepa-1 cells. Esterase-2, an anodally migrating esterase band controlled by the *Es-2* locus, was found to be present in both Hepa and Hepa-1.

DISCUSSION

The chromosome numbers of Hepa cells extended over a wider range than did those of the subpopulation Hepa-1, although variation in the total chromosome number was apparent in Hepa-1 as well. The mean number of chromosomes in Hepa-1 cells was less than that in Hepa cells, whereas the number of banded chromosomes increased. Possibly, some of the banded chromosomes resulted from centric fusion of telocentrics; those of Hepa appeared from their banding patterns to be isochromosomes.

The production of albumin by rat hepatoma cells in culture has been observed by several investigators (27, 28). Our results showed that Hepa and Hepa-1 secreted ceruloplasmin, transferrin, and AFP in addition to albumin. In contrast to the Fu-5 cells of Bertolotti and Weiss (29), the enzymatic traits of Hepa and Hepa-1 (alcohol dehydrogenase, esterase-1, TAT inducibility, aldolase B, and XOX) were diminished or absent, whereas the production of serum proteins was more extensive and has remained stable over several years in culture.

Pseudocholinesterase activities in mouse serum showed some differences from the electrophoretic patterns found in human serum on starch gel (30). In our acrylamide system, we resolved 10 bands of pseudocholinesterase activity in normal mouse serum as opposed to 7 bands in man. These additional bands may reflect species differences and/or increased resolution by polyacrylamide electrophoresis.

The hepatoma cells did not produce the entire spectrum of cholinesterases that were observed in serum; however, both AChE and pseudocholinesterase

activities were present. The two most cathodal bands corresponded in mobility to serum isoenzymes. The remaining two bands migrated to unique positions. Differences in the migration of secreted albumin have been observed in concentrated medium from Hepa cells versus mouse serum (Darlington GJ: Unpublished observation). Mobility differences also appeared between the transferrin and AFP derived from different sources. Several possibilities exist to explain these results. 1) The concentration procedure could introduce this artifact. 2) Transferrin and AFP could each contain varying amounts of sialic acid and these modifications would alter their electrophoretic mobilities (22). Cholinesterase may be similarly affected. Alternatively, it has been suggested that the various forms of cholinesterase in man result from the aggregation of homopolymers (31). The aggregation of the enzyme subunits from Hepa-1 might not be identical to that in serum. Finally, the unique cholinesterase bands of Hepa-1 possibly represent a fetal product. A number of fetal proteins are known to be expressed by tumors [cf. (32)], and the presence of AFP demonstrates that Hepa and Hepa-1 express fetal genes *in vitro*.

TAT activity has been described in cell lines derived from rat hepatomas. The uninduced level of TAT activity in Hepa-1 cells was less than that of the MH₁C₁ line reported by Tashjian et al. (33) or of the HTC cells described by Thompson et al. (34). These two rat hepatoma lines had approximately 1 unit of enzyme activity per milligram protein. Uninduced Hepa-1 cells had about 10% of that level. Maximal induction of TAT in Hepa-1 required at least 48 hours, although the two lines just mentioned required only 12-18 hours. Gelehrter et al. (35) showed that serum and insulin both increased TAT levels in HTC cells. Neither serum, insulin, nor dexamethasone affected the inducing capacities of the other agents; i.e., the stimulation by each was additive. Possibly, the Hepa-1 cells were partially induced by the insulin and serum present in complete MAB 87/3 medium. We did not determine TAT activity in the absence of insulin or serum.

TAT activity was measured in the cultured cells within 1 year of the isolation of Hepa. Upon retesting at 18 months in culture, we found that TAT activity was no longer inducible in either Hepa or Hepa-1. Loss of this enzyme activity could not be correlated with any single event in the history of the cells.

One hepatic enzyme, XOX, was found in the hepatoma but not in the cultured cell lines. The apparent loss of a liver-specific function could be explained by heterogeneity among the cells that made up the original BW7756 tumor. A cell type that did not have XOX activity might have become predominant in the Hepa line. Another possibility is that the cells at the time of explantation were producing XOX but that the conditions of *in vitro* culture did not permit synthesis of this enzyme, either because of a physical loss of genetic material or because of the action of regulatory mechanisms to prevent its expression.

TABLE 2.—Summary of hepatic phenotypes tested

Hepatic phenotypes	Designation ^a
Glycogen storage	—
Pseudocholinesterase	+
AHH	+
Inducibility of AHH by phenobarbital	+
AFP	+
Transferrin	+
Ceruloplasmin	+
Albumin	+
C3	—
Fibrinogen	—
Esterase-2	+
Aldolase A	+
Aldolase B	—
Alcohol dehydrogenase	—
Esterase-1	—
XOX	—
TAT	+
Inducibility of TAT	± ^b

^a —, Absent; +, present.

^b Initially present in the cells but subsequently lost.

Esterase activity governed by the *Es-2* locus is high in kidney and liver tissues (16), although little or no activity is seen in cultured fibroblast lines such as LM(TK⁻) (7). The renal adenocarcinoma cell line RAG has retained esterase-2 activity after many generations in culture; Hepa and Hepa-1 also expressed esterase-2 activity.

Aldolase B is the major electrophoretic form of the enzyme in adult rabbit liver (36, 37). Two other forms, A and C, are found primarily in muscle and brain, respectively. Aldolase A is present in mouse fetal liver, but the B-form predominates later in development. Aldolase A, rather than B, was observed in cultured cells derived from fetal mouse liver (5) and in murine hepatomas (38). Hepa and Hepa-1 resembled the fetal and tumor tissues in that they did not express aldolase B, the form characteristic of adult hepatocytes.

In summary, a murine hepatoma cell line that has retained many hepatic traits (table 2) has been adapted to *in vitro* culture. Such a line would be a useful parental strain for the production of somatic cell hybrids (39) as well as for the study of hepatocyte function *in vitro*.

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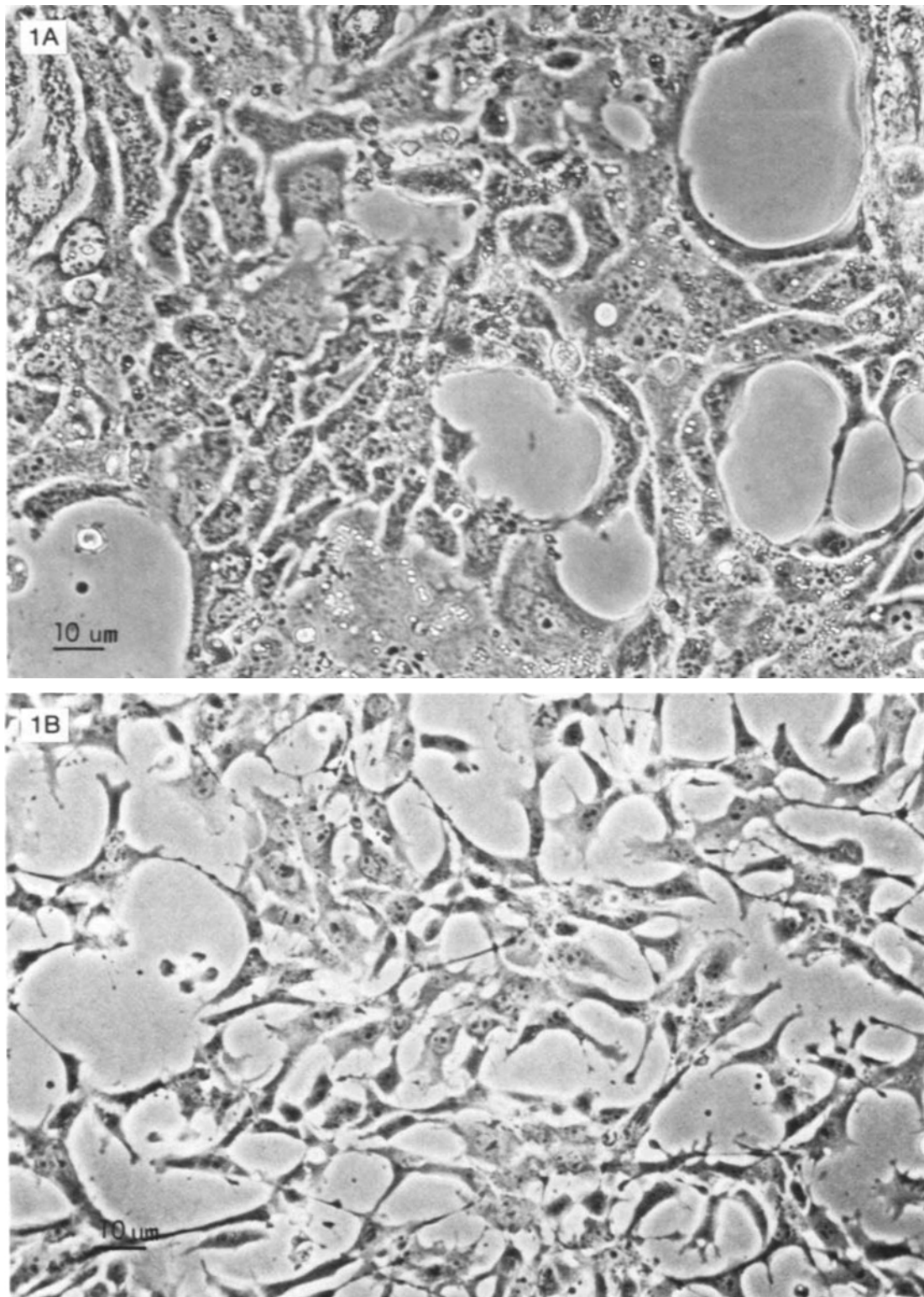


FIGURE 1.—A) Hepa cells and B) Hepa-1 cells. Phase-contrast photograph of cell monolayers.

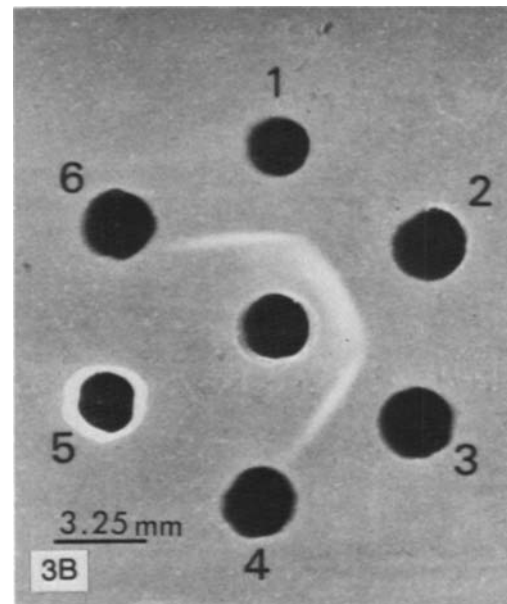
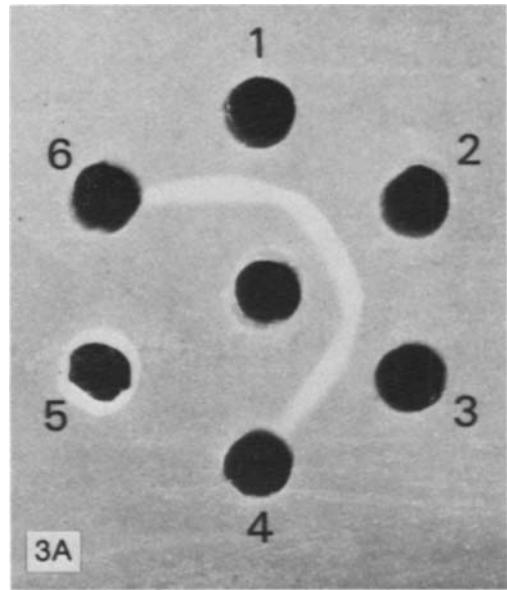
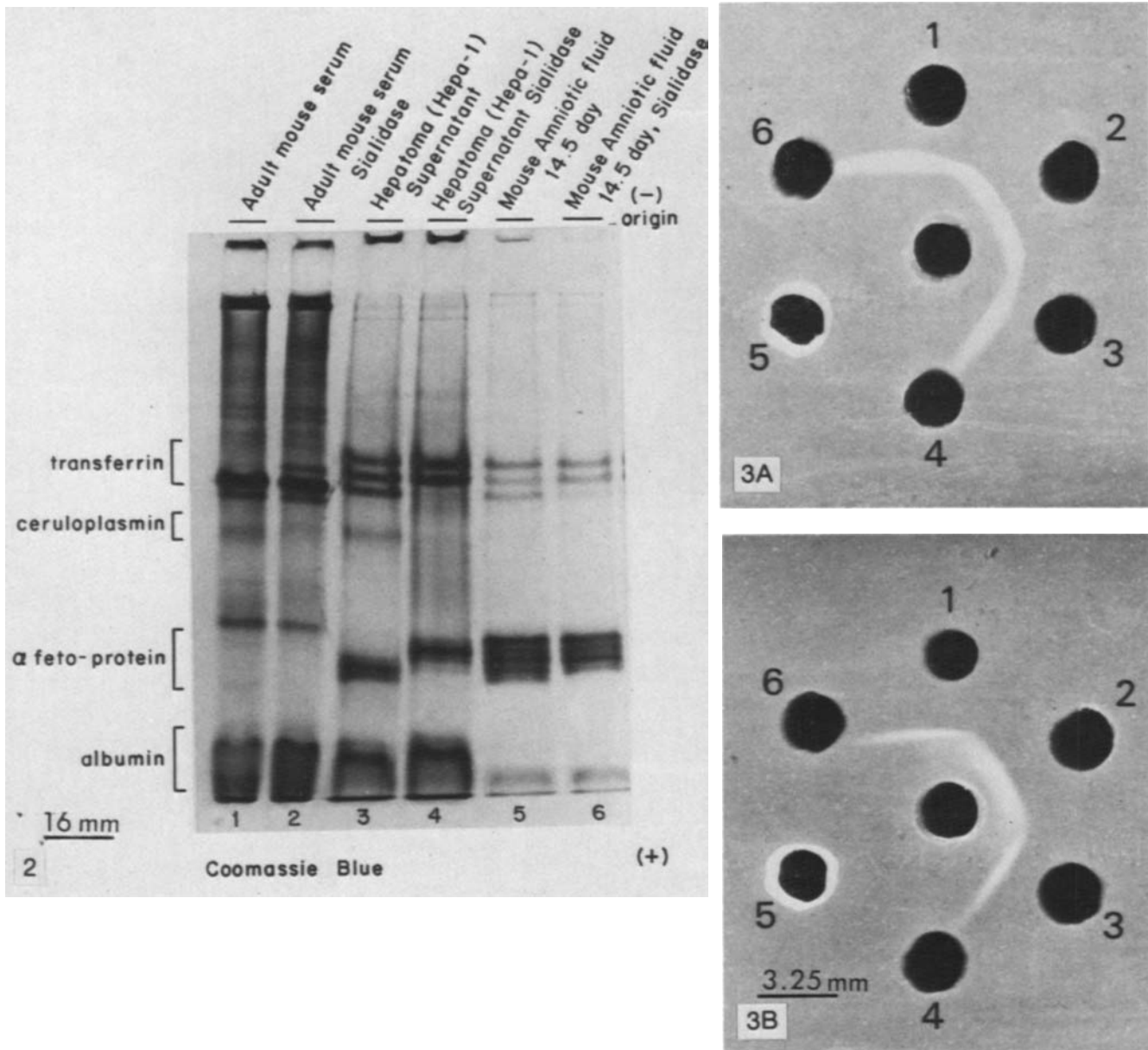


FIGURE 2.—Polyacrylamide gel analysis of Hepa-1-secreted proteins and controls. Channel 1, normal mouse serum from adult animal. Channel 2, normal adult mouse serum treated with sialidase. Channel 3, Hepa-1 supernatant concentrated ≈ 100 times. Channel 4, Hepa-1 supernatant concentrated ≈ 100 times and treated with sialidase. Channel 5, mouse amniotic fluid from 14½-day fetus. Channel 6, mouse amniotic fluid treated with sialidase.

FIGURE 3A.—Transferrin detection by Ouchterlony double diffusion. 1) Hepa supernatant concentrated ≈ 10 times; 2) mouse serum diluted 1:20; 3) Hepa-1 supernatant concentrated ≈ 20 times; 4) FCS; 5) cell-free supernatant medium concentrated ≈ 40 times; and 6) blank.

FIGURE 3B.—AFP detection by Ouchterlony double diffusion. 1) Hepa supernatant concentrated ≈ 25 times; 2) mouse amniotic fluid; 3) Hepa-1 supernatant medium concentrated ≈ 5 times; 4) FCS; 5) cell-free supernatant medium concentrated ≈ 40 times; and 6) mouse serum diluted 1:20.

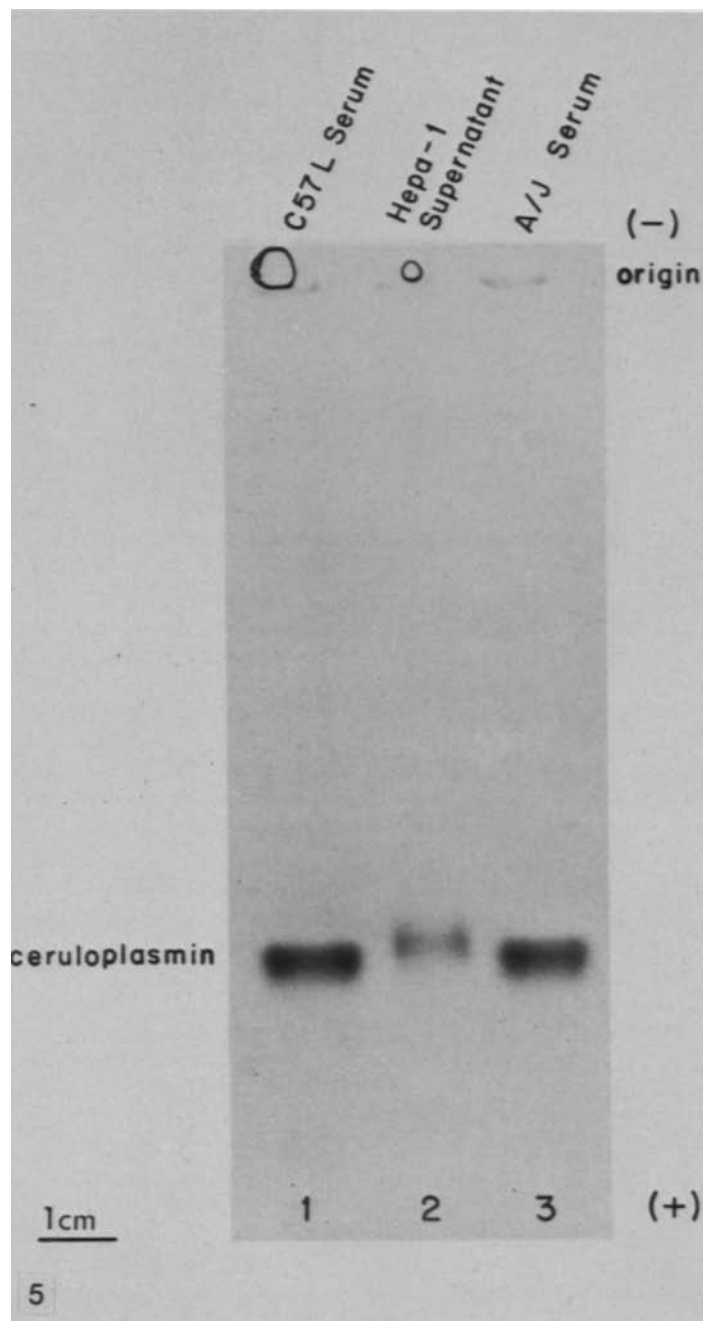
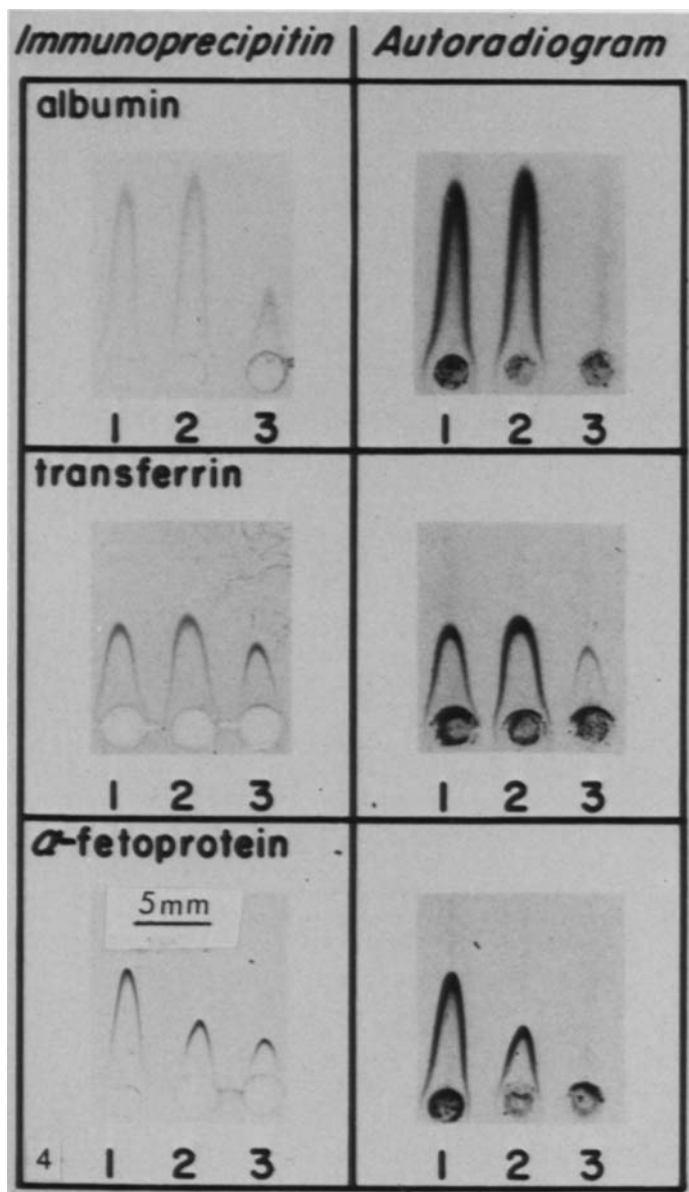


FIGURE 4.—Synthesis and secretion of albumin, transferrin, and AFP in Hepa and Hepa-1 cells. Carrier protein was added to all supernatants before electrophoresis; albumin, 10 $\mu\text{g}/\text{ml}$; transferrin, 6.35 $\mu\text{g}/\text{ml}$; and AFP, 1:1 dilution of mouse amniotic fluid. Well 1 contained supernatant from Hepa cells; well 2 contained Hepa-1; well 3 contained RAG.

FIGURE 5.—Polyacrylamide gel stained for ceruloplasmin.



FIGURE 6.—Polyacrylamide gel electrophoretic analysis of cholinesterase activity. FCS (1), Hepa-1 supernatant (3), and mouse serum (4) with acetylthiocholine iodide (ACT) as substrate. Hepa-1 supernatant (5) and mouse serum (6) incubated with BW284C51 prior to addition of substrate. Hepa-1 supernatant (7) and mouse serum (8) with butyrylthiocholine iodide (BTC) as substrate. Hepa-1 supernatant (9) and normal mouse serum (10) incubated in 10^{-4} M eserine prior to addition of ACT as substrate.