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METHOD OF OBTAINING A PRIMARY MONOLAYER CULTURE OF RAT HEPATOCYTES AND ESTIMATING ITS FUNCTIONAL ACTIVITY

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Many workers have shown that the action of different hormones on synthesis and secretion of serum proteins and the activity of certain enzyme systems in the liver *in vitro* are most satisfactorily studied in primary monolayer cultures of hepatocytes [3, 4, 7-9, 12]. In the last 10 years hepatocytes have been isolated mainly by perfusion of the liver with a saline solution of collagenase. The use of trypsin as dispersing agent gives a much lower percentage yield of viable hepatocytes than collagenase [6]. Nevertheless, regardless of the method of their isolation, when cultured the cells become completely adapted to their new conditions after 24-48 h and they restore their receptor apparatus, when damaged to some degree or other in the course of enzymic disintegration [10]. It has been shown that hepatocytes, whether obtained with the aid of trypsin or of collagenase, are functionally equally active during the first 4 days of culture, after which they gradually undergo degradation [3, 5].

Grisham et al. [6] found that the cell ultrastructure is virtually unchanged as a result of the relatively strong but reversible action of trypsin on membrane receptors.

It was accordingly decided to attempt to obtain viable and functionally active rat hepatocytes by a simpler method than perfusion of the liver. Techniques widely used and described in the literature were chosen as the basis. The more readily available trypsin was used as dispersing agent. The functional activity of the cells in culture was estimated by their ability to synthesize and secrete serum albumin. To assess the hormonal sensitivity of the hepatocytes in culture, the action of growth hormone on albumin synthesis was determined.

EXPERIMENTAL METHOD

To obtain cultures of hepatocytes the liver of female Wistar rats aged 1-2 months and weighing under 100 g was used. According to Ricca et al. [11], it is at this age that the maximal incorporation of labeled precursor into albumin is observed in rats. The liver was removed with sterile precautions, washed in 0.02% Versene solution (to remove ions of heavy metals), containing 50 i.u./ml each of penicillin and streptomycin, and about 500 mg of tissue (always the same region) was taken from the middle of greater lobe and transferred to a sterile penicillin flask. In the flask the tissue was carefully cut into small pieces with scissors, after which it was washed 3 or 4 times with 10 ml of 0.02% Versene solution to remove blood. The minced and washed tissue was covered with 7 ml of 0.25% trypsin solution and incubated at 37°C for 5-8 min. The trypsin was removed, 7 ml of medium No. 199 with 10% embryonic calf serum was added, and the tissue was dispersed by pipeting 8-10 times. The resulting primary suspension was allowed to stand at room temperature to sediment the cells. A white, light residue of hepatocytes appeared after 15-20 min at the bottom of the flask. The supernatant was removed with a syringe and the residue diluted with fresh medium No. 199 with 10% embryonic calf serum to obtain the final suspension. The suspension containing 5×10^6 cells/ml was poured in a volume of 1 ml into ordinary sterile glass tubes. The medium was changed every 24 h for 2 days. The cells were cultured at 37°C in an atmosphere with 95% air and 5% CO_2. A 48-h

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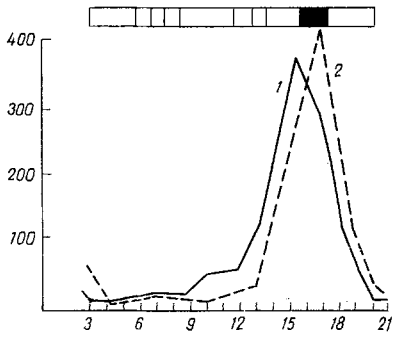

Fig. 1

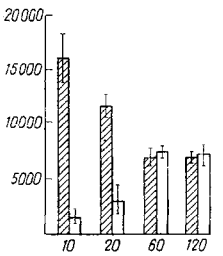

Fig. 2

Fig. 1. Character of incorporation of ^{14}C-L-leucine into proteins secreted by hepatocytes into culture medium and contained in cells. Abscissa, length of gel (in mm); ordinate, incorporation of ^{14}C-L-leucine into proteins (in cpm/mg protein). 1) Culture medium; 2) cell contents. Top: electrophoresis of rat plasma proteins.

Fig. 2. Rate of synthesis and secretion of albumin in primary monolayer hepatocyte culture. Abscissa, incubation time (in min); ordinate, incorporation of ^{14}C-L-leucine into albumin (in cpm/mg protein). Shaded columns represent rate of synthesis; unshaded columns rate of secretion.

culture was used for the experiments.

Fractionation of Labeled Proteins. Fractionation of labeled proteins occurred in the cells during incubation for 1 h with the labeled precursor (^{14}C-L-leucine), and also secreted into the culture medium during the same period, was carried out by electrophoresis in polyacrylamide gel. The curve of distribution of labeled proteins along the whole length of the gel was obtained after cutting the gel into disks 2 mm thick followed by extraction of the native proteins with 0.5 ml of 0.3% sodium dodecylsulfate [2] for 24 h at 37°C.

Preliminary electrophoresis of the radioactive proteins in the medium and cell supernatant was carried out by comparison with rat blood plasma. The stained band of the largest serum albumin fraction was found to coincide completely with the largest radioactive peak (Fig. 1). Hence it was concluded that the larger fraction of the labeled leucine was present in the main albumin fraction.

The distribution of labeled proteins in the test material and the rate of synthesis and secretion of albumin were determined by the method normally used in the writers' laboratory for adenohypophyseal proteins [2].

Determination of the Rate of Synthesis and Secretion of Albumin. To determine the rate of synthesis, after the last change of medium the cells were covered with fresh medium without serum, containing 5 μCi/ml ^{14}C-L-leucine (from Czechoslovakia, specific activity 240 mCi/mmole) and incubated for 10, 20, 60, and 120 min. At the end of incubation the medium with excess of label was removed by rinsing 4 times with Hanks' solution containing an increased concentration of unlabeled leucine (50 mg%). The cells were then covered with 0.5 ml water and were frozen quickly in a mixture of dry ice and ethanol and quickly thawed 3 times. After centrifugation for 10 min at 3000 rpm radioactivity was counted in the supernatant.

To determine the rate of secretion of albumin the liver cells were preincubated for 17 h with 5 μCi/ml of ^{14}C-L-leucine and, after removal of excess of label by the method described above, the hepatocytes were incubated for the same time intervals in fresh medium No. 199 without serum. Labeled albumin secreted into the medium was then estimated.

The Sensitivity of Hepatocytes to Growth Hormone. This was determined after incubation of the cells for 3 h with human growth hormone in a dose of 500 ng/ml. The hepatocytes were isolated from the liver of intact and hypophysectomized rats, and the ability of the cultured cells to synthesize albumin was determined while under the influence of growth hormone (from Kaunas Endocrine Preparations Factory). Hypophysectomy was performed through a transauricular approach on an apparatus constructed in the Biological Standardization of Hormones Laboratory [1]. Radioactivity was counted on an Intertechnique scintillation counter and the results were expressed in cpm/mg protein. Protein was determined by Lowry's method.

EXPERIMENTAL RESULTS

Optimal conditions were chosen for the action of trypsin, which would, on the one hand, be favorable for dissociation of liver tissues and, on the other hand, would not cause digestion of a high proportion of the cells. The primary suspension was allowed to stand for 15-20 min for passive sedimentation of the heavier hepatocytes, thereby ensuring that

TABLE 1. Effect of Growth Hormone (STH) on Rate of Albumin Synthesis in Primary Monolayer Culture of Rat Hepatocytes (M ± m)

Series of experiments	Group of animals	Rate of albumin synthesis, cpm/mg protein	P
I	Intact	1150±220	
	Intact + STH	1870±88	<0,05
	Hypophysectomized	1100±120	
	Hypophysectomized + STH	4580±500	<0,05
II	Intact	5340±353	
	Intact + STH	6520±350	<0,05
	Hypophysectomized	520±60	
	Hypophysectomized + STH	2910±800	<0,01
III	Intact	2050±173	
	Intact + STH	2310±125	>0,05
	Hypophysectomized	1400±300	
	Hypophysectomized + STH	2950±620	<0,05

more of the viable cells were preserved. The relatively low percentage yield of uninjured hepatocytes was compensated by using a knowingly larger number of cells for seeding ($5 \cdot 10^6$ per ml medium). Unattached and dying hepatocytes were removed after the first change of medium.

The study of the character of distribution of labeled leucine in protein fractions of the medium and cell supernatant by electrophoresis in polyacrylamide gel showed that synthesized and secreted labeled albumin accounted for 75-80% of the total of other radioactive proteins (Fig. 1). Accordingly the very small degree of incorporation of the isotope into other protein fractions compared with albumin was disregarded and its content was estimated from the total count of the medium or cell supernatant tested.

Determination of the rate of albumin synthesis revealed that labeled protein appeared in large quantities during the first 10 min of incubation. Later its concentration in the cells decreased, and after 1 h the rate of albumin synthesis became constant (Fig. 2). Albumin secretion, on the other hand, was low in the first 10 min. After 1 h the values of the rate of synthesis and secretion of albumin were about equal and were unchanged 2 h or more after the beginning of incubation (Fig. 2). The main characteristics of synthesis and secretion of albumin coincided with those described by other workers [3, 11]. Consequently, hepatocytes isolated and cultured by the method described above are functionally active as regards production of the principal serum protein, albumin.

Determination of hormonal sensitivity of the hepatocytes revealed that the cells in culture could respond to the action of growth hormone by an increase in the rate of albumin synthesis. In cells taken from the liver of intact rats this effect was much less marked than in cells from hypophysectomized animals (Table 1).

By means of the suggested method it is thus possible with comparatively little effort to obtain a primary monolayer culture of rat hepatocytes, completely suitable for use in the study of the direct action of natural and synthetic hormones.

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