PRIMARY CULTURE OF NORMAL ADULT RAT LIVER CELLS WHICH MAINTAIN STABLE UREA CYCLE ENZYMES*

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SUMMARY - Normal adult rat liver cells have been successfully cultured as monolayers without subjecting donor animals to a partial hepatectomy before cell isolation. Coating plastic tissue culture dishes with acid soluble calf skin collagen increases the efficiency of cell attachment. Hepatocytes form a monolayer in 24 hr in serum-free L-15 medium although 10% fetal calf serum for 24 hr increases efficiency of attachment. In serum-free medium the monolayer remains viable for at least one week without added insulin. Cultured cells maintain tyrosine transaminase and four of the five urea cycle enzymes at levels above or equal to freshly isolated cells for 72 hr. Adenylyl cyclase activities are maintained for at least 72 hr, and are stimulated by epinephrine, glucagon and fluoride. Tyrosine transaminase activity is increased in cultured cells by glucagon and dexamethasone, but urea cycle enzymes are not.

The factors which control enzyme activities in liver cells are difficult to study in the intact animal. Many variables cannot be controlled and direct vs indirect effects of diets, chemicals and hormones on enzyme activities cannot be separated experimentally. We have been studying the factors which induce or suppress the urea cycle enzymes in rat liver, and have developed a suitable cell culture system which allows us to study this cycle *in vitro*. The culture method should be ideal for investigators wishing to prepare large numbers of stable monolayer cultures in serum-free medium for any studies of liver cell metabolism over a period of one to seven days.

Recently Bissell et al. (1) and Bonney et al. (2) have successfully cultured in monolayers hepatocytes isolated from regenerated adult rat liver. Such monolayer cells exhibit minimal mitotic activity and demonstrate several major metabolic functions characteristic of liver *in vivo*.

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We have improved upon their culture methods in three ways: 1) cells can be isolated from normal adult rats; 2) coating the culture dishes with acid soluble calf skin collagen greatly improves plating efficiency; 3) serum-free medium without added insulin is able to maintain healthy cells for seven days. These cell cultures maintain high levels of urea cycle enzymes and of tyrosine transaminase for 96 hr.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats (250-350 g) were used for cell preparations. Leibovitz' L-15 medium and fetal calf serum (FCS) were purchased from Grand Island Biological Co., Grand Island, N. Y. HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) was purchased from Calbiochem, San Diego, Calif., glucagon from Eli Lilly and Co., Indianapolis, Ind., dexamethasone from Merck, Sharp & Dohme, West Point, Pa., acid soluble calf skin collagen from Sigma Chemical Co., St. Louis, Mo., bacitracin from Nutritional Biochemical Co., Cleveland, Ohio. Petri dishes were obtained from Falcon Plastics, Oxnard, Calif.

Rat liver parenchymal cells were isolated with collagenase (Worthington Biochemicals) using an *in vitro* perfusion technique under strictly aseptic conditions. The method of Ingebretsen and Wagle (3) was employed except that bovine serum albumin was omitted from the perfusion medium. After the parenchymal cells were separated from liver debris, the cells were washed four times with Hank's buffer and one time with culture medium in order to remove traces of collagenase. Viability of cells was checked by exclusion of trypan blue dye. Cells were counted in duplicate with a hemocytometer. Only the preparations with at least 80% viability were used in cell culture. Standard culture medium was Leibovitz' L-15 containing 28 mM HEPES, pH 7.4, 100 u/ml penicillin and 100 µg/ml streptomycin. Sterile fetal calf serum or hormones were added when indicated.

Calf skin collagen was dissolved in triple distilled water (1 mg/ml) in a boiling water bath and sterilized by passage through a Millipore filter (0.45 µm pore size). Then 0.5 ml of collagen solution was pipetted into 100 mm plastic petri dishes. After the dishes were shaken gently until the bottom surfaces were coated with collagen, they were then placed in a humidified incubator at 37° for 48 hr or until dry.

Suspensions of 3-5 x 10^6 cells were placed in coated 100 mm plastic petri dishes in 7.0 ml of culture medium. The plates were then placed in a humidified incubator at 37° under 95% O_2-5% CO_2 mixture. Medium was changed every day, at which time loosely attached cells were removed by irrigating the surface of the plate by drawing the medium in and out of a pipet.

To prepare cultured cells for enzyme assay the culture medium was drawn off a petri dish, approximately 5 ml of 0.9% NaCl was added to the dishes, swirled gently and drawn off. This washing was repeated three times to remove loosely attached cells. Approximately 1 ml of 0.075 M KCl was added to the dish and the cells were scraped free with a rubber policeman. The cell suspension was homogenized with a Polytron for 15 seconds, and used for enzyme assays without further purification.

To determine the viability of cultured cells, the monolayer was detached

by incubating with 0.25% trypsin and examined for its exclusion of trypan blue dye.

Urea cycle enzymes were assayed according to Richardson et al. (4), tyrosine transaminase according to Spencer and Gelehrter (5), adenylyl cyclase according to White et al. (6), and protein was measured by Lowry's method (7).

RESULTS

At 48 hr of culture with 10% FCS added, the efficiency of cell attachment was 47.8 ± 3.6% (n = 15) in collagen-coated dishes and only 28.3 ± 5.2% (n = 7) in non-coated dishes. When serum-free medium was used, the efficiency was 40.4 ± 4.1% (n = 10) in collagen-coated dishes and 19.0 ± 8.7% (n = 5) in non-coated dishes. When cultured in 10% FCS, cells that bound tightly to the collagen-coated dishes were flat and polygonal in shape (Fig. 1a). However, when cells were cultured in serum-free medium, many had a rounded shape (Fig. 1b); they gradually detached and were lost at the subsequent changes of medium. If the cells were cultured in 10% FCS-medium for the first 24 hr, and shifted to no-serum medium thereafter (10 $\longrightarrow$ 0), the cells remained polygonal after medium shifting. The number of cells attached decreased 10% at the time of shifting medium and thereafter remained constant for at least three days.

Table 1 compares the urea cycle enzymes and tyrosine transaminase activities of whole rat liver homogenate with freshly isolated rat hepatocytes. Specific activities of CPS, OCT and AL were higher in isolated cells whereas AS, arginase and TAT were lower. The cultured cells maintained all these enzyme activities over a period of four days (Fig. 2). CPS and AL declined in all three media. OCT and AS were best maintained in the 10 $\longrightarrow$ 0 serum medium. Arg and TAT were stable in all three media. By the seventh day of culture, all of the enzyme activities except arginase fell to 0-10% of control values.

Stimulation of tyrosine transaminase activity was carried out by adding serum-free media containing hormones to cells which had been cultured in serum for 24 hr. Enzyme was assayed 24 hr after hormone addition. Tyrosine

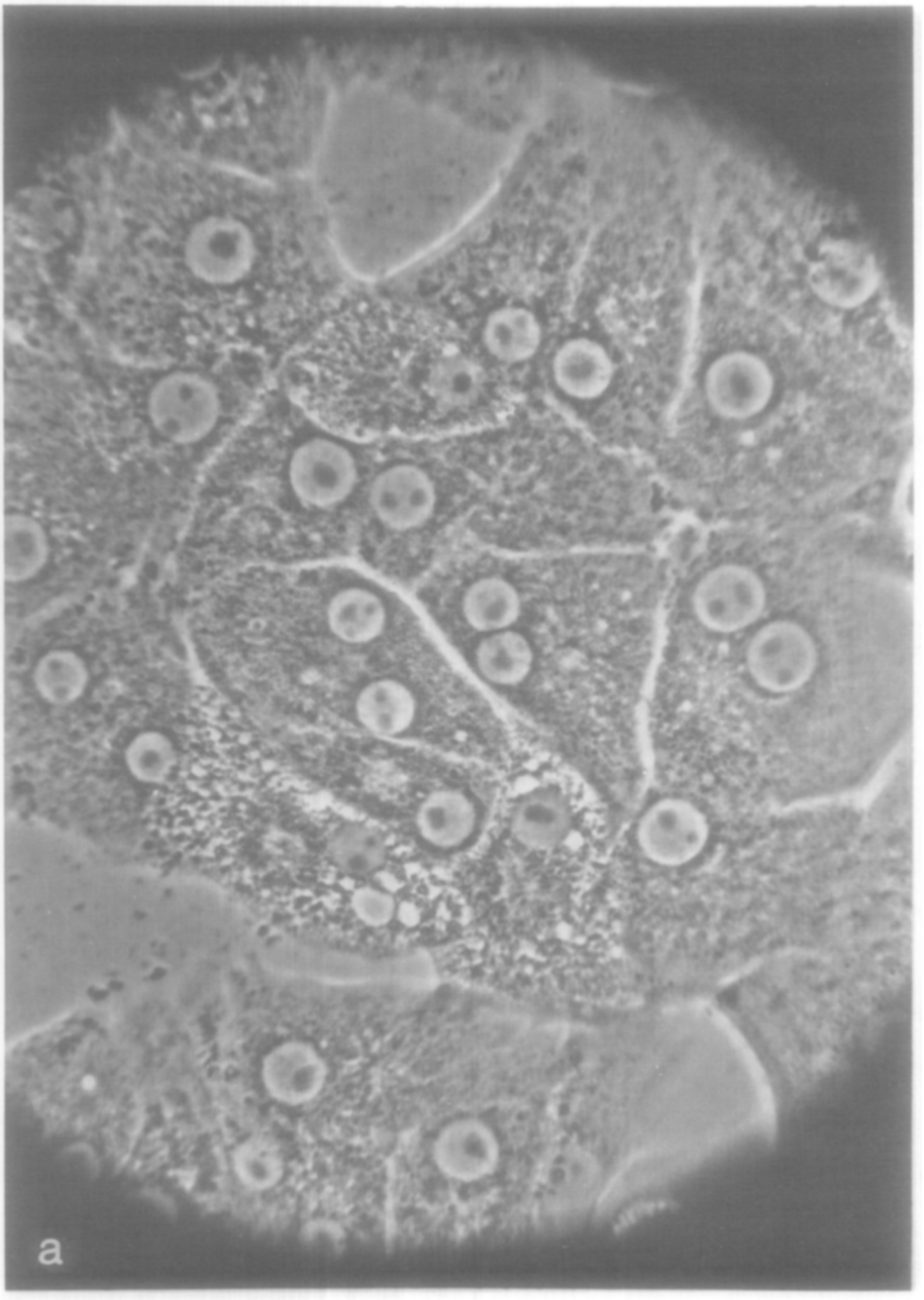
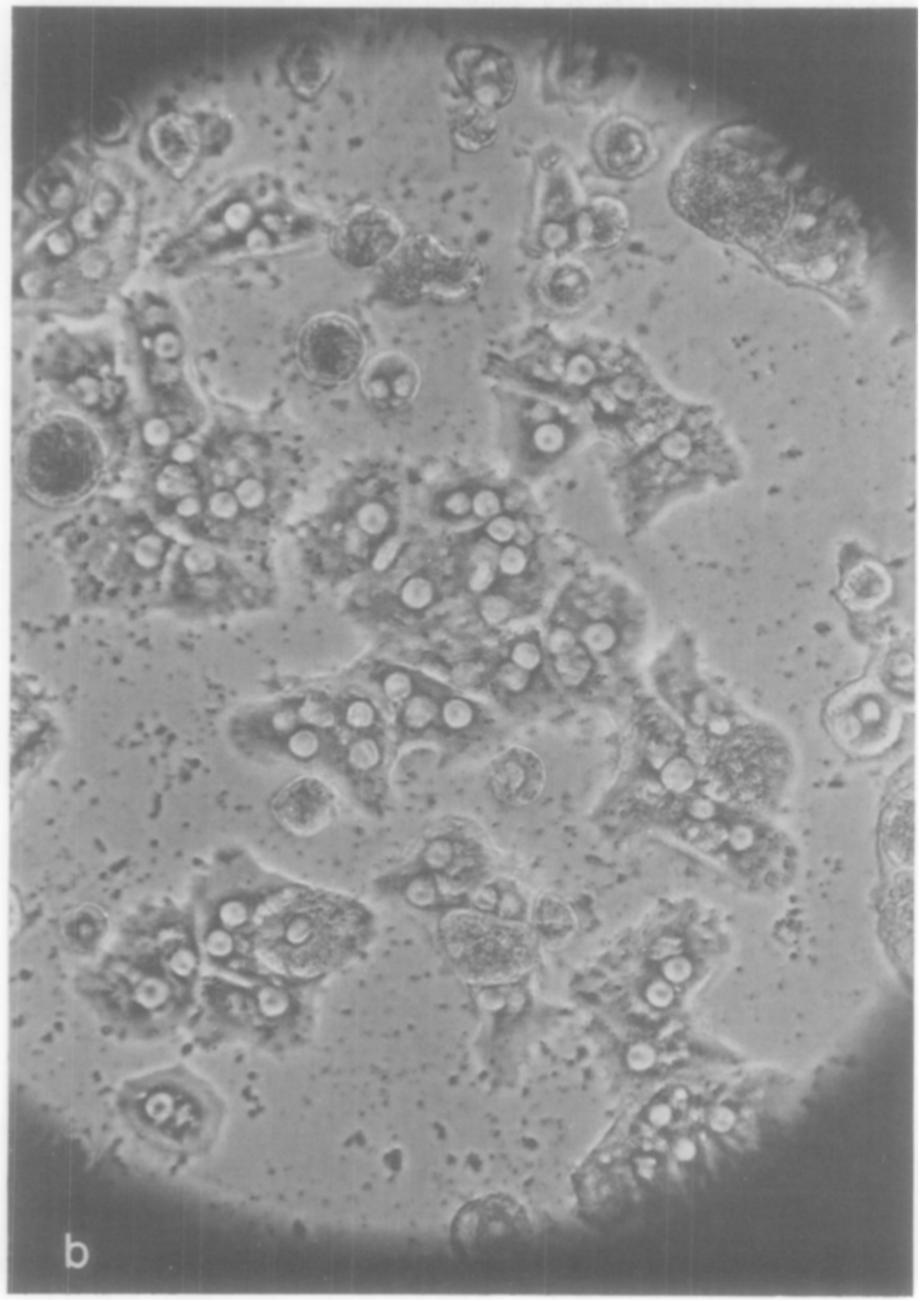


Fig. 1. Rat hepatocytes in monolayer after 2 days of incubation. (a) standard L-15 culture medium supplemented with 10% FCS; (b) standard culture medium, no serum added.



transaminase activity increased 2, 10 and 3.5 fold respectively with 8×10^{-7} M insulin, 1×10^{-5} M dexamethasone and 1×10^{-4} M glucagon. 500 µg/ml bacitracin (8) was added to the medium to inhibit the degradation of glucagon by hepatocytes (9).

TABLE 1

Urea Cycle Enzymes and Tyrosine Aminotransferase Activities in Whole Rat Liver and Isolated Rat Hepatocytes

	unit‡/mg protein (mean±SEM)					milliunit‡‡/mg protein (mean±SEM)
	CPS	OCT	AS	AL	ARG	TAT
Whole rat liver (n=20) (4)	2.46±0.10	52.2± 2.9	0.97±0.06	1.35±0.05	655±49	20.7±1.3 (n=7)*
Isolated rat hepatocytes (n=11)*	4.88±0.37	92.8±10.2	0.60±0.08	1.50±0.17	380±38	10.6±0.9 (n=6)*

* Liver or cell preparations from n donor animals

Abbreviations: CPS Carbamyl phosphate synthetase (E.C.2.7.2.a)
OCT Ornithine carbamyl transferase (E.C.2.1.3.3)
AS Argininosuccinate synthetase (E.C.6.3.4.5)
AL Argininosuccinate lyase (E.C.4.3.2.1)
ARG Arginase (E.C.3.5.3.1)
TAT Tyrosine aminotransferase (E.C.2.6.1.5)

‡ A unit of urea cycle enzyme activity is defined as the amount catalyzing the formation of one micromole of product per hour at 37°

‡‡ One milliunit of TAT catalyzes the formation of one n mole of p-hydroxyphenylpyruvate per minute at 37°

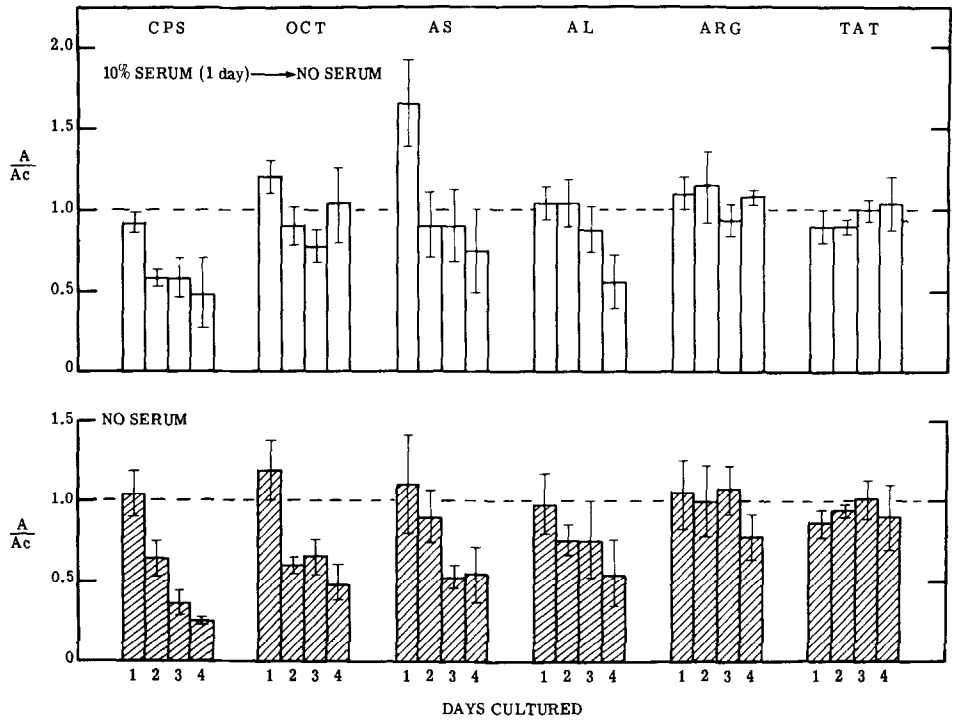


Fig. 2. Time course of urea cycle enzymes and tyrosine aminotransferase activities in cultured cells. The results are expressed as the ratios of enzyme activities of cultured cells (A) to the activities of control cells (Ac) which were isolated from the same livers but not cultured (see Table 1). Brackets indicate ± SEM of the means of 3-9 donor animals. Abbreviations of enzymes as in Table 1.

Upper panel: cells were cultured in L-15 medium containing 10% fetal calf serum for the first 24 hr and shifted to no serum-containing medium at 24 hr (10 ⟶ 0) for the rest of culturing time.

Lower panel: cells were cultured as monolayer in culture medium containing no serum from 0 to 96 hr.

Adenylyl cyclase activities of freshly isolated hepatocytes were comparable to those in whole liver and maintained activities up to at least 72 hr in culture (Table 2). The enzymes could be activated by epinephrine, glucagon and fluoride at 24 and 72 hr.

DISCUSSION

The method developed here for culturing adult rat liver cells is convenient and simple. Anatomy is distorted by a previous hepatectomy which

TABLE 2

Adenylyl Cyclase Activity

		p moles C-AMP formed/mg protein/15 min (mean±SEM)			
		Basal	+Epinephrine (10^{-4}M)	+Glucagon (10^{-5}M)	+NaF (10 mM)
Normal adult rat liver (n=5)		233±28	689±34	1,320±31	1,272±42
Isolated rat hepatocytes (n=3)		212±14		1,247±23	1,143±20
Rat hepatocytes cultured in monolayer (n=3)					
Culture medium	Culture time				
Standard culture medium, no serum throughout	24 hr	147±13	213±13	1,995±24	1,259±38
	72 hr	111±18	170±20	318±15	560±21
Standard culture medium, 10% FCS for 24 hr and shifted to no serum medium (10 → 0)	24 hr	167±17	254±20	1,463±68	1,077±54
	72 hr	139± 6	168± 5	542±12	1,446±29

makes liver perfusion to isolate cells difficult. The high efficiency of attachment (50%) using petri dishes coated with collagen and 10% fetal calf serum for the first 24 hr allows us to prepare one 100 mm dish covered with a complete monolayer when 5 x 10^6 cells are inoculated per dish. Thus a 7-12 g adult rat liver is sufficient for 30-80 dishes depending on yields of viable cells. Switching to no-serum medium at 24 hr maintains normal cell morphology and attachment while obtaining the advantages of a completely synthetic medium.

Isolated liver cells are viable in liquid culture medium for 6-24 hr (10-11) and allow convenient study of short-term metabolic changes. For study of slower adaptive processes like enzyme induction or repression, a stable cell culture method is needed so that steady-states can be studied. One of the uncontrollable variables in mammalian cell cultures has been the need for serum in the otherwise defined media. The factors present in serum which help cells form monolayers, and which promote growth or stability of function and composition are poorly understood. The present liver cell culture system is stable from 24 hr to 72 or even 96 hr in serum-free synthetic medium and added hormones like insulin are not necessary for cell survival. This means that effects of hormones, drugs, or nutrients as possible inducers are not complicated by the presence of other hormones or metabolites bound to serum proteins, or by binding of the test substances to these proteins.

The maintenance of high activities of a complete enzyme cycle (urea) which includes mitochondrial (CPS, OCT) and cytoplasmic enzymes (AS, AL, ARG) allows study of genetic control mechanisms and coordinate induction or repression of this cycle similar to those performed in the bacterial arginine biosynthetic pathway (12). The presence of hormone sensitive adenylyl cyclase activity means this culture system can be used to study the role of cyclic AMP in enzyme induction, a subject which has remained obscure because of methodologic difficulties. Stimulation of TAT activity by glucagon, dexamethasone and insulin demonstrates the presence of normal induction mechanisms in these

cells. Whether the entire urea cycle can be induced *in vitro* as has been done *in vivo* with casein hydrolysate and with glucagon (13) is currently under study.

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