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Albumin, Fibrinogen and Transferrin Synthesis in Isolated Rat Hepatocyte Suspensions

A MODEL FOR THE STUDY OF PLASMA PROTEIN SYNTHESIS

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A system using hepatocyte suspensions *in vitro* was developed for studying the synthesis of albumin, fibrinogen and transferrin. Conditions for optimum survival of the hepatocyte and for synthesis of these plasma proteins were defined for this system. These conditions included the use of horse serum (17.5%, v/v, heat-inactivated), an enriched medium (Waymouth's MB 752/1), an O_2 tension of between 18.7×10^3 and 26.7×10^3 Pa and constant stirring. Albumin, fibrinogen and transferrin synthesis rates were obtained of 0.32 ± 0.094 (10), 0.12 ± 0.030 (11) and 0.097 ± 0.017 (10) [mean $\pm$ S.D. (n)] mg/h per g of hepatocytes respectively. These rates were maintained for the first 12h of study and synthesis continued at a diminished rate up to 48h. The synthesis of albumin was decreased in a medium containing less amino acids and glucose, but that of fibrinogen was substantially unaffected. ATP concentrations up to 12h and RNA/DNA ratios up to 24h were comparable with values *in vivo*. The ability to study cells up to 48h permitted us to find that the addition of a mixture of hormones consisting of glucagon, cortisol, triiodothyronine and growth hormone enhanced fibrinogen synthesis. Addition of insulin to the above mixture resulted in increased synthesis for albumin and transferrin but not for fibrinogen.

Hitherto the isolated-cell model derived from normal rat liver cells has been unsatisfactory for the measurement of plasma protein synthesis because unfortunately it has not been structurally or functionally comparable with the isolated perfused liver for the study of plasma protein synthesis (East *et al.*, 1973; Weigand *et al.*, 1971). Bissel *et al.* (1973) have described a monolayer derived from regenerating rat liver cells in which albumin synthesis was reported to be 1 μg/h per mg of liver protein (0.22mg/h per g of liver) but synthesis rates of other plasma proteins and a detailed study of plasma protein synthesis in this model were not made. Further, since other studies have indicated that the synthesis of some plasma proteins is enhanced in the regenerating rat liver (Gordon, 1970; Majumdar *et al.*, 1967) cells derived from regenerating liver may not be an appropriate model for the study of normal plasma protein synthesis.

The present paper describes a method of incubating isolated hepatocytes obtained from normal liver in which the mean albumin synthesis was 0.32mg/h per g of liver. This synthesis rate and those observed for fibrinogen and urea are equal to or exceed those reported for the isolated perfused liver over a study period of 12h. More than 70% of the cells present after 24h incubation exclude Trypan Blue. Further, ATP concentrations and the RNA/DNA ratio are in the range found *in vivo*. Ultrastructurally the cells appear to be normal when studied for up to 48h of incubation.

This model would seem to be an improvement on similar earlier ones and more suitable for the study of plasma protein synthesis and hepatocyte function in general.

Materials and Methods

Animals

Wistar rats weighing 160–220g were used for the studies. Purina rat chow and water were allowed *ad libitum* until the time of liver perfusion.

Materials

Media, including Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution, Ham's F10 and Waymouth's MB 752/1, were obtained in concentrated or powdered form from the Grand Island Biological Co. (Grand Island, N.Y., U.S.A.) and diluted with distilled water to an appropriate volume. Collagenase, derived from *Clostridium histolyticum* (type I) and hyaluronidase, derived from bovine testis, were

obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and used without further chemical purification. The collagenase was found to contain bacteria and all media solutions were passed through a 0.20 μm Nalgene filter before use. L-[G-^{3}H]Valine (specific radioactivity 2–10 Ci/mmol) was obtained from the International Chemical and Nuclear Corporation, Irvine, Calif., U.S.A.

Table 1. *Composition of media used*

For perfusion of liver:

Modified Hanks' balanced-salt solution

Component	Concentration (mg/l)	Component	Concentration (mg/l)
NaCl	8000	Glucose	1000
KCl	400	$NaHCO_3$	2100
$Na_2HPO_4,2H_2O$	60	Phenol Red	10
KH_2PO_4	60		

For incubation of hepatocytes:

(i) Modified Waymouth's medium MB 752/1

Component	Concentration (mg/l)	Component	Concentration (mg/l)
NaCl	6000	L-Arginine hydrochloride	75
KCl	150	L-Cystine	15
Na_2HPO_4	300	L-Histidine	128
$CaCl_2,2H_2O$	120	L-Leucine	50
$MgCl_2,6H_2O$	240	L-Lysine hydrochloride	240
$MgSO_4,7H_2O$	200	L-Methionine	50
Glucose	5000	L-Cysteine hydrochloride	90
KH_2PO_4	80	L-Phenylalanine	50
L-Isoleucine	25	Folic acid	0.40
L-Threonine	75	Choline hydrochloride	250
L-Tryptophan	40	Nicotinamide	1
L-Tyrosine	40	Calcium pantothenate	1
L-Valine	65	Pyridoxine hydrochloride	1
L-Glutamic acid	150	Thiamine hydrochloride	10
L-Aspartic acid	60	Riboflavin	1
Glycine	50	Ascorbic acid	17.50
L-Proline	50	Isoinositol	0.90
L-Glutathione	15	Vitamin B_{12}	0.20
L-Hypoxanthine	25	Phenol Red	10
L-Glutamine	350	$NaHCO_3$	2900
Biotin	0.02		

(ii) Ham's nutrient mixture F10

Component	Concentration (mg/l)	Component	Concentration (mg/l)
NaCl	7400	$MgSO_4,7H_2O$	153
KCl	285	Glucose	1100
$Na_2HPO_4,7H_2O$	290	Sodium pyruvate	110
KH_2PO_4	83	L-Arginine hydrochloride	211
$FeSO_4,7H_2O$	0.83400	L-Histidine hydrochloride	21
$CuSO_4,5H_2O$	0.00249	L-Lysine hydrochloride	29
$ZnSO_4,7H_2O$	0.02880	L-Tryptophan	0.60
$CaCl_2,2H_2O$	44	L-Methionine	4.48
L-Phenylalanine	5	Hypoxanthine	4
L-Tyrosine	1.81	Biotin	0.02400
L-Alanine	9	Folic acid	1.32
Glycine	7.51	Riboflavin	0.37600
L-Serine	10.50	D-Calcium pantothenate	0.71500
L-Threonine	3.57	Choline chloride	0.69800
L-Isoleucine	2.60	Isoinositol	0.54100
L-Leucine	13	Niacinamide	0.61500
L-Proline	11.50	Pyridoxine hydrochloride	0.20600
L-Valine	3.50	Thiamine hydrochloride	1
L-Aspartic acid	13	Thymidine	0.70
L-Glutamic acid	14.70	Vitamin B_{12}	1.36
L-Asparagine	13	Lipoic acid	0.20
L-Glutamine	146	Phenol Red	1.20
L-Cysteine	25	$NaHCO_3$	2400

Preparation of isolated hepatocytes

The method used was a modification of the method of Berry & Friend (1969). The rats were anaesthetized with sodium pentobarbital (60mg/kg) and given 2000 U.S.P. units of sodium heparin solution to prevent coagulation of blood during cannulation. Both these agents were injected intraperitoneally. After the rats were shaved and washed with an aqueous solution (1:750) of benzalkonium chloride all procedures, until the final harvesting of hepatocytes, were carried out in a laminar-flow hood (Microvoid IIC, Air Control Inc., Norristown, Pa., U.S.A.) in a room at 37 ± 1°C. Instruments, previously sterilized by boiling for 30min, were used and the abdomen was opened and the inferior vena cava tied above the renal veins. The portal vein was cannulated with an 18-gauge Argyle Medicut intravenous plastic cannula (Aloe Medical, St. Louis, Mo., U.S.A.). The liver was cleared of blood by washing through with up to 300ml of a Ca^{2+}- and Mg^{2+}-free modified Hanks' balanced salt solution containing 25mmol of $NaHCO_3$/litre (Table 1). The solution was kept at 39°C and oxygenated by bubbling through a mixture of $O_2 + CO_2$ (95:5). The solution was pumped through the liver with a Harvard peristaltic pump (model 1215, Harvard Apparatus, Millis, Mass., U.S.A.) at the rate of 10ml/min and the perfusate was drained away by opening the right atrium and sucking out and discarding the effluent with a Pasteur pipette. After the liver was washed free from blood another 18-gauge Argyle plastic cannula was inserted into the inferior vena cava through the right atrium to the level of the hepatic veins and tied in place. Then the liver was perfused with 80ml of fresh solution of the above composition to which collagenase (0.05%) and hyaluronidase (0.1%) were added. These enzymes were conserved by recycling the effluent to a reservoir that was immersed in a water bath which kept the solution at 39°C. Oxygenation was again achieved by bubbling $O_2 + CO_2$ (95:5) through the enzyme solution. The rate of perfusion was about 10ml/min. Perfusion of all lobes was ensured by periodically momentarily occluding the effluent tubing connected to the plastic cannula inserted through the right atrium. After about 20min of perfusion the liver was swollen with blebs on the surface, and oozing of solutions from its surface was seen. At this point the liver was cut away from the abdomen with scissors and forceps. The liver was placed on a Petri dish and snipped with scissors into about ten pieces. The pieces were transferred to a 250ml beaker containing 100ml of the same enzyme–salt solution with which the liver had been perfused, to which was added bovine serum albumin (Sigma cat. no. A-5128) to a final concentration of 1% (w/v) in the enzyme–salt solution. The beaker was kept at 39°C in a water bath and the contents of the beaker were agitated by swirling and continuous vigorous bubbling with $O_2 + CO_2$ (95:5) by using a hand-held Pasteur pipette to deliver the gas mixture. After about 20min of the above treatment to release cells from the liver pieces, the contents of the beaker were strained through four layers of surgical cotton gauze (placed in a funnel) into a 1 litre beaker. The liver pieces were gently stirred with a glass rod and a few drops of the Hanks' solution were added to wash through loose cells. This procedure allowed cells and debris to pass through but held back pieces of liver and strands of tissue. Further procedures were carried out at about 21°C.

The cell suspension was then left for 10 ± 1min to permit cells to sediment by gravity alone. The supernatant medium was carefully sucked off leaving the cells in 3–4ml of solution. Fresh incubating medium (450ml) containing 50μg of gentamicin/ml was added to the sedimented cells. The cells were not washed or centrifuged as described by Berry & Friend (1969) since we found that centrifugation increased the number of cells stained by Trypan Blue. When the sedimented cells and supernatant were examined after addition of Trypan Blue, we found that 99% of all unstained hepatocytes had sedimented and that the supernatant contained much of the debris, almost all the non-hepatocytic cells and 95% of the total stained hepatocytes. Since the sedimented cells were in a volume of 3–4ml, from an original volume of 100–150ml, less than 4% of the suspended debris and enzymes was carried over owing to this lack of washing, and the concentration of these contaminants in the final working suspension, owing to dilution by the incubating medium, was less than 0.04%. Hence the above method permitted harvesting of cells without centrifugation or much manipulation both of which we had found to result in subsequently decreased survival. All these above manipulations up to the final harvesting were carried out at 37°C to minimize K^+ loss. In line with our findings, Quistorff *et al.* (1973), have noted loss of K^+ and adenylate during centrifugation as well as the importance of a close-to-physiological rate of perfusion.

Incubation of cells

Maintenance of asepsis. The initial cleaning of the animal with benzalkonium chloride after shaving, sterilization of all instruments, perfusion of vessels and tubing (made of Tygon which withstands autoclaving) were undertaken to minimize bacterial contamination. All solutions were sterilized by being filtered through a 0.20μm Nalgene filter unit. Incubation vessels were cleansed with chromic acid and a laminar-flow hood was used as described above. Finally gentamicin (final concentration 50μg/ml of

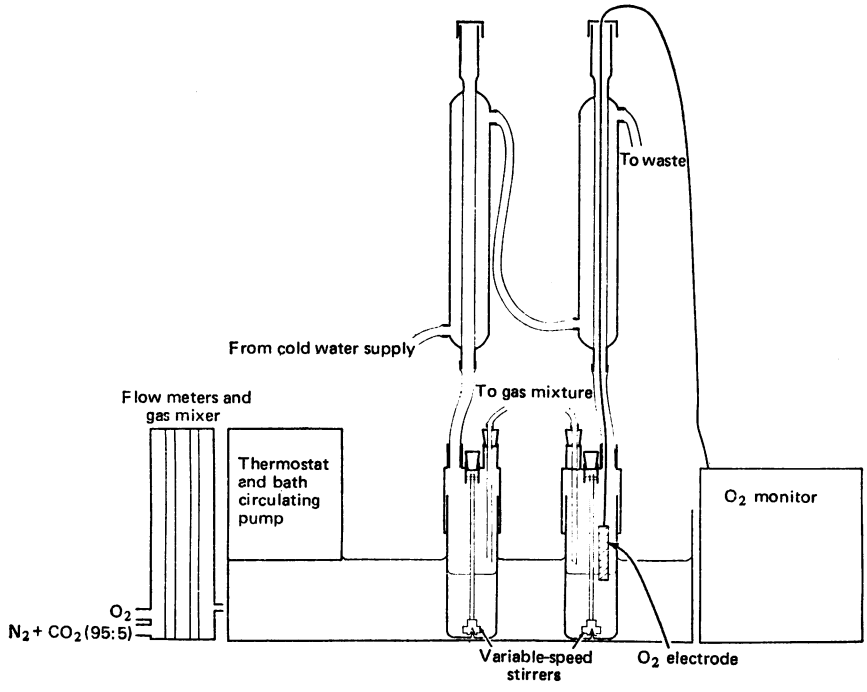


Fig. 1. *System for incubation of hepatocyte suspensions in spinner flasks in a water bath*

The arrangements for return of condensate and monitoring of oxygen tension (and of pH similarly) are shown. Sampling is carried out through the gassing ports.

medium) was added to prevent growth of bacteria during incubation. Preliminary experiments showed no effect of gentamicin (at this concentration) on protein synthesis, urea synthesis or ATP concentrations in the suspensions. By using this system we have maintained the suspension free from bacteria for up to 48h of incubation.

Media for incubation. For optimum results the cells were incubated in Waymouth's MB 752/1 medium (Waymouth, 1959) (Table 1) containing in addition, to a final concentration of 17.5% (v/v), horse serum that had been inactivated by heating at 56°C for 30min. The proportion of cells to medium was adjusted to between 9 and 15mg/ml of medium, corresponding to about 1000–2000 cells/mm^3 of medium. In some experiments Ham's F10 medium (Ham, 1963) (Table 1) was used. This last medium also contained the 17.5% (v/v) heat-inactivated horse serum. Heparin was added to both media to give a final concentration of 7 units of heparin/ml to prevent fibrinogen from clotting.

Technique of incubation. The cells, suspended in the media, were placed in special Pyrex spinner flasks of 250ml capacity (Johns Scientific, Toronto, Ont., Canada) (Fig. 1), fitted with magnetically stirred Teflon spinners and reflux condensers. The O_2 tension was carefully monitored with an O_2 electrode (Radiometer, Copenhagen, Denmark) and kept at between 18.7×10^3 and 26.7×10^3 Pa. The O_2 tension was controlled by varying the O_2/N_2 ratio in the gasses passed into the flasks. This was done by mixing two gasses, O_2 and $N_2 + CO_2$ (95:5) in a proportion suitable to provide the desired O_2 tension. [Empirically it had been found that better pH control was achieved, under our conditions of use, when the $N_2 + CO_2$ (95:5) was mixed with O_2 alone rather than with the $O_2 + CO_2$ (95:5) that might have been expected theoretically.] Preliminary experiments showed that the O_2 tension was relevant in determining the rates of synthesis of albumin and fibrinogen, and further, it was observed that with time the O_2 needs of the cells decreased, so that for a given O_2 flow rate the O_2 tension in the medium was observed to rise unless the flow rate of the gasses was adjusted. Since reproducible results required moderately uniform O_2 tension it was necessary to monitor and adjust the ratio of O_2/N_2 at 3h intervals for the first 12h and again after 24h of incubation. Similarly the pH value was repeatedly monitored at the same intervals and maintained between 7.3 and 7.4.

Sampling

Two 3ml samples were taken at intervals. A sample (0.1ml) was removed from one for cell counting, the remainder being slowly frozen (to −20°C over the space of an hour or two) until homogenization after which it was centrifuged to remove insoluble cell debris. Measurements for synthesis were made on portions of culture that had been slowly frozen and thawed (in a 37°C incubator). The homogenization was carried out in a suitably sized glass mortar with a Teflon pestle (cat. no. 3431-E45-F15, Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.) inserted about six times at about 500rev./min.

The other portion was centrifuged, the supernatant separated and the drained pellet weighed. At the end of the experiment only one 3ml sample of the suspension was taken for homogenization, and the remaining volume measured. This remaining suspension was centrifuged and the hepatocyte pellet drained and weighed. All samples were frozen at −20°C until required for analysis. For ATP determinations separate samples of 1ml were taken during the course of the experiments, immediately frozen in liquid N_2 and stored below −30°C until the measurements were made.

Determination of staining with Trypan Blue

A sample (0.1ml) of cell suspension was mixed with 0.1ml of 0.134% Trypan Blue in 0.9% NaCl and counted in a Neubauer chamber. Four 1 mm^2 areas were counted.

Measurement of urea

A micro Conway method was used (Obrink, 1955).

Synthesis of albumin, fibrinogen and transferrin

Albumin, fibrinogen and transferrin were measured in homogenized samples by the solid-phase radioimmunoassay method, with antibody-coated tubes, of Askenase & Leonard (1970). The albumin and fibrinogen antigens were prepared by the method of Ho *et al.* (1974) and checked for purity by immunoelectrophoresis against polyvalent anti-(rat plasma protein) antiserum. Antigens were labelled with ^{125}I by the choramine-T method (Hunter & Greenwood, 1962). Antibodies were prepared in rabbits with these pure antigens by subcutaneously injecting 10mg of antigen with 0.5ml of complete Freunds adjuvant three times at intervals of 1 week. The animals were bled 1 week after the last injection and γ-globulin was separated from the serum by the method of Askenase & Leonard (1970). The specificity of the antibody was established by showing that there was no cross-reaction against horse serum and also by showing that there was no cross-displacement of ^{125}I-labelled antigen from specific antibody in the radioimmunoassay by a non-specific rat plasma protein, e.g. transferrin, and also that fibrinogen did not displace ^{125}I-labelled albumin from its specific antibody. The calibration curve contained medium and the relevant heterologous serum in the same proportion as the test samples. The precision of immunoassay, assessed as described by Rodbard *et al.* (1968), gave a coefficient of variation of 2.0, 8.1 and 8.0% for albumin, fibrinogen and transferrin respectively. Rat transferrin and antibody were obtained from Dr. A. S. Tavill (Clinical Research Centre, Northwick Park, London, U.K.).

The net increase in the amount of albumin, fibrinogen and transferrin in the homogenized samples over the value observed at the start of the culture was taken as synthesis. Thus these values are felt to correspond to synthesis rather than simple release, because of representing both intra- and extra-cellular albumin, fibrinogen and transferrin.

Incorporation of L-[G-^3H]valine into protein

The method used was that described by Mans & Novelli (1960). Suitable volumes of supernatants from homogenates were dried on to discs of filter paper and the protein was precipitated by placing each discs for a period of 1.5h in 3ml of 10% (w/v) trichloroacetic acid containing carrier valine (0.1M). The discs were washed with 5% trichloroacetic acid and then boiled in 5% trichloroacetic acid for 15min. They were treated with methanol–ether (1:1, v/v) at 37°C for 15min, washed with diethyl ether and air-dried. The discs were then placed in 1ml of NCS (Amersham–Searle, Don Mills, Ont., Canada) in glass counting vials and left for 24h at room temperature to dissolve the precipitated protein in the NCS. Scintillation fluid was added and vials were counted for radioactivity in a liquid-scintillation counter (Mark I, Nuclear–Chicago, Des Plaines, Ill., U.S.A.). [The composition of the scintillation fluid was 2,5-diphenyloxazole (scintillation grade), 5g, 1,4-bis-(5-phenyloxazol-2-yl)benzene (scintillation grade), 0.3g, both from Nuclear–Chicago, and 1 litre of toluene (certified A.C.S.) from Fisher Scientific Co., Toronto, Ont., Canada.] Since acidified methanol may dissolve albumin, control experiments were done with ^{125}I-labelled albumin and with ^3H-labelled hepatocyte protein to show that radioactivity was not lost by the methanol or ether treatment in this procedure.

RNA and DNA determinations

These were measured in the pellets obtained at the end of the experiment by the method of Maggio *et al.* (1963).

ATP determination

This was done by the method of Lowry & Passoneau (1972).

Simultaneous measurement of intracellular and extracellular amino acid concentration

In three experiments after 24h of incubation a 40ml portion of the suspension was centrifuged at 5000*g* for 10min in a Sorval refrigerated centrifuge at 4°C. The supernatant was pipetted off, the cell pellet drained of any supernatant fluid and the net weight noted. An equal volume of cold 20% (w/v) trichloroacetic acid was added to both pellet and supernatant and the pellet homogenized in a Potter–Elvehjem-type homogenizer. Both the trichloroacetic acid-treated pellet and the treated supernatant were then centrifuged to separate the protein precipitate and the trichloroacetic acid extract containing the amino acids. The protein precipitate was extracted twice more with 10% (w/v) trichloroacetic acid and the combined extracts were treated thrice with an equal volume of diethyl ether to remove the trichloroacetic acid. The acid-free extracts were freeze-dried, reconstituted with lithium citrate buffer pH 2.2 and analysed in an automatic amino acid analyser (Anderson *et al.*, 1974). No attempt was made to compensate for dilution of intracellular amino acid by trapped medium in the pellet.

Light and electron microscopy

Cell suspension (1ml) was added to 5ml of 3% (v/v) glutaraldehyde at 4°C temperature, left to fix for 15min, centrifuged to form a pellet and rinsed in buffer and post-fixed for 1h at 4°C in 1% (w/v) OsO_4 buffered with 0.1M-sodium phosphate buffer (pH 7.2). The blocks were dehydrated in a graded series of ethanol solutions, transferred to propylene oxide and then to a mixture of propylene oxide and resin. The embedding medium was prepared as described by Mollenhauer (1964). Sections from the plastic-embedded tissue were cut with glass knives by using Porter Blum or LKB microtomes. Sections 1.0μm thick were stained with Toluidine Blue (Trump *et al.*, 1961) and examined by light microscopy. Ultrathin sections of grey interference colour were picked up on uncoated grids and stained at room temperature with Reynolds' solution of lead citrate for 10min (Reynolds, 1963). The sections were examined in a Philips 200 electron microscope.

Effect of hormones

The cell suspensions in Waymouth's medium containing 17.5% (v/v) heat-inactivated horse serum were divided into three equal volumes of 150ml and placed in spinner flasks. The cell suspensions were incubated for 6h without any additions. This was done to show that albumin, fibrinogen and transferrin synthesis were initially comparable in the three suspensions. At 6h of incubation a mixture of hormones was added to give a concentration of hormone in the medium as follows. Cortisol (0.25μg/ml), glucagon (0.75ng/ml), tri-iodothyronine (0.09μg/ml) and bovine growth hormone (0.5μg/ml) were added to one flask, the above mixture plus insulin to give 20 U.S.P. μunits/ml was added to a second flask and the third flask was a control with only the equivalent diluent added. The hormone mixture in the same quantity was again added to the appropriate flasks at 12, 18 and 24h of incubation. The results were statistically evaluated by testing pair differences between control and hormone-treated flasks, using the Wilcoxon's Rank test for pair differences (Wilcoxon, 1945; Documenta Geigy, 1962).

Results

The evolution of the method given above required preliminary experiments to determine, first, the effect of O_2 tension, secondly, the effect of supplementation with different sera and, thirdly, the effect of two different modes of agitating hepatocytes in the medium. Shaking cell suspensions in a water bath was compared with stirring in spinner flasks. The preliminary experiments were all performed with Ham's F10 medium and except for the spinner-flask studies the cell suspensions were studied by using paired suspensions agitated in a shaking water bath. Paired observations were used to compare two variables with each other so that differences between hepatocyte batches would not cause artifacts in the results.

Preliminary experiments

Effect of O_2 tension. The cells were suspended in Ham's F10 medium in paired 250ml polycarbonate Erlenmeyer flasks shaken in a water bath. The flasks were gassed to give an O_2 tension in the medium of about 45.3×10^3Pa in one, and of about 2.7×10^3Pa in the other. The composite results of the studies in paired hepatocyte suspensions are given in Fig. 2.

The results show that there is greater albumin synthesis with a higher O_2 tension. The incorporation of [^{3}H]valine into hepatic protein was also significantly better ($0.02 > P > 0.05$) in cells gassed to a higher O_2 tension. The incorporation [mean $\pm$ S.D. (*n*)] was $3.92 \times 10^6 \pm 1.05 \times 10^6$ (4) and $2.62 \times 10^6 \pm 1.05 \times 10^6$ (4) d.p.m./4h per g of hepatocytes for cells gassed at an O_2 tension of 45.3×10^3 and 2.7×10^3 Pa respectively. In contrast the permeability of the hepatocyte membrane to Trypan Blue was

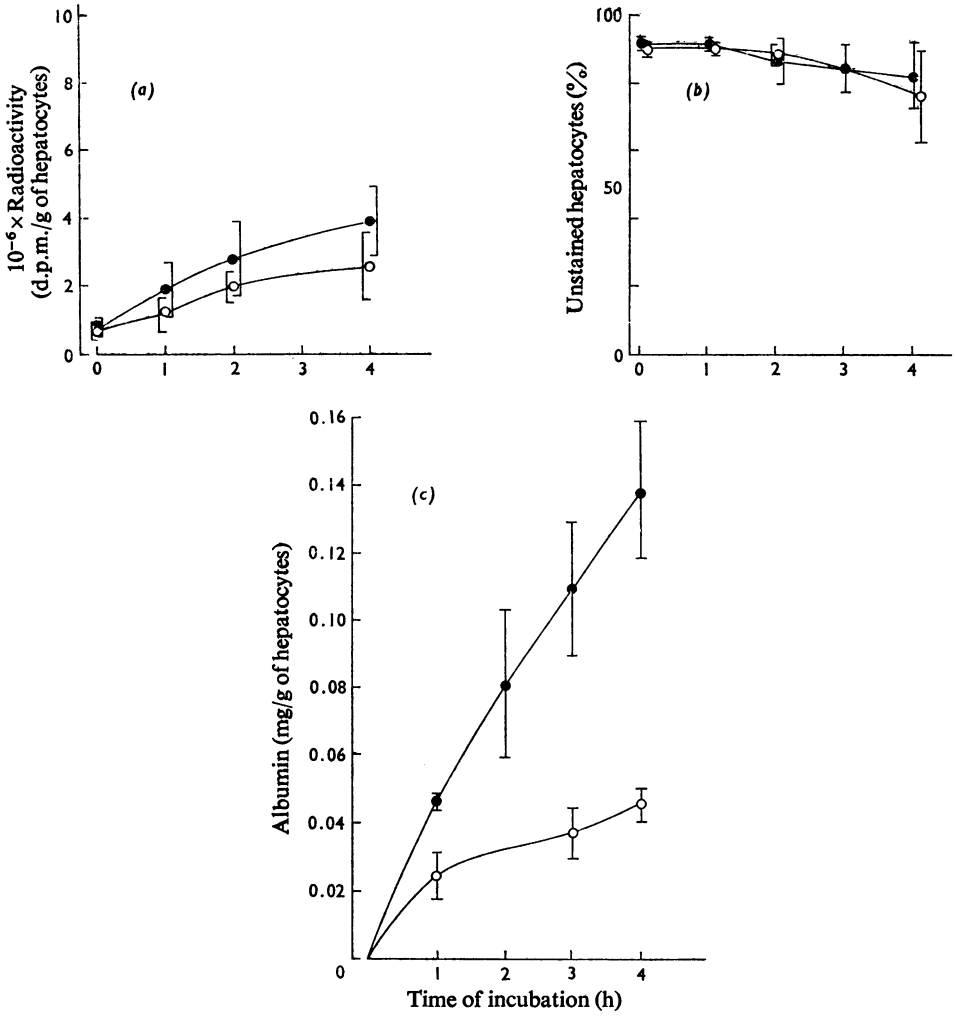


Fig. 2. *Initial demonstration in early studies of effect of low* (2.7 *Pa*) (○) *and high* (45.3 *Pa*) (●) *oxygen tension throughout the incubation on* (*a*) [^{3}*H*]*valine incorporation into total hepatocyte protein,* (*b*) *percentage of unstained cells and* (*c*) *synthesis of albumin*

For details see the text. Bars denote ±S.D. for three experiments.

not significantly different in the two groups after 4h of incubation. The proportions of cells present that were unstained at that time were 82.9 ± 9.9% and 77.0 ± 14.4% for cells gassed at O_2 tensions of 45.3×10^3 and 2.7×10^3 Pa respectively. These findings were corroborated in the optimal system by using spinner flasks and serum supplementation as shown in Fig. 3.

Effect of serum supplementation. With no serum supplementation more than 90% of the cells became stained with Trypan Blue after 6h of incubation. On supplementation with 17.5% (v/v) foetal calf serum, all cells were stained after 12h of incubation and incorporation of [^{3}H]valine into total hepatocyte protein ceased after 4–6h of incubation. In contrast supplementation with 17.5% (v/v) rat serum or heat-inactivated horse serum resulted in 50% of cells remaining unstained after 12h of incubation and incorporation of [^{3}H]valine continuing up to 12h of incubation. A representative experiment in which cells were incubated in paired Erlenmeyer flasks in Ham's F10 medium, where one flask contained 17.5% (v/v) foetal calf serum and the other 17.5% (v/v) horse serum, is illustrated in Fig. 4. Both flasks were gassed with O_2 to maintain an O_2 tension of about 45.3×10^3 Pa in the medium.

Comparison of mode of agitation o, hepatocyte suspension (shaking versus stirring). By using the same medium (Ham's F10) with 17.5% (v/v) heat-inactivated horse serum, results from nine suspensions agitated by shaking in polycarbonate Erlenmeyer flasks were compared with those of eight stirred in Pyrex spinner flasks. Up until 6h of study the two methods gave comparable results, but from 6 to 12h incubation the stirring gave superior results. The proportion of cells left unstained with Trypan Blue after 12h of incubation was significantly lower ($P < 0.01$) with shaking [being $44.0 \pm 12.7\%$ (9), mean ± S.D. (*n*)] than with stirring [$76.6 \pm 9.1\%$ (8)]. Similarly incorporation of [^{3}H]valine into total hepatocyte protein after 12h of incubation was significantly lower ($P < 0.01$) in the shaken suspensions, being $25.58 \times 10^6 \pm 7.06 \times 10^6$ (9) and $46.34 \times 10^6 \pm 8.46 \times 10^6$ (8) [mean ± S.D. (*n*)] d.p.m./g of hepatocytes with shaking and stirring techniques respectively. Albumin synthesis however was 0.13 ± 0.026(9) and 0.21 ± 0.12(8) mg/h per g of hepatocytes for shaken and stirred cells respectively. Although numerically the mean was greater with stirred cells, the difference was not statistically significant.

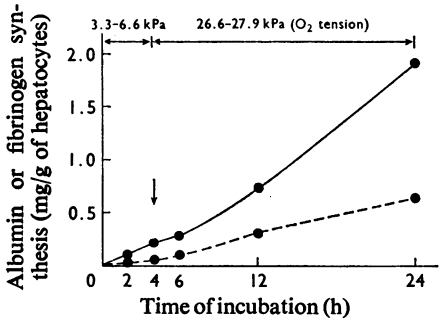


Fig. 3. *Effect of increasing the oxygen tension on the synthesis of albumin* (——) *and of fibrinogen* (– – – –) *in the optimal system of Fig.* 1 *by using Waymouth's medium MB* 752/1

Note response lag of about 2h. O_2 tension was increased at the point marked by the arrow.

Functional studies with hepatocyte suspensions by using the optimum technique

Recovery of cells. This was tested by comparing the weight of cells obtained with the weight of the liver, assuming the latter to be 4.1% of body weight. The latter percentage had been found to be valid in separate studies. We obtained values between 32 and 68, with a mean of 50% of the initial liver weight as hepatocytes, amounting to between 32×10^6 and 68×10^6 cells/g of liver.

Trypan Blue staining. Membrane integrity of the cell was judged in part by Trypan Blue staining. Fig. 5 shows that 91.9 ± 3.1 (14) and $76.6 \pm 9.1\%$ (14) [mean ± S.D. (*n*)] cells were unstained after 6 and 12h of incubation. The finding that, even after 24h of incubation, $77.9 \pm 4.1\%$ of the cells present were unstained was noteworthy. However, at that time only four cultures out of 16 could be evaluated since in the remainder there were well-developed reaggregations of cells into clumps, making cell counting difficult (see the sections on light and electron microscopy).

Light microscopy. At the beginning of each experiment the cell suspension examined showed large numbers of hepatocytes. The vast majority of cells occurred singly but occasionally they occurred in pairs. These hepatocytes were round with central nuclei, prominent nucleoli and abundant cytoplasm. A small number of cells appeared vacuolated or pale, the remainder had well-stained cytoplasm (Plate 1*a*). Other types of cells (biliary cells, fibroblasts and endothelial cells) were not seen and cell debris was

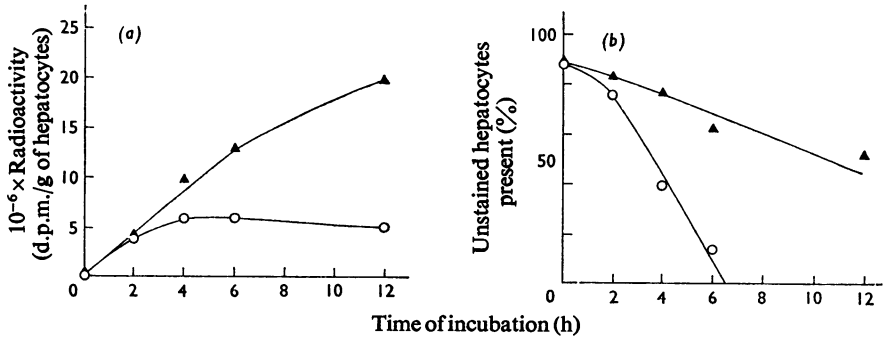
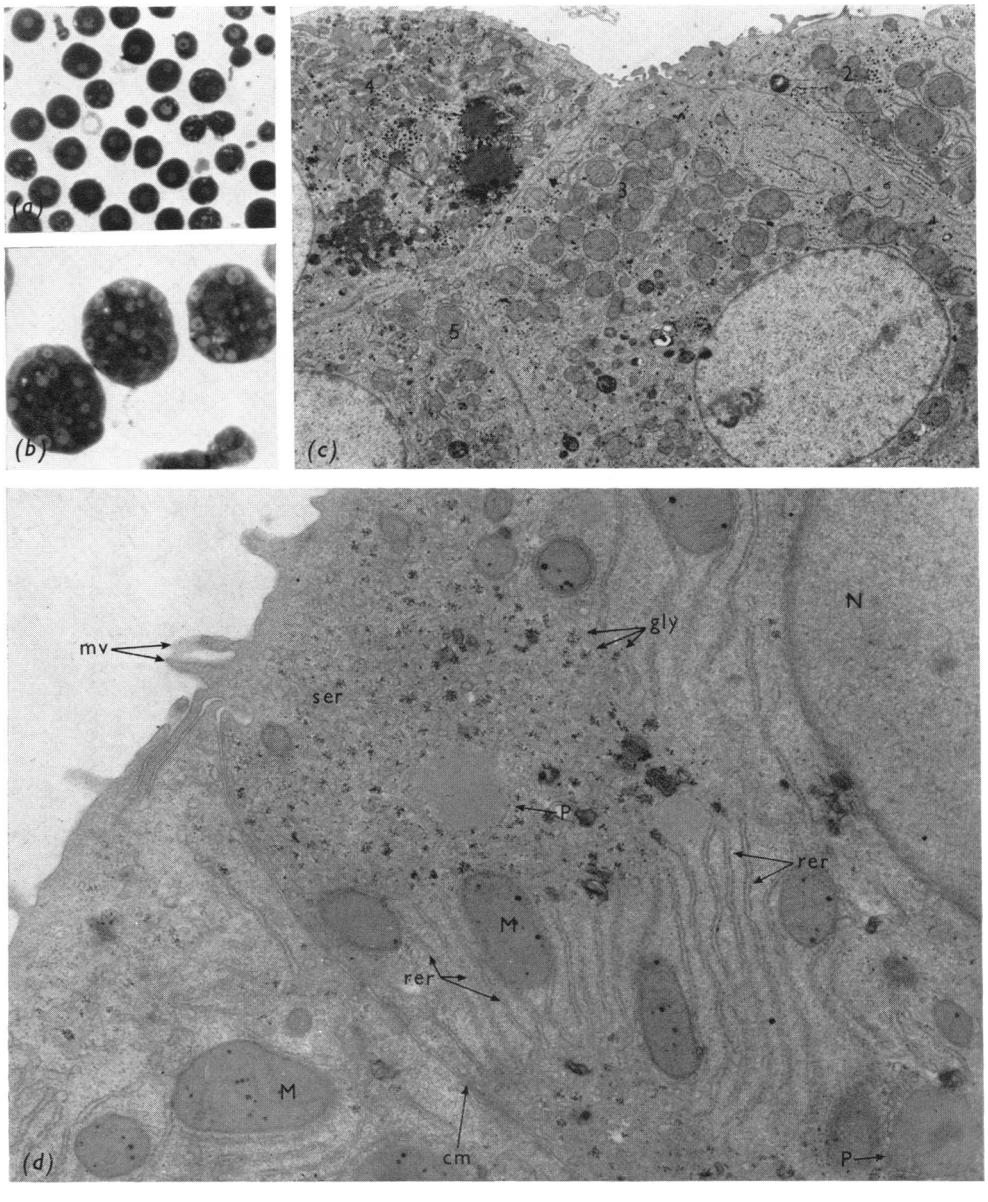


Fig. 4. *Representative effect of* 17.5% (*v*/*v*) *serum supplementation*

In this case heat-inactivated horse serum (▲) is compared with foetal calf serum (○), with regard to its effect on (*a*) the incorporation of [^{3}H]valine into total hepatocyte protein and on (*b*) the percentage of cells unstained by Trypan Blue.



EXPLANATION OF PLATE 1

Morphology of isolated liver cells

(*a*) Hepatocytes 1 h after isolation. Note the liver cells are round and have vesicular nuclei, prominent nucleoli and abundant cytoplasm. A 1 μm section stained with Toluidine Blue, magnification ×170. (*b*) Hepatocytes 24 h after isolation. The liver cells are now disposed in multicellular spheroidal aggregates. A 1 μm section stained with Toluidine Blue, magnification ×100. (*c*) Electron micrograph of hepatocytes 24 h after isolation showing part of five liver cells from a multicellular aggregate. Note the excellent preservation of liver cell structure. Stained with lead citrate, magnification ×3500. (*d*) Higher magnification of liver cells from a multicellular aggregate. Cisternae of rough endoplasmic reticulum (rer) are arranged in stacks; glycogen rosettes (gly) are disposed between the membranes of the smooth endoplasmic reticulum (ser). Microvilli (mv) are present at the surface plasma membrane. N, nucleus; M, mitochondrion; P, peroxisome; cm, cell membrane. Stained with lead citrate, magnification ×18 200.

(*Facing p.* 148)

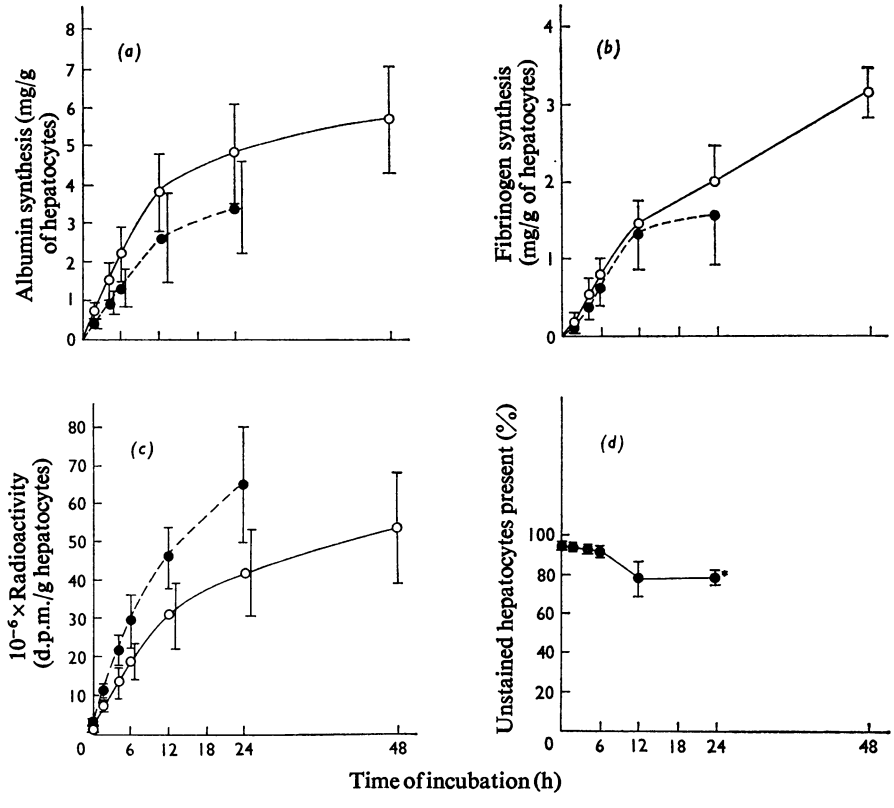


Fig. 5. *Composite plots of all control incubations save those of experiments dealing with hormone effects*

The results obtained with Ham's F10 (- - - -) are compared with those obtained with Waymouth's MB 752/1 (——) media, for (*a*) albumin synthesis, (*b*) fibrinogen synthesis, (*c*) [^{3}H]valine incorporation into total hepatocyte protein and (*d*) percentage of unstained cells.

* Result formed on the basis of only four of 16 suspensions (see the text for details).

minimal. After 12h of incubation intact cells were numerous and stained normally. At 24 and 48h most of the hepatocytes were aggregated (Plate 1*b*) in groups of 10–50 or more cells; in serial thin sections the clumps appeared circular or ovoid and solid suggesting that they were spheroidal cell masses.

Electron microscopy. The results shown were obtained by random examination of multiple fields of hepatocytes obtained from several different studies to avoid bias. The large round cells observed by light microscopy were easily identified as hepatocytes by the general cytological organization and the abundance of cytoplasmic organelles including stacks of rough endoplasmic reticular cisternae, glycogen rosettes, peroxisomes and bile canaliculi. All the cells examined were hepatocytes; no other cell types were present in any of the grids examined.

At 12, 24 and 48h, intact viable hepatocytes were abundant. These cells were still easily identifiable as hepatic parenchymal cells with normal or nearly normal fine structure. Mitochondria were numerous and normally disposed in the cytoplasm. Stacks of rough endoplasmic reticulum cisternae were notable as were the Golgi apparatus, smooth endoplasmic reticulum, peroxisomes, lysosomes and other normal cytoplasmic structures (Plates 1*c* and 1*d*). Lipid droplets were small. Cytolysosomes were found in some cells. Glycogen content was variable.

At 24 and 48h, the clumps of liver cells were composed of highly differentiated hepatocytes similar to those observed at 12h. They differed only in their arrangement. The cell membranes between adjacent hepatocytes were separated by the usual 10nm (100Å) intercellular space. Bile canaliculi and junctional complexes were also noted.

Albumin synthesis (*Fig.* 5). This was almost linear up to 12h and then continued at a lesser rate to 48h. The synthesis rate over the first 12h period was 0.22 ± 0.10 (7) [mean ± S.D. (*n*)] mg/h per g of hepatocytes for cells suspended in Ham's F10 medium and

Table 2. *Effect of hormones on the synthesis of albumin, fibrinogen and transferrin*

The previous control values (shown for comparison with the present control values) are from the numerical data displayed in part in Fig. 5 (under synthesis of the corresponding plasma protein) and include all incubations using Waymouth's medium MB 752/1 before these experiments on hormone effects, i.e. those detailed in this Table. (*n*) is the number of determinations on which a given mean is based. a, b and c represent separate vessels containing portions of the same suspension incubated concurrently in any given experiment. Numbers refer to the same given experiment. Hormone effect is determined by comparing mixture c (control) with mixture a (hormone mixture without insulin) or mixture b (hormone mixture with insulin) in an experiment bearing the same number.

	Albumin, fibrinogen or transferrin synthesis (mg/g of hepatocytes)				
Time of incubation (h) ...	6	12	18	24	48
Expt. A, albumin synthesis					
(i) Control					
1c	2.81	5.36	—	6.59	8.20
2c	1.76	2.61	3.20	3.20	—
3c	1.78	3.03	3.58	4.92	5.00
4c	4.12	5.60	5.92	6.47	—
5c	1.49	2.24	2.70	2.89	3.70
6c	2.70	3.87	5.58	5.94	5.90
Mean ± S.D. (*n*)	2.44 ± 0.98 (6)	3.79 ± 1.42 (6)	4.20 ± 1.46 (5)	5.00 ± 1.63 (6)	5.70 ± 1.90 (4)
Previous control values	2.21 ± 0.69 (11)	3.79 ± 1.12 (10)		4.80 ± 1.35 (10)	5.67 ± 1.39 (3)
(ii) Hormone mixture including insulin					
1b	3.48	7.33	—	9.35	9.90
2b	0.94	1.70	2.33	2.41	—
3b	2.29	4.37	6.37	6.17	7.80
4b	3.71	5.34	6.94	7.37	—
5b	1.70	2.50	4.30	4.30	4.80
6b	2.78	3.90	5.55	5.90	7.30
Mean ± S.D. (*n*)	2.48 ± 1.06 (6)	4.19 ± 2.02 (6)	5.10 ± 1.84 (5)	5.92 ± 2.40 (6)	7.45 ± 2.10 (4)
(iii) Hormone mixture without insulin					
1a	3.97	6.25	—	6.69	6.20
2a	2.13	3.26	3.73	3.89	—
3a	2.12	3.67	5.30	5.30	7.20
4a	3.25	5.13	5.79	6.11	—
5a	0.90	1.80	2.52	2.94	3.60
6a	3.90	4.12	5.18	5.72	6.06
Mean ± S.D. (*n*)	2.58 ± 1.08 (6)	4.04 ± 1.54 (6)	4.50 ± 1.35 (5)	5.11 ± 1.42 (6)	5.77 ± 1.53 (4)
Expt. B, fibrinogen synthesis					
(i) Control					
1c	0.63	1.87	—	2.74	3.27
2c	0.32	0.65	0.76	0.85	—
3c	0.52	0.98	1.41	1.82	2.39
4c	0.60	1.55	1.84	2.90	—
5c	0.32	0.38	0.69	0.83	1.56
6c	0.18	0.72	0.99	0.89	2.52
Mean ± S.D. (*n*)	0.43 ± 0.18 (6)	1.03 ± 0.57 (6)	1.14 ± 0.48 (5)	1.84 ± 0.48 (6)	2.44 ± 0.70 (4)
Previous control values	0.81 ± 0.23 (11)	1.43 ± 0.36 (11)		1.99 ± 0.58 (9)	3.31 ± 0.30 (3)
(ii) Hormone mixture including insulin					
1b	0.57	1.78	—	2.96	3.84
2b	0.15	0.40	0.73	1.38	—
3b	0.48	1.46	3.83	5.33	6.34
4b	0.30	0.71	1.99	2.83	—
5b	0.48	0.92	1.86	3.15	4.36
6b	0.14	1.52	3.55	3.82	6.96
Mean ± S.D. (*n*)	0.35 ± 0.18 (6)	1.13 ± 0.54 (6)	2.39 ± 1.29 (5)	3.25 ± 1.30 (6)	5.38 ± 1.51 (4)
(iii) Hormone mixture without insulin					
1a	0 61	1.92	—	3.30	4.68
2a	0.29	0.68	1.33	1.71	—
3a	0.68	1.22	3.34	5.08	7.64
4a	0.31	0.67	2.01	3.06	—
5a	0.37	0.55	1.27	2.38	3.93
6a	0.35	1.07	3.52	4.55	7.83
Mean ± S.D. (*n*)	0.44 ± 0.17 (6)	1.18 ± 0.51 (6)	2.06 ± 1.57 (5)	3.35 ± 1.28 (6)	6.02 ±2.01 (4)

Table 2.—Continued

Time of incubation (h) ...	Albumin, fibrinogen or transferrin synthesis (mg/g of hepatocytes) 6	12	18	24	48
Expt. C, transferrin synthesis					
(i) Control					
1c	0.76	1.48	—	2.63	3.93
3c	0.45	0.84	1.02	1.29	1.86
4c	0.26	0.61	0.95	1.48	2.30
5c	0.59	0.86	1.30	1.51	2.30
Mean ± S.D. (*n*)	0.52 ± 0.20 (4)	0.95 ± 0.37 (4)	1.09 ± 0.18 (3)	1.72 ± 0.61 (4)	2.60 ± 0.91 (4)
Previous control values	0.66 ± 0.15 (11)	1.16 ± 0.21 (10)		1.56 ± 0.41 (10)	2.33 ± 0.79 (3)
(ii) Hormone mixture including insulin					
1b	0.88	1.64	—	2.10	4.25
3b	0.41	0.95	1.33	1.59	3.84
4b	0.63	0.86	1.25	2.04	3.40
5b	0.66	0.96	1.41	1.81	3.22
Mean ± S.D. (*n*)	0.62 ± 0.16 (4)	1.10 ± 0.36 (4)	1.33 ± 0.08 (3)	1.88 ± 0.22 (4)	3.68 ± 0.45 (4)
(iii) Hormone mixture without insulin					
1a	0.63	1.36	—	2.27	2.85
3a	0.57	1.13	1.17	1.81	2.43
4a	0.31	0.47	0.90	1.20	2.06
5a	0.74	1.06	1.64	1.86	2.64
Mean ± S.D. (*n*)	0.56 ± 0.17 (4)	1.02 ± 0.37 (4)	1.24 ± 0.37 (3)	1.79 ± 0.44 (4)	2.50 ± 0.33 (4)

0.32 ± 0.094 (10) mg/h per g of hepatocytes for cells in Waymouth's medium MB 752/1. The difference continued to increase and was statistically significant ($P < 0.05$) after 24h of incubation.

Fibrinogen synthesis (*Fig.* 5). This was linear up to 12h of incubation and continued at a lower rate up to 48h. In contrast with albumin synthesis there was no difference between cells incubated in Ham's F10 and in Waymouth's MB 752/1 media. The fibrinogen synthesis for the first 12h period was 0.11 ± 0.05 (7) and 0.12 ± 0.03 (11) [mean ± S.D. (*n*)] per g of hepatocytes for Ham's and Waymouth's media respectively.

Transferrin synthesis (*Table* 2). In Waymouth's medium, transferrin synthesis was rapid up to 12h and then continued at a lower rate up to 48h. Transferrin synthesis for the first 12h period was 0.097 ± 0.017 (10) [mean ± S.D. (*n*)] mg/h per g of hepatocytes in the batches of hepatocytes used for the studies displayed in Fig. 5.

Incorporation of [^{3}H]valine into total hepatocyte protein (*Fig.* 5). When the hepatocytes were incubated with Ham's F10 medium this incorporation amounted to $29.3 \times 10^6 \pm 6.69 \times 10^6$ (8) and $46.3 \times 10^6 \pm 8.46 \times 10^6$ (7) [mean ± S.D. (*n*)] d.p.m./h per g of hepatocytes after 6 and 12h incubation respectively. When Waymouth's medium replaced Ham's medium, incorporation of [^{3}H]valine into total hepatocyte protein appeared to be less, but it must be noted that Waymouth's medium contains 18.6 times as much valine as Ham's F10 medium, so that the specific radioactivity of the precursor was diluted in the extracellular fluid by this factor. That the incorporation of [^{3}H]valine was decreased to only $31.15 \times 10^6 \pm 9.07 \times 10^6$ d.p.m./g of hepatocytes after 12h (i.e. a factor of 1.5) indicates that incorporation of valine into liver protein was actually much enhanced during incubation in Waymouth's medium. The extent of enhancement cannot be stated exactly since the precise specific radioactivities of valine intracellularly and in protein were not determined.

Effect of hormones on the synthesis of albumin, fibrinogen and transferrin. The results are set out in Table 2. (i) Reproducibility of results. There was no difference in treatment of the three flasks until after 6h of incubation. Table 2 shows that there was no significant difference between the three flasks up to 6h in the synthesis of albumin, fibrinogen and transferrin. Further, the results of albumin and transferrin synthesis in the controls (Table 2) were similar to those observed in another series (Fig. 5) with Waymouth's medium thus indicating good reproducibility of the results with respect to different batches as a whole for albumin and transferrin, and to triplicate determinations of individual cell suspensions for all three proteins. However, there were differences in fibrinogen synthesis in the two groups. In the studies shown in Fig. 5 synthesis was higher initially and was linear only up to 12h whereas in the second series (Table 2) synthesis was almost linear up to 24h of incubation. (ii) Effect of hormones on fibrinogen synthesis. The addition of a mixture of cortisol, tri-iodothyronine, glucagon and bovine growth hormone with and without insulin resulted in a significant rise in fibrinogen synthesis, first noted between 12 and 18h of incubation (i.e. between 6 and

Table 3. *ATP concentrations in hepatocytes*

Values are means ±S.D. for the number of observations in parentheses.

Duration of incubation (h)	ATP concentration (μmol/g of hepatocytes in given sample)
0	2.95 ± 0.33 (9)
2	3.10 ± 0.26 (9)
4	3.06 ± 0.42 (9)
6	3.04 ± 0.41 (10)
12	3.13 ± 0.45 (14)
24	2.33 ± 0.62 (17)
48	1.72 ± 0.27 (6)

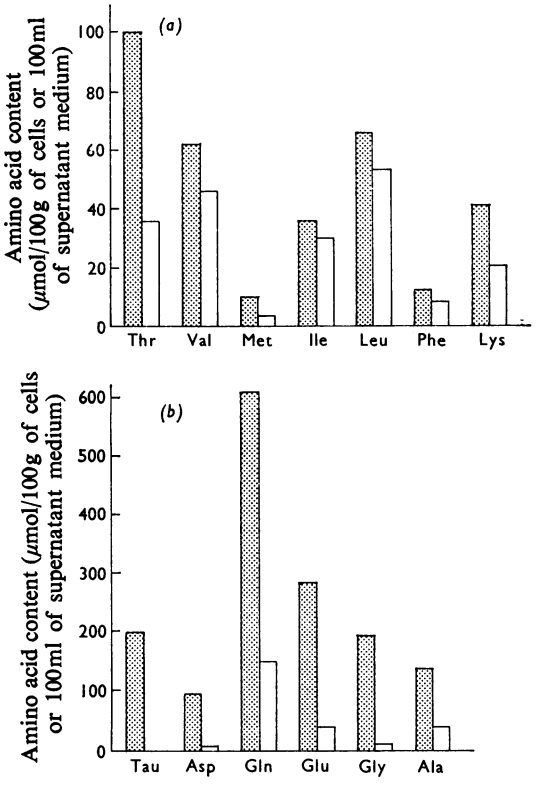


Fig. 6. *Free amino acid content in hepatocyte pellet and medium*

▨, Hepatocytes; □, medium (Ham's F10 and horse serum). (*a*) Essential free amino acids; (*b*) non-essential free amino acids.

12h after first adding the hormones). In hormone-treated suspensions the synthesis of fibrinogen, after 48h of incubation, was more than twice the control value ($P < 0.05$) between 24 and 48h of incubation (18 and 42h after the start of hormone addition).

ATP concentrations. ATP concentrations are given in Table 3. The ATP concentrations whether expressed per weight of cells or in terms of cell numbers are comparable with those obtained *in vivo* (Bloxam, 1971; Bucher *et al.*, 1964). After this 12h, the ATP concentrations gradually fell over the next 36h to about 55% of the peak value.

RNA/DNA ratio. This ratio reflects the RNA concentration per cell. The values were 3.06 ± 0.36 (6) and 3.69 ± 0.88 (8) [mean ± S.D. (*n*)] after 24h of incubation in Ham's F10 and Waymouth's MB 752/1 media respectively.

Concentrations of intra- and extra-cellular amino acids. A representative result is given in Fig. 6. The medium used for incubation in these studies was Ham's medium (Table 1) with 17.5% (v/v) horse serum. These results show that there is a higher concentration of all amino acids in the cell pellet than in the medium. This difference was especially evident for non-essential amino acids. Considering that the cell pellet included some trapped medium the intracellular concentration of free amino acid is likely to have been even greater.

Discussion

With regard to the specific problem of the rates of plasma protein synthesis the use of isolated hepatocyte preparations from normal (non-regenerating) rat liver has hitherto given disappointing results (East *et al.*, 1973; Weigand *et al.*, 1971) or has not been studied (Kaighn & Prince, 1971; Schreiber & Schreiber, 1972; Howard *et al.*, 1973). In the study of East *et al.* (1973) 80% of the cells became stained with Trypan Blue after 4h of incubation. The albumin synthesis in this preparation amounted to 8.5mg/h per 100g of hepatocytes (assuming that 10^6 cells weigh about 10mg, as in our studies) which is only 10% of the value found *in vivo*. Weigand *et al.* (1971) have claimed that the ratio of radioactivity in total protein to that in albumin by using [^{14}C]leucine was comparable with the value found *in vivo*, but incorporation of radioactivity was linear for only 60min and no information about absolute synthesis rates and other parameters of cell structure or membrane integrity at the end of incubation were given. In a review of isolated hepatocyte systems by Schreiber & Schreiber (1973) an attempt was made to derive the optimum conditions for protein synthesis but their studies show linear synthesis only up to 60–80min. In contrast the system described in this paper shows essentially linear synthesis up to 12h. Bissel *et al.* (1973) have described a system consisting of hepatocyte monolayers derived from regenerating liver. The rate of albumin synthesis was reported to be about 1 μg/h per mg of cellular protein which when recalculated in terms of wet liver weight, by using a conversion factor given by the authors, comes to

0.22mg/h per g of hepatocytes. However, other studies with regenerating liver have indicated that synthesis of a number of proteins, especially fibrinogen and transferrin (Gordon, 1970) and perhaps albumin (Majumdar *et al.*, 1967), may be stimulated, making it doubtful whether a system derived from regenerating cells would be appropriate for the study of the synthesis of fibrinogen or other acute phase proteins.

The system described in the present paper has several features which are indicated below.

Preparation and incubation of cells

Although the method of preparing cells was essentially that of Berry & Friend (1969) the following differences were found to be important in obtaining a good preparation. The initial washout of blood was as complete as possible and the perfusion rate was very much slower, being 10ml/min. Isolated cells were separated by gravity sedimentation permitting a better separation of cells stained and unstained by Trypan Blue. For optimum survival and function during incubation, Waymouth's MB 752/1 medium containing 17.5% (v/v) rat serum or heat-inactivated horse serum was required. Further, a fairly constant moderately high O_2 tension with continuous spinning of the suspensions was superior to shaking cells gassed with an $O_2 + CO_2$ (95:5) mixture where O_2 tension varied by reason of changing requirements.

Structural characteristics of the cells

The cells showed excellent viability with 70% remaining unstained up to 24h of incubation, and normal ultrastructure being maintained up to 24 and even 48h of incubation. After 12h of incubation the cells reaggregate so that bile canaliculi and junctional complexes reappear. Such reaggregation may indicate the syntheses of mucopolysaccharide (Pessac & Defendi, 1972) and cell-surface glycoprotein (Humphreys, 1967) and may also indicate that this model could be developed to study surface protein synthesis and other surface phenomena in the hepatocyte.

Membrane permeability to amino acids

In a recent review Schreiber & Schreiber (1973) have indicated that their isolated hepatocytes did not concentrate amino acids, suggesting the presence of a leaky membrane. After 24h of incubation the cells studied in our system did have a higher concentration of amino acids in the cell pellet indicating intracellular concentration of amino acids. The true intracellular concentration would have been even greater as the pellet contained some trapped medium. Further, some amino acids such as taurine and aspartic acid were almost exclusively intracellular as would be expected. These results suggest that cells in the system described here possess greater integrity of their walls than those in the cell-suspension studies reported by Schreiber & Schreiber (1973). This difference seems likely to be due to the longer viability of cells in our system, resulting in resynthesis of surface glycoproteins. Indirect evidence of synthesis of surface glycoproteins was obtained by observing the phenomenon of reaggregation after 12h of incubation (Pessac & Defendi, 1972).

Reproducibility and control of experiments

Since duplicate or triplicate suspensions from a batch of hepatocytes give comparable results it is possible to control each study by dividing the cell suspension into two or more portions in which variables can be tested against each other and compared with a standard control. This makes it possible to bring out rather subtle effects which might otherwise be overshadowed by variability in batches of hepatocytes. This is exemplified in the effect of insulin on albumin synthesis (Table 2).

Albumin, fibrinogen and transferrin synthesis

The query may be raised as to whether our method of homogenization extracts all the intracellular albumin since it includes neither ultrasonification nor the deoxycholate treatment deemed necessary by Peters (1962) to release most of the microsomal albumin and thus affect the synthesis rates reported herein. In answer it may be noted that although our extraction of intracellular plasma protein may indeed be incomplete, it appears to us that unextracted amounts are, by 12h, relatively small (10–15%) with regard to the amounts measured and would not grossly affect the outcome. In any case, rates of synthesis reported are based on net increase or the difference between measurements of the given protein at any two intervals of time. (These intervals include a zero-hour measurement at which point a small but measurable concentration is usually present.) Any error due to unextracted plasma protein should be absolute and be present to approximately the same extent each time. Therefore we should not expect any such error to affect at all the rate of synthesis that we report here.

The mean albumin synthesis of 0.32mg/h per g of hepatocytes with Waymouth's medium amounts to about 40% of the value found *in vivo* in rats [recalculated from the data of Jeejeebhoy *et al.* (1972)] but is 30% higher than that observed in hepatocyte monolayers (Bissel *et al.*, 1973). Further, this value is comparable with the results in perfused-liver experiments with amino acid supplementation

but without hormones (John & Miller, 1969) which give a synthesis of 0.29 mg/h per g of liver [recalculated by assuming a 300 cm^2 rat has a liver weighing 10 g (L. L. Miller, personal communication)]. Fibrinogen synthesis was 0.12 mg/h per g of hepatocytes, or up to twice the rate *in vivo* of 0.06 mg/h per g of liver [recalculated from the data of Jeejeebhoy *et al.* (1973)]. The mean transferrin synthesis was 0.097 mg/h per g of hepatocytes which is about 74% of the value *in vivo* (Morgan & Peters, 1971).

The fact that the synthesis of albumin and transferrin is below the value *in vivo* may indicate one of two possibilities. Either the cell preparation is generally inferior to the state *in vivo* with regard to 'export protein' synthesis or there is a difference between controlling factors *in vivo* compared with the state *in vitro*. The data for fibrinogen synthesis favour the latter explanation. It is noted that plasma protein synthesis is not uniformally depressed for albumin, fibrinogen and transferrin in the state *in vitro* as would be expected if the cells were poorly viable. On the contrary, albumin synthesis is 40% of the control value *in vivo*, fibrinogen synthesis is stimulated above it and transferrin synthesis is 74% of this value. Also, too, our results for fibrinogen are comparable with those of John & Miller (1969). Hence it appears that the state *in vitro* is distinguished by a change in controlling factors resulting in decreased albumin and increased fibrinogen synthesis.

The availability of nutrients may be important in the case of albumin synthesis since albumin but not fibrinogen synthesis is better in cells incubated in Waymouth's medium which has higher content of amino acids and glucose than Ham's F10 medium (Table 1). However, the exact factors remain to be determined.

ATP concentrations were comparable with values found *in vivo* (Bloxam, 1971; Bucher, *et al.*, 1964) and increased during up to 12 h of incubation (Table 3). Even after 24 h of incubation, ATP concentrations were above 2 μmol/g of hepatocytes indicating that the energy reserve of the cell was adequate for protein synthesis.

RNA/DNA ratios (an index of the amount of RNA/cell) were comparable with the value found *in vivo* (Munro, 1970) and point to cells having a full complement of the nucleotides necessary for protein synthesis.

Effect of hormones on plasma protein synthesis

John & Miller (1969) have demonstrated stimulation of plasma protein synthesis by the addition of cortisol and insulin to the perfusing medium *in vitro* and have pointed out that demonstration of such stimulation *in vitro* was favoured by a 12 h duration of study. Hitherto the ability of isolated hepatocytes to respond to hormones has not been studied (East *et al.*, 1973; Weigand *et al.*, 1971; Bissell *et al.*, 1973). The isolated hepatocyte model described above did show an increase of fibrinogen synthesis above the control value after the addition *in vitro* of a mixture of hormones. These results of fibrinogen synthesis were comparable with those of John & Miller (1969) and to our own studies *in vivo* (Jeejeebhoy *et al.*, 1973). From Table 2 it is clear that in the control experiment mean fibrinogen synthesis over the first 12 h was about 1 mg/g of hepatocytes for all three groups of cells (control and hormone mixture, with and without insulin). During the next 12 h (i.e. 12 to 24 h) the mean value in the control was 0.84 mg/g of hepatocyte which is only 16% lower than that in the previous 12 h period. In contrast the hormone-treated cells synthesized about 2.0 mg/g of hepatocytes indicating stimulation compared with the initial 12 h period. It is noteworthy that from 24 to 48 h the mean synthesis per 12 h period for hormone-stimulated cells without insulin was 1.35 mg/g of hepatocytes which is still 35% above the initial 12 h control value.

Albumin and transferrin synthesis rates in insulin-treated cells were higher than the control value between 24 and 48 h of incubation. Transferrin and albumin synthesis rates in the control incubation declined after the first 12 h of incubation suggesting that the hepatocytes were becoming depleted of factors (possibly remaining from those found *in vivo*) required for optimum synthesis. During this phase insulin increased synthesis of transferrin so that it was almost linear over the 48 h period (mean synthesis was 1.1 mg/g of hepatocyte for the first 12 h period and 0.9 mg/g of hepatocyte for the 12 h period from 24 to 48 h of incubation in insulin-treated cells). These findings indicated that relative or absolute insulin deficiency may be a possible cause for a fall in transferrin synthesis after the first 12 h of incubation. Albumin synthesis, although enhanced by insulin, did not become linear, indicating that other factors were also operative. The alternative explanation for a fall in albumin and transferrin synthesis, namely a 'dying preparation' is unlikely as the synthesis of another plasma protein, fibrinogen, was almost linear up to 24 h and could be stimulated above the initial control value. Further, the cells displayed excellent ultrastructure up to 48 h of incubation.

The results of studies with hormones indicated first, that the model showed stimulation with hormones comparable with the state *in vivo*; second, that a single hormone may increase synthesis of a defined protein without influencing another; and finally that the role of a hormone in maintaining optimum synthesis may take up to 48 h to become evident in such a model.

In conclusion, this paper describes a system *in vitro* for studying the synthesis of plasma proteins in the liver, a system which gives results comparable with those obtained with the long-term perfused liver

and defines the conditions necessary to obtain optimum synthesis rates. Further, the cells respond to hormonal stimuli. This system is an advance on previously published methods.

Such a system has the advantage of providing direct access to the hepatocyte and its membrane and to the extracellular milieu which can be closely controlled. Theoretically it could also be used to test the presence of hepatotrophic factors in sera (in the way that calf and horse sera were tested), a subject which is rapidly becoming of interest in the study of the control of liver regeneration.

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