IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY
Volume 25, Number 9, September 1989

IMPROVED MAINTENANCE OF ADULT RAT HEPATOCYTES IN A NEW SERUM-FREE MEDIUM IN THE PRESENCE OR ABSENCE OF BARBITURATES

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(Received 27 December 1988; accepted 12 May 1989)

SUMMARY

For serum-free primary culture of adult rat hepatocytes, a synthetic medium DM-160 and rat-tail collagen were selected for the basal medium and for the culture substratum, respectively. Barbiturates, such as phenobarbital and 1-ethyl-5-isobutylbarbiturate, efficiently supported survival of hepatocytes and maintained their morphologic features at lower concentrations under the serum-free conditions than under the serum-supplemented conditions. However, the hepatocyte survival rates under the serum-free conditions were lower than those under the serum-supplemented conditions in the presence or absence of barbiturates. Supplementation of the basal medium with a combination of five groups of factors (5Fs), such as eight amino acids (Ala, Arg, Gly, Ile, Met, Phe, Pro, and Trp), two unsaturated fatty acids (linoleate and oleate), a protease inhibitor (aprotinin), three vitamins (A, C, and E), and five trace elements (Mn, Fe, Cu, Zn, and Se), improved the hepatocyte survival under the serum-free conditions in the presence or absence of barbiturates. In other words, the serum could be completely substituted by the 5Fs. Hepatocyte cultures maintained in the 5Fs-supplemented basal medium showed excellent induction of tyrosine aminotransferase activity in response to dexamethasone in the presence or absence of barbiturates. The efficiency of the 5Fs-supplemented basal medium for maintaining hepatocytes was not inferior to those of other media in common use with hepatocytes, such as Williams' medium E and Waymouth's medium MB-752/1. In conclusion, maintenance of functional hepatocytes in serum-free primary culture could be improved by use of the new medium preparation (the 5Fs-supplemented DM-160) in the presence of barbiturates.

Key words: adult rat hepatocytes; development of a new serum-free medium; barbiturates; hepatocyte survival; tyrosine aminotransferase.

INTRODUCTION

Primary hepatocyte cultures would be useful for studies on hepatic metabolism, regeneration, and carcinogenesis. However, hepatocytes, like other differentiated cell types, are not easily maintained in culture without loss of many of their in vivo functions. Therefore, a number of attempts have been made to increase the functional longevity of hepatocytes in primary culture. Mainly three different approaches, namely selective supplementation of medium with various factors (10,13,14,26,27,39), use of appropriate substrata (15,18,19,29,30,34), and co-cultivation with other liver epithelial cells (6,7) have been made. By these efforts, the culture conditions have been improved.

In connection with the improvement of culture conditions for maintenance of functional hepatocytes, we have also reported that phenobarbital (PB), one of barbiturates, efficiently supported survival of functional hepatocytes from adult rats for a long time, i.e., at least 1.5 mo. in primary culture (22). In this study it has also been found that PB is not unique in such action, and that a number of commercially available barbiturates show the same action on primary cultured hepatocytes. (23). A correlation exists between the barbiturate structure and the potency for supporting hepatocyte survival (23). On the basis of these findings, we have succeeded in synthesizing a more efficient barbiturate, 1-ethyl-5-isobutylbarbiturate (C-9), as compared with the commercially available ones such as PB and hexobarbital (23,24).

The methodology for maintenance of functional hepatocytes in primary culture is now quite reliable. However, most of these studies included serum-containing media (6,13-15,19,22-24,26,27,29,30,34,39). Serum contains hormones, growth factors, and many other undefined substances. The complex and undefined nature of serum complicates the design and interpretation of experiments aimed at understanding the interac-

TABLE 1

COMPARISON OF ATTACHMENT EFFICIENCY OF ADULT RAT HEPATOCYTES IN VARIOUS SYNTHETIC MEDIA UNDER SERUM-FREE CONDITIONS[a]

Medium	Attachment Efficiency, %
DM-160	82.9 ± 5.0
WE	81.1 ± 4.4
MB-752/1	78.4 ± 8.0
M199	77.5 ± 2.8
BME	76.6 ± 5.1
McCoy-5a	76.4 ± 4.0
MEM	72.9 ± 1.7*
RPMI-1640	72.2 ± 2.2*
F12	35.2 ± 5.9**
L15	30.2 ± 1.0**

[a]Isolated hepatocytes were inoculated at a cell number of 1.4×10^6 cells onto plastic dishes (60 mm in diameter) containing 4 ml of various serum-free media supplemented with dexamethasone (10 μM), insulin (10 μg/ml), and HEPES (5 mM, pH 7.2). Attachment efficiency is expressed as the percentage of viable inoculum cells attached after a 24-h attachment period. Results are expressed as mean ± SD from three dishes. *Asterisks* denote significant differences against DM-160 as follows: *, $P < 0.05$, and **, $P < 0.005$.

tion of hormones, growth factors, and so forth. In the present study, we developed a new serum-free medium for long-term maintenance of functional hepatocytes from adult rats in primary culture, using the typical and efficient barbiturates such as PB and C-9. We found that the developed serum-free medium was not at all inferior to the serum-containing medium in supporting survival of functional hepatocytes in the presence or absence of barbiturates in primary culture.

Materials and Methods

Materials. Male Donryu rats (3 mo. old) inbred in this laboratory were used in the present experiments. Bovine serum (BS) was prepared as reported previously (22). 1-Ethyl-5-isobutylbarbituric acid sodium salt (C-9) was synthesized as reported previously (24). The following were purchased: DM-160 medium from Kyokuto Pharmaceutical Industrial Co., Ltd. (Tokyo, Japan); Eagle's minimal essential medium (MEM) and RPMI 1640 medium from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan); Williams' medium E (WE), Waymouth's medium MB-752/1 (MB-752/1), Medium 199 (M199), basal medium Eagle (BME), McCoy-5a medium, Ham's medium F12 (F12), and Leibovitz' 15 (L15) medium from Flow Laboratories, Inc. (McLean, VA); dexamethasone sodium phosphate (dexamethasone) from Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan); $MnCl_2 \cdot 4H_2O$, $FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, H_2SeO_3, and ethanol from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); PB and linoleic acid sodium salt (linoleate) from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan); oleic acid sodium salt (oleate) from Ishizu Pharmaceutical Co., Ltd. (Osaka, Japan); α-cyclodextrin and DL-α-tocopherol (vitamin E) from Nakarai Chemicals, Ltd. (Kyoto, Japan); Cellmatrix type IV (collagen type IV, C-IV) from Nitta Gelatin Co., Ltd. (Osaka, Japan); Vitrogen 100 (95% collagen type I:5% collagen type III, C-I) from Collagen Corporation (Palo Alto, CA); laminin (LN) from Collaborative Research, Inc. (Lexington, MA); fibronectin (FN), gelatin (GN), poly-L-lysine (mol. wt. 70 000 to 150 000, PL), aprotinin, trypsin inhibitor, retinol acetate (vitamin A), L-ascorbic acid (vitamin C), EDTA, HEPES, L-alanine (Ala), L-arginine (Arg), glycine (Gly), L-isoleucine (Ile), L-methionine (Met), L-phenylalanine (Phe), L-proline (Pro), L-tryptophan (Trp), insulin and collagenase type I from Sigma Chemical Co. (St. Louis, MO); and trypsin (1:250) from Difco (Detroit, MI). Other ordinary chemicals from various sources were of reagent grade.

Attachment factors. Rat-tail collagen (RTC, type I) gels were prepared by a slight modification of the method of Michalopoulos and Pitot (18), as reported previously (25).

TABLE 2

EFFECT OF NATURAL AND ARTIFICIAL MATRIX SUBSTANCES ON ATTACHMENT AND SURVIVAL OF ADULT RAT HEPATOCYTES IN SERUM-FREE PRIMARY CULTURE[a]

Matrix Substance		Optimal Concentration	Attachment Efficiency, %	Surviving Hepatocyte Number, %
None		—	60.3 ± 3.0[b]	25.7 ± 3.4[c]
RTC	(C)	1.0 mg/ml	84.5 ± 2.4****	38.6 ± 3.7**
C-I	(C)	0.5 mg/ml	81.6 ± 1.3****	35.6 ± 2.5**
C-IV	(C)	1.0 mg/ml	83.7 ± 2.7****	37.9 ± 0.7****
GN	(C)	0.5 mg/ml	79.7 ± 1.5****[d]	37.2 ± 0.8****
FN	(A)	1.0 μg/ml	79.7 ± 0.8****[d]	37.1 ± 2.0***
LN	(A)	5.0 μg/ml	84.4 ± 6.8***	38.0 ± 0.7****
PL	(A)	1.0 μg/ml	70.4 ± 5.0*[c]	25.8 ± 4.8[c]

[a]Isolated hepatocytes were inoculated at a cell number of 1.4×10^6 cells per dish (60 mm in diameter) per 4 ml of the basal medium (DM-160). Attachment efficiency is expressed as the percentage of viable inoculum cells attached after a 24-h attachment period. Surviving hepatocyte number is expressed as the percentage of the attached cells 24 h after the inoculation, which survived on Week 1. Results are expressed as mean ± SD from three dishes.

Asterisks and *letters* denote significant differences against *None* and *RTC*, respectively, as follows: * and [d], $P < 0.05$; ** and [c], $P < 0.025$; ***, $P < 0.01$; **** and [b], $P < 0.005$.

(C) = tested by coating dishes at the indicated concentrations.

(A) = tested by direct addition to the culture medium at the indicated concentrations.

Falcon plastic dishes were flooded with 1 ml (for 60-mm dish) or 2.5 ml (for 100-mm dish) of the solutions of RTC, C-I, C-IV, or GN at the indicated concentrations, and left to stand for 1 h at room temperature. The excess solutions were then aspirated off, and the dishes were dried overnight at 37° C. Before use for cell culture, the coated dishes were rinsed once with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS).

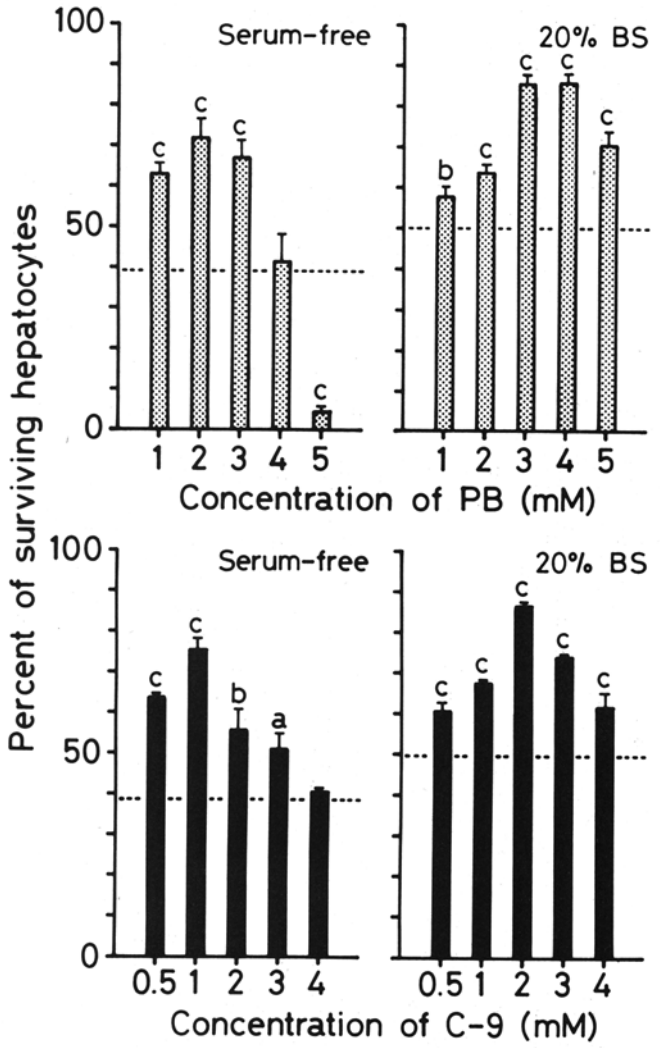


FIG. 1. Effect of barbiturates on survival of hepatocytes under the serum-free and serum-supplemented conditions in primary culture. Isolated hepatocytes were inoculated at a cell number of 1.4×10^6 cells in 4 ml of the serum-free or 20% BS-containing basal medium (DM-160) onto RTC-coated dishes in the presence of dexamethasone (10 μM) and insulin (10 μg/ml). After a 24-h attachment period, the culture media were replaced by the serum-free or 20% BS-containing basal medium with or without PB or C-9 at the indicated concentrations, and thereafter renewed every 2 d. Viable hepatocyte numbers were expressed as percentages of the attached cells 24 h after the inoculation, which survived on Week 1. *Horizontal dotted lines* show the numbers of surviving hepatocytes in the control cultures. Results are expressed as mean from three dishes. *Vertical lines* show SD. *Letters* over the vertical lines show significant differences against control as follows: $a = P < 0.05$; $b = P < 0.01$, and $c = P < 0.005$.

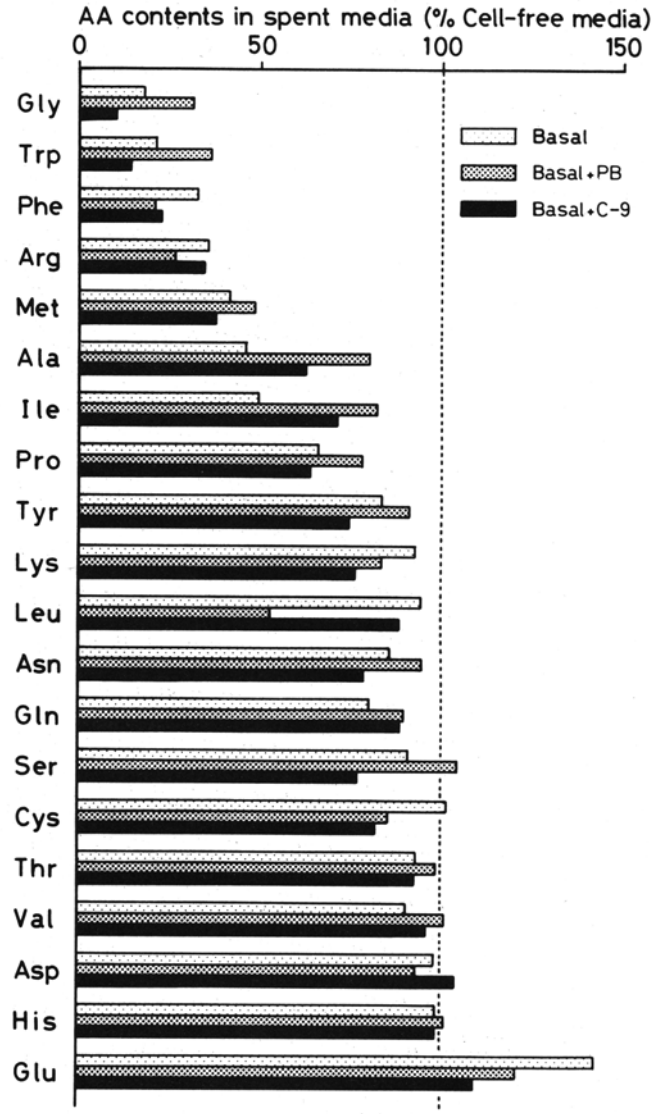


FIG. 2. Amino acid patterns in the spent serum-free basal medium (DM-160) with or without barbiturates. Concentrations of amino acids were measured with the spent media in which hepatocytes from 3-d cultures were incubated for 48 h. Amino acid contents in the spent media were expressed as percentages of the respective ones in the media which were incubated for 48 h without cells. Results are expressed as mean from three dishes. *AA* = amino acid.

Other attachment factors, such as FN, LN, and PL, were added to the culture medium to give the final concentrations indicated in the text.

Supplementation of medium with various factors. PB, C-9, amino acids, trypsin inhibitor, and vitamin C were directly dissolved in the culture medium to give the indicated final concentrations. Linoleate and oleate were dissolved in double-distilled water (DDW) with α-cyclodextrin at a ratio of 1:50. Aprotinin was dissolved in PBS. Vitamins A and E were dissolved in ethanol. $FeSO_4 \cdot 7H_2O$ was dissolved in 0.1 *N* HCl. Other metal salts were dissolved in DDW. The solutions of unsaturated fatty acids, protease inhibitor, vitamins, and metal salts

were added to the culture medium to give the final concentrations indicated in the text. The final concentrations of α-cyclodextrin and ethanol were 25 mg/liter and 0.1%, respectively. The media supplemented with or without various factors were finally adjusted to pH 7.2.

Isolation and primary culture of adult rat hepatocytes. As reported previously, hepatocytes, having an initial viability of 86 to 95% as measured by trypan blue exclusion, were isolated from normal male adult rats by a collagenase-liver-perfusion technique (22). The isolated hepatocytes suspended in cold Hanks' balanced salt solution (HBSS) were sedimented by centrifugation at 17 $\times g$ for 2 min. The hepatocytes were further washed twice by resuspending in cold HBSS followed by recentrifugation. Then, to inhibit further proteolytic activity included in the collagenase preparation (type I) (32), the hepatocytes were rinsed once with the culture medium supplemented with BS at 5% or aprotinin at 8000 Kallikrein Units (KU)/liter. Inasmuch as the attachment efficiency of the hepatocytes rinsed with the BS- and aprotinin-containing medium did not differ from each other in serum-free primary culture, the latter medium was used for rinsing hepatocytes in the following experiments (data not shown).

The thoroughly washed hepatocytes were finally suspended in the culture medium, inoculated at a density of 7×10^4 cells·cm^{-2}·ml^{-1} into the coated or uncoated dishes, and cultured in a humidified atmosphere of 5% CO_2:95% air at 37° C, unless otherwise indicated. After the 1-d attachment period, the culture medium was replaced by the various factor-supplemented media or by the media supplemented with or without ethanol at 0.1% or α-cyclodextrin at 25 mg/liter or both, for control cultures. Thereafter, the culture media were renewed every 2 d.

Determination of viable hepatocyte number. Preparations of the suspensions of hepatocytes cultured on the plastic dishes or cultured on the collagen- or gelatin-coated plastic dishes was carried out as reported previously (22,25). Briefly, all the cultures were first washed once with 4 ml of PBS. Then the cultures were incubated with 2 ml of 0.1% trypsin in PBS containing 0.02% EDTA for 5 min at 37° C. The cultures on the collagen- or gelatin-coated dishes were further incubated for 10 min after addition of 2 ml of 0.05% collagenase in Ca^{2+}- and Mg^{2+}-free HBSS supplemented with 5 m*M* $CaCl_2$ and 10 m*M* HEPES (pH 7.5) at 37° C. Immediately after the final enzyme treatments, 2 ml (for plastic dish) or 4 ml (for collagen- or gelatin-coated dish) of cold culture medium supplemented with 20% BS was added to each dish to inhibit further proteolytic activity of the enzymes. Then the suspensions were gently pipetted to disperse the hepatocytes. The finally obtained hepatocyte suspensions were kept in an ice-water bath until determination of viable cell number. The numbers of viable hepatocytes were determined by trypan blue exclusion in a hemacytometer and expressed as the mean from three dishes.

Amino acid analysis. To remove proteins, 1-ml aliquots of the spent culture media were treated with 100 μl of 50% 5-sulfosalicylic acid, and centrifuged at 6150 $\times g$ for 5 min. The supernatants were analyzed on an automatic amino acid analyzer (model A5500, Irika Kogyo Co., Ltd., Tokyo, Japan). The contents of amino acids in the spent media were expressed as mean from three dishes.

TABLE 3

EFFECT OF SUPPLEMENTATION OF THE BASAL MEDIUM (DM-160) WITH VARIOUS FACTORS ON HEPATOCYTE SURVIVAL IN SERUM-FREE PRIMARY CULTURE[a]

Added Factor	Optimal Concentration	Surviving Hepatocyte Number, % Control		
		Added Barbiturate		
		None	2 m*M* PB	1 m*M* C-9
8 Amino acids[b]	x 2[c]	121.5 ± 9.8*	118.5 ± 1.5****	116.1 ± 3.4**
Linoleate	0.1 mg/l	133.5 ± 5.6***	127.9 ± 2.7****	124.4 ± 6.9***
Oleate	0.1 mg/l	118.3 ± 8.1**	126.1 ± 1.6****	116.3 ± 3.5****
Aprotinin	4000 KU/l	122.1 ± 7.1***	127.5 ± 7.6****	116.9 ± 1.9****
Trypsin inhibitor	200 mg/l	114.7 ± 5.7**	124.8 ± 5.3****	122.1 ± 8.2**
Vitamin A	1 μg/l	115.5 ± 2.1****	116.4 ± 6.2**	111.2 ± 6.0*
Vitamin C	20 mg/l	112.4 ± 6.8*	124.1 ± 0.6****	114.8 ± 4.9**
Vitamin E	1μg/l	121.5 ± 4.7****	123.5 ± 2.1****	121.1 ± 6.2***
$MnCl_2 \cdot 4H_2O$	100 n*M*	119.5 ± 6.0***	114.3 ± 1.4****	112.0 ± 5.8*
$FeSO_4 \cdot 7H_2O$	100 n*M*	114.6 ± 7.9*	111.2 ± 0.5****	107.0 ± 2.2*
$CuSO_4 \cdot 5H_2O$	100 n*M*	118.6 ± 3.6***	114.5 ± 2.1****	116.3 ± 2.9***
$ZnSO_4 \cdot 7H_2O$	100 n*M*	116.7 ± 5.2**	122.9 ± 5.7****	119.5 ± 3.7***
H_2SeO_3	10 n*M*	122.3 ± 5.2****	126.7 ± 2.8****	115.1 ± 5.9**

[a] Isolated hepatocytes were inoculated at a cell number of 1.4×10^6 cells in 4 ml of the basal medium (DM-160) supplemented with dexamethasone (10 μ*M*) and insulin (10 μg/ml) onto RTC-coated dishes (60 mm in diameter). After a 24-h attachment period, the culture medium was replaced by the basal medium with or without various factors at each optimal concentration, and thereafter the culture media were renewed every 2 d. Surviving hepatocyte numbers were determined on Week 1 and expressed as the percentages of those in control cultures. Results are expressed as mean ± SD from three dishes. *Asterisks* denote significant differences against control as follows: *, $P < 0.05$; **, $P < 0.025$; ***, $P < 0.01$, and ****, $P < 0.005$.

[b] 8 Amino acids = Ala, Arg, Gly, Ile, Met, Phe, Pro, and Trp.

[c] x 2 = twofold higher concentration than the formula of DM-160 medium (*see* Table 4).

Assay of tyrosine aminotransferase activity. The preparation of cell supernatants and the assay of tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5, TAT) activity (5) were carried out as reported previously (20,21). Specific activities were expressed in milliunits (nanomole of product formed per minute) per milligram protein of supernatants. Protein contents were determined by the method of Lowry et al. (16) with bovine serum albumin as standard.

Statistical evaluation. For statistical evaluation, the numerical data were analyzed using Student's t test. The level of significance was chosen at $P < 0.05$.

Results and Discussion

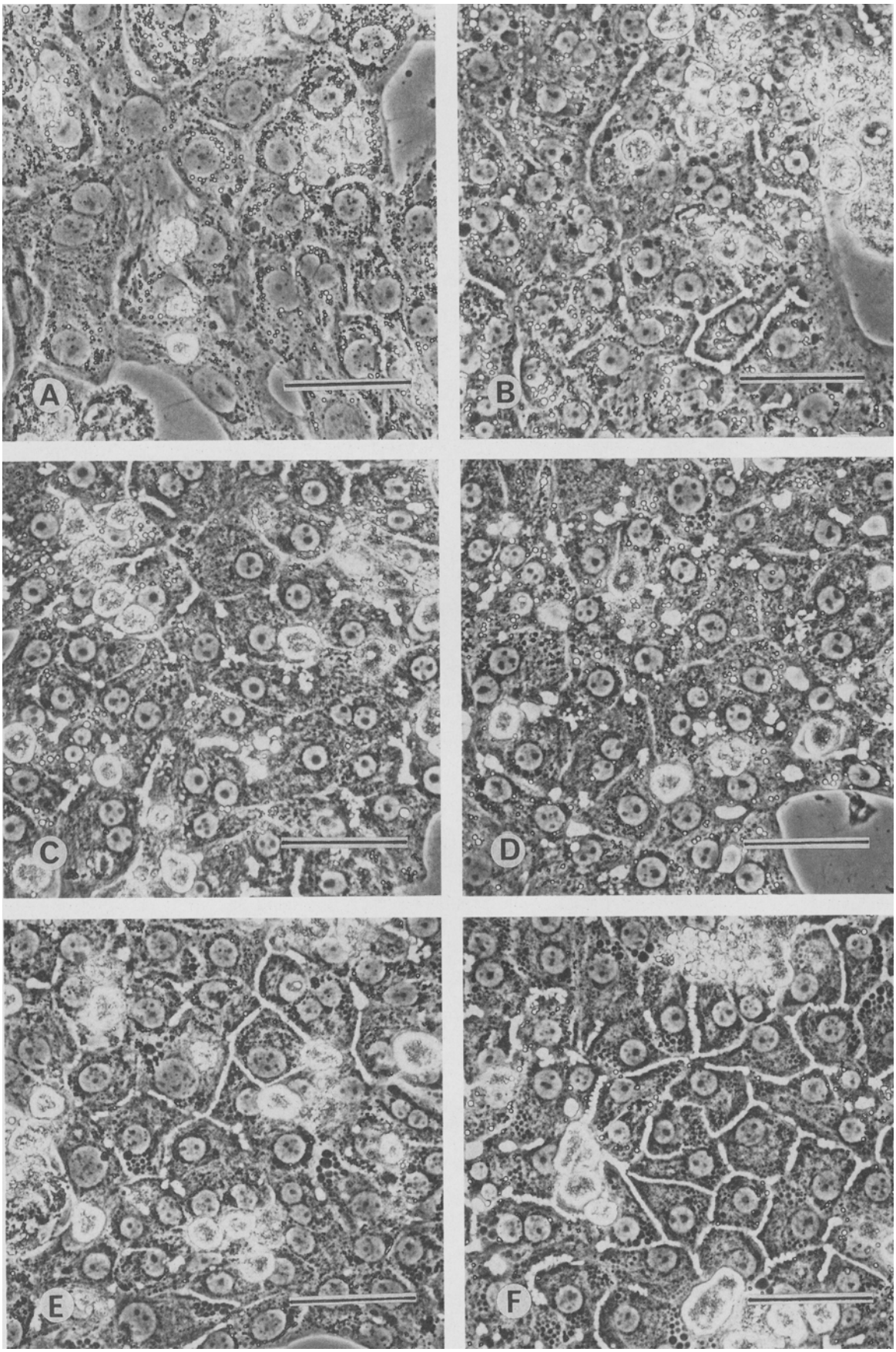
Determination of basal culture conditions. To select a basal medium, we tested 10 commercially available synthetic media for their efficiency for allowing hepatocytes to attach to plastic substratum under the serum-free conditions. Efficiency of attachment was reduced with the use of four of these media (MEM, RPMI 1640, F12, and L15) but was unaffected by the other six media (DM-160, WE, MB-752/1, M199, BME, and McCoy-5a) we tested (Table 1). Then a homemade

TABLE 4

COMPOSITION OF DM-160 MEDIUM SUPPLEMENTED WITH OR WITHOUT 5Fs

Ingredient	DM-160 Without 5Fs		DM-160 With 5Fs	
	mg/liter	(M)	mg/liter	(M)
L-Alanine	400	(4.489 −3)	800*	(8.978 −3)*
L-Arginine	100	(5.740 −4)	200*	(1.148 −3)*
L-Aspertic acid	25	(1.878 −4)	25	(1.878 −4)
L-Asparagine	25	(1.892 −4)	25	(1.892 −4)
L-Cystein · HCl	80	(5.075 −4)	80	(5.075 −4)
L-Glutamic acid	150	(1.019 −3)	150	(1.019 −3)
L-Glutamine	300	(2.052 −3)	300	(2.052 −3)
Glycine	15	(1.998 −4)	30*	(3.996 −4)*
L-Histidine	30	(1.933 −4)	30	(1.933 −4)
L-Isoleucine	150	(1.143 −3)	300*	(2.286 −3)*
L-Leucine	400	(3.049 −3)	400	(3.049 −3)
L-Lysine	100	(6.840 −4)	100	(6.840 −4)
L-Methionine	80	(5.362 −4)	160*	(1.072 −3)*
L-Phenylalanine	80	(4.842 −4)	160*	(9.684 −4)*
L-Proline	12	(1.042 −4)	24*	(2.084 −4)*
L-Serine	80	(7.612 −4)	80	(7.612 −4)
L-Threonine	100	(8.394 −4)	100	(8.394 −4)
L-Tryptophan	40	(1.958 −4)	80*	(3.916 −4)*
L-Tyrosine	50	(2.759 −4)	50	(2.759 −4)
L-Valine	85	(7.255 −4)	85	(7.255 −4)
Retinol acetate	—	—	0.001*	(3.044 −9)*
Thiamine	1.0	(3.261 −6)	1.0	(3.261 −6)
Riboflavine	1.0	(2.657 −6)	1.0	(2.657 −6)
Pyridoxine	1.0	(5.835 −6)	1.0	(5.835 −6)
Cyanocobalamine	0.005	(3.688 −9)	0.005	(3.688 −9)
Pantothenic acid	1.0	(4.561 −6)	1.0	(4.561 −6)
Nicotinamide	1.0	(8.188 −6)	1.0	(8.188 −6)
Biotin	0.1	(4.093 −7)	0.1	(4.093 −7)
Choline · HCl	5.0	(3.171 −5)	5.0	(3.171 −5)
Ascorbic acid	1.0	(5.677 −6)	21.0*	(1.192 −4)*
Folic acid	1.0	(2.265 −6)	1.0	(2.265 −6)
Inositol	5.0	(2.775 −5)	5.0	(2.775 −5)
DL-α-Tocopherol	—	—	0.001*	(2.321 −9)*
Sodium linoleate	—	—	0.1*	(3.306 −7)*
Sodium oleate	—	—	0.1*	(3.284 −7)*
Aprotinin	—	—	4000 KU/l*	
NaCl	6800	(1.163 −1)	6800	(1.163 −1)
KCl	400	(5.364 −3)	400	(5.364 −3)
$CaCl_2$	200	(1.801 −3)	200	(1.801 −3)
$MgSO_4 \cdot 7H_2O$	200	(8.114 −4)	200	(8.114 −4)
$NaH_2PO_4 \cdot H_2O$	125	(9.058 −4)	125	(9.058 −4)
$NaHCO_3$	1000	(1.190 −2)	1000	(1.190 −2)
Glucose	1000	(5.550 −3)	1000	(5.550 −3)
Phenol red	6	(1.693 −5)	6	(1.693 −5)
$MnCl_2 \cdot 4H_2O$	—	—	0.0198*	(1.000 −7)*
$FeSO_4 \cdot 7H_2O$	—	—	0.0278*	(1.000 −7)*
$CuSO_4 \cdot 5H_2O$	—	—	0.0250*	(1.000 −7)*
$ZnSO_4 \cdot 7H_2O$	—	—	0.0288*	(1.000 −7)*
H_2SeO_3	—	—	0.0013*	(1.000 −8)*

(4.489 −3) = (4.489 × 10^{-3}). *Altered.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medium, DM-160, which was devised by Katsuta and Takaoka (12) for the cultivation of various kinds of cells, with or without serum (12), was selected for the basal medium in the present study.

Extracellular matrix components (C-I, C-IV, FN, and LN), each of which is uniquely distributed in normal rat liver (17), are known to promote hepatocyte attachment in primary culture (18,31). Also in the present study, all of the natural and artificial matrix substances tested, such as RTC, C-I, C-IV, GN, FN, LN, and PL, consistently enhanced the hepatocyte attachment under the serum-free conditions (Table 2). A previous study demonstrated that C-IV was more effective than C-I or C-III in promoting hepatocyte attachment (1). However, in the present study there was no difference between the efficiency of C-I and C-IV. Except for PL, the other six matrix substances were effective not only in promoting hepatocyte attachment but also supporting survival of the cells (Table 2). Because there was no difference in the efficiencies, the laboratory-made RTC was chosen for coating dishes.

A combination of dexamethasone (10 μM) and insulin (10 μg/ml) further enhanced the hepatocyte attachment to RTC-coated dishes under the serum-free conditions (data not shown), as similarly reported previously under the different experimental conditions (26). Therefore, in the following experiments, these hormones were added to the cultures only during the first 24 h after inoculation.

Effect of barbiturates on survival of hepatocytes. Both barbiturates PB and C-9 efficiently supported survival of hepatocytes and maintained their morphologic features at lower concentrations under the serum-free conditions than under the serum-supplemented conditions (Figs. 1 and 3 *A, C, E*). The optimal concentrations of PB under the serum-free and serum-supplemented conditions were 2 to 3 mM and 3 to 4 mM, respectively (Fig. 1); similarly, those of C-9 were 1 mM and 2 mM (Fig. 1). Requirement of higher doses of barbiturates under the serum-supplemented conditions seems to result from trapping of the chemicals by substance(s) in the serum.

In the presence of barbiturates at each optimal concentration, the survival rates of hepatocytes under the serum-free conditions were, however, lower than those under the serum-supplemented conditions on Week 1 of primary culture (Fig. 1). The reduced effect of barbiturates on hepatocyte survival under the serum-free conditions seems to result from deprivation of some serum components that are required for the survival of hepatocytes in primary culture.

Effect of supplementation of basal medium with various factors on hepatocyte survival. To enhance the survival rates of hepatocytes under the serum-free conditions, we modified the basal medium by supplementing with various factors, as follows.

During culture in the serum-free basal medium in the presence or absence of barbiturates, the concentrations of Gly, Trp, Phe, Arg, and Met in the medium decreased greatly, and those of Ala, Ile, and Pro decreased moderately (Fig. 2). The pattern of amino acids found in the spent medium seems to be determined by cell permeability, incorporation of amino acids into proteins, release of amino acids from proteins, decomposition of amino acids, and formation of amino acids, as suggested previously (36). After supplementation of the basal medium with the well-consumed eight amino acids to give the twofold higher concentrations than the formula of the medium, the survival rates of hepatocytes significantly increased in the presence or absence of barbiturates in serum-free primary culture (Table 3). However, the basal medium supplemented with the eight amino acids at the fourfold higher concentrations was toxic to hepatocytes (data not shown). It has been reported that synthesis of extracellular proteins by hepatocytes decreased when the cells were cultured in the medium deficient in amino acids, and that the reduced production could be restored by addition of amino acid mixture to the medium (35). Similarly in our experiments, albumin production by hepatocytes was enhanced by supplementation of the basal medium with the eight amino acids (data not shown).

Unsaturated fatty acids have been shown to be necessary nutrients for maintenance and propagation of mammalian cells in culture, and to be supplied on the cells from serum albumin in an ordinary serum-supplemented medium (41). Also in the present study, supplementation of the basal medium with linoleate or oleate consistently enhanced the survival rates of hepatocytes in the presence or absence of barbiturates in serum-free primary culture (Table 3).

A unique, trypsinlike protease has been demonstrated to be present on the surface of hepatocytes (37). Under the serum-supplemented conditions, protease inhibitors in the serum seem to protect hepatocyte membrane from being damaged by the protease (9). On the other hand, under the serum-free conditions, this enzyme seems to be activated and to damage hepatocytes (28). Thus in the present study, both protease inhibitors added, such as aprotinin and trypsin inhibitor, significantly prolonged survival of hepatocytes in the presence or absence of barbiturates under the serum-free conditions (Table 3).

Retinol bound to retinol-binding protein, which is synthesized in the liver (3), circulates in the plasma (11).

FIG. 3. Appearance of primary hepatocyte cultures on Week 1. Culture media are as follows: *A*, basal (DM-160); *B*, 5Fs-supplemented basal; *C*, PB-supplemented basal; *D*, PB and 5Fs-supplemented basal; *E*, C-9-supplemented basal, and *F*, C-9 and 5Fs-supplemented basal medium. Concentrations of PB and C-9 were 2 mM and 1 mM, respectively. The 5Fs consisted of eight amino acids (Ala, Arg, Gly, Ile, Met, Phe, Pro, and Trp), two unsaturated fatty acids (linoleate and oleate), a protease inhibitor (aprotinin), three vitamins (A, C, and E), and five trace elements (Mn, Fe, Cu, Zn, and Se). For concentration of each factor in the 5Fs *see* Table 4. Both barbiturates, PB and C-9, efficiently prevented hepatocytes from morphologic degeneration, such as enlargement of cell surface, degranulation of cytoplasm, increase in nuclear:cytoplasm ratio, and multinucleation, which were observed in the control cultures (*A, C, E*). In the absence of barbiturates, morphologic maintenance of hepatocytes was improved by supplementation of the 5Fs (*A, B*). Phase contrast micrographs. *Bars* = 50 μm.

It has become increasingly clear that vitamin A and its analogs are necessary for cell growth, differentiation, and maintenance of differentiated functions (2,4). Simonian et al. (33) have demonstrated that a combination of antioxidants, such as vitamin C, vitamin E, and Na_2SeO_3, was responsible for the prolonged survival of bovine adrenocortical cells in serum-free primary culture. Also in the present study, supplementation of the basal medium with vitamins (A, C, and E) significantly enhanced the survival of hepatocytes in the presence or absence of barbiturates in serum-free primary culture (Table 3).

Because inorganic trace elements tended to be ubiquitous contaminants of chemicals used in the preparation of media, their requirements tended to be masked by contaminants (8). However, due to increased purity of chemicals, supplementation of medium with trace elements is thought to be necessary for growth of cultured cells, especially under the serum-free conditions (8). In the present study, supplementation of the basal medium with trace elements, such as Mn, Fe, Cu, Zn, and Se, also significantly enhanced the hepatocyte survival rates in the presence or absence of barbiturates in serum-free primary culture (Table 3).

Effect of combined supplementation of various factors on survival and function of hepatocytes. As shown in Table 4, five groups of factors (5Fs), such as eight amino acids (Ala, Arg, Gly, Ile, Met, Phe, Pro, and Trp), two unsaturated fatty acids (linoleate and oleate), a protease inhibitor (aprotinin), three vitamins (A, C, and E), and five trace elements (Mn, Fe, Cu, Zn, and Se), were simultaneously supplied at each optimal concentration to the basal medium. The survival rates of hepatocytes were significantly increased by the supplementation of 5Fs in the presence or absence of barbiturates (Table 5). The hepatocyte survival rates in the 5Fs-supplemented serum-free basal medium were not at all inferior to those in the serum-supplemented basal medium in the presence or absence of barbiturates (Table 5). Thus, the serum could be completely substituted by the 5Fs. The efficiency of the 5Fs-supplemented basal medium for supporting hepatocyte survival was compared with those of other media preparations in common use with hepatocytes, i.e. WE (13,14,40) and MB-752/1 (19,38) under serum-free conditions. Except for the slightly higher survival rate of hepatocytes in MB-752/1 in the absence of barbiturates, there was no difference in the survival rates of hepatocytes in the three different media in the presence or absence of barbiturates (Table 5).

In the presence of barbiturates, further effect on morphologic maintenance of hepatocytes was hardly observed after supplementation of the 5Fs (Fig. 3 *D*, *F*). However, in the absence of barbiturates, morphologic maintenance of hepatocytes was improved by supplementation of the 5Fs (Fig. 3 *B*).

Inducibility of TAT activity by dexamethasone (10 μM), which is a well-accepted expression of differentiated liver cell function, was examined in the hepatocyte cultures maintained in the various serum-free media. The hepatocyte cultures maintained in the basal medium showed remarkable induction of TAT activity in response to the hormone in the presence or absence of barbiturates (Table 6). Furthermore, the supplementation of the basal medium with the 5Fs significantly enhanced the induction rates of TAT activity in the presence or absence of barbiturates (Table 6). On the other hand, under the serum-supplemented conditions, no induction of TAT activity was observed in the absence of barbiturates, as reported previously (23,24). In the presence of barbiturates, TAT activity was induced, but the induction rates were much lower as compared with those under the serum-free conditions (22-24). Therefore, it seems that hepatocytes cultured under serum-free conditions respond more sensitively to hormones than those cultured under the serum-supplemented conditions, and that the 5Fs are effective for maintenance of higher sensitivity of hepatocytes to hormones.

As compared with WE and MB-752/1, the basal activities of TAT in the hepatocyte cultures maintained in the 5Fs-supplemented basal medium were lower than

TABLE 5

SURVIVAL OF HEPATOCYTES MAINTAINED IN VARIOUS SERUM-FREE OR SERUM-CONTAINING MEDIA IN THE PRESENCE OR ABSENCE OF BARBITURATES IN PRIMARY CULTURE[a]

Medium	Surviving Hepatocyte Number, %
DM-160[b]	38.6 ± 3.7***
DM-160 + 20% BS[c]	49.5 ± 1.0*
DM-160 + 5Fs[d]	54.5 ± 2.8
WE[e]	53.8 ± 0.9
MB-752/1[f]	60.4 ± 2.4*
DM-160 + 2 m*M* PB[b]	71.3 ± 4.6**
DM-160 + 20% BS + 3 m*M* PB[c]	85.1 ± 2.1
DM-160 + 5Fs + 2 m*M* PB[d]	82.9 ± 2.7
WE + 2 m*M* PB[e]	83.1 ± 3.4
MB-752/1 + 2 m*M* PB[f]	86.2 ± 2.2
DM-160 + 1 m*M* C-9[b]	75.5 ± 2.6***
DM-160 + 20% BS + 2 m*M* C-9[c]	86.4 ± 0.9
DM-160 + 5Fs + 1 m*M* C-9[d]	85.6 ± 1.6
WE + 1 m*M* C-9[e]	83.8 ± 0.9
MB-752/1 + 1 m*M* C-9[f]	83.5 ± 1.4

[a]Isolated hepatocytes were inoculated at a cell number of 1.4×10^6 cells in 4 ml of DM-160 ([b] and [d]), 20% BS-containing DM-160 ([c]), WE ([e]) or MB-752/1 ([f]) onto RTC-coated dishes (60 mm in diameter) in the presence of dexamethasone (10 μM) and insulin (10 μg/ml). After a 24-h attachment period, the culture media were replaced by the indicated media, and thereafter renewed every 2 d. Surviving hepatocyte number is expressed as the percentage of the attached cells 24 h after the inoculation, which survived on Week 1. Results are expressed as mean ± SD from three dishes. *Asterisks* denote significant differences against the respective 5Fs-supplemented DM-160 in the presence or absence of barbiturates as follows: *, $P < 0.05$; **, $P < 0.025$, and ***, $P < 0.005$.

[d]5Fs consisted of eight amino acids (Ala, Arg, Gly, Ile, Met, Phe, Pro, and Trp), two unsaturated fatty acids (linoleate and oleate), a protease inhibitor (aprotinin), three vitamins (A, C, and E), and five trace elements (Mn, Fe, Cu, Zn, and Se). For concentration of each factor in the 5Fs *see* Table 4.

[b–f]These media contained aprotinin (4000 KU/liter) and HEPES (5 m*M*, pH 7.2). These cultures were maintained in a atmosphere of 7.5% CO_2 to prevent elevation of pH.

TABLE 6

INDUCTION OF TAT ACTIVITY BY DEXAMETHASONE IN PRIMARY HEPATOCYTE CULTURES MAINTAINED IN VARIOUS SERUM-FREE MEDIA IN THE PRESENCE OR ABSENCE OF BARBITURATES[a]

Medium	TAT Activity, mU/mg protein		
	Dexamethasone, 10 μM		
	−	+	
DM-160	4.2 ± 1.2	20.5 ± 1.5****	(4.9)
DM-160 + 5Fs	5.2 ± 1.0	41.3 ± 9.1	(7.9)
WE	6.4 ± 0.6	18.7 ± 6.2**	(2.9)
MB-752/1	9.9 ± 2.0**	32.9 ± 7.0	(3.3)
DM-160 + 2 mM PB	6.4 ± 0.5	40.2 ± 2.0*	(6.3)
DM-160 + 5Fs + 2 mM PB	6.3 ± 0.7	49.8 ± 4.6	(7.9)
WE + 2 mM PB	7.5 ± 1.6	39.4 ± 7.8	(5.3)
MB-752/1 + 2 mM PB	10.9 ± 1.0****	40.8 ± 8.4	(3.7)
DM-160 + 1 mM C-9	10.8 ± 1.4	79.6 ± 4.6*	(7.4)
DM-160 + 5Fs + 1 mM C-9	11.2 ± 1.6	106.3 ± 16.0	(9.5)
WE + 1 mM C-9	7.6 ± 2.6	56.3 ± 4.4***	(7.4)
MB-752/1 + 1 mM C-9	11.0 ± 1.8	42.4 ± 1.6****	(3.9)

[a]Isolated hepatocytes were inoculated at a cell density of 3.5×10^6 cells·10 ml^{-1}·100 mm^{-1} RTC-coated dish. Dexamethasone (10 μM) was added to the cultures 18 h before cell harvest. *Numbers in parentheses* show the induction rates of TAT activity by dexamethasone. Results are expressed as mean ± SD from three experiments (three perfusions). *Asterisks* denote significant differences against the respective 5Fs-supplemented DM-160 in the presence or absence of barbiturates as follows: *, $P < 0.05$; **, $P < 0.025$; ***, $P < 0.01$, and ****, $P < 0.005$. For other details *see* footnote for Table 5.

those in the hepatocyte cultures maintained in MB-752/1 in the presence or absence of PB (Table 6). In all other cases, there was no difference in the basal activities. However, the induction rates of TAT activity by dexamethasone were higher in the hepatocyte cultures maintained in the 5Fs-supplemented basal medium than in WE and MB-752/1 in the presence or absence of barbiturates (Table 6).

In conclusion, the supplementation of DM-160 medium with the combination of 5Fs and barbiturate (PB or C-9) enables us to maintain functional hepatocytes for extended periods of time in serum-free primary culture. The new 5Fs-supplemented DM-160 medium is not at all inferior to the popular media, such as WE and MB-752/1, which are in common use with hepatocytes.

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This work was supported by a grant no. 61771923 from the Ministry of Education, Science and Culture of Japan.