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METABOLISM OF RAT LIVER CELLS CULTURED IN SUSPENSION: INSULIN AND GLUCAGON EFFECTS ON GLYCOGEN LEVEL*

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Several methods for the preparation of isolated liver cells have been described. We tried some of them and cultured the cells in monolayers or in suspension, because of our interest to develop a system of dispersed rat liver cell as a primary explant, able to live for long periods of time; to study dedifferentiation and hormonal effects on metabolism.

Most of the cultured cells did not survive, and the methods were cumbersome and difficult to perform under sterile conditions.

Then a paper describing a technique to dissociate mouse liver sodium tetraphenylboron, a potassium chelating agent came to our attention (Rappaport et al., 1966). Using this chemical we have developed a simple and highly repeatable method to obtain viable isolated liver cells.

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They were kept cultured in suspension for periods of over 2 months, during which time it was observed that the cells were able to fulfill some of the liver functions: glycogen, urea and bilirubin synthesis (GERSCHENSON et al.-submitted for publication). The technique to obtain the cells and some findings concerning their carbohydrate metabolism and the effect of insulin and glucagon upon them, form the basis of the present communication.

Three to 7 days old rats of the Long-Evans strain were used throughout. All the operations were performed under sterile conditions. The rats were killed by decapitation and the livers were dissected and kept in a Petri dish with 10 ml of the dissociating solution or D.S. made of: 5 mM sodium tetraphenylboron (Aldrich Chemical Co.), 11 mM glucose, 130 mM NaCl, 0.4 mM $MgSO_4 \cdot 7\ H_2O$, 0.49 mM $MgCl_2 \cdot 6\ H_2O$, 0.33 mM $Na_2HPO_4 \cdot 2\ H_2O$, 0.44 mM $NaH_2PO_4 \cdot H_2O$, 4.1 mM $NaHCO_3$ and 0.02 g/l phenol red (All these drugs were purchased either from J.T. Baker Chemical Co. or B.D.H.), pH 7.2, at room temperature. The pooled livers were then transfered to another Petri dish and carefully minced with small surgical scissors or blades to give pieces of about 1 mm^3. Afterwards the minced tissue was transfered to an Erlenmeyer flask and suspended in D.S., the added volume of D.S. per liver was 0.5 ml.

The suspended tissue was then subjected to magnetic stirring, using a Pyrex glass or Teflon coated magnet at 300 r.p.m. and at 37°C. After 20 minutes the cell suspension was decanted into autoclavable plastic centrifuge tubes and centrifuged at 150 x g during 10 minutes at room temperature; after discarding the supernatant the pellet was resuspended in 10 ml of incubation medium or I.M. and kept at 37°C. The small pieces of tissue that remained in the flask were resuspended in 40 ml of D.S. and the whole procedure was repeated 6 to 8

times until the tissue was completely dissociated. The obtained cells were pooled. The I.M. used was made with Hanks' balanced salt solution (1949), Eagles' glucose and vitamin concentration (1955) and M.E.M. amino acids mixture (Eagle, 1959) plus 10 % calf serum, supplemented with 50000 U.I. of penicillin and 0.1 g of streptomycin per liter.

The cells were resuspended in I.M. at a concentration of 100000 to 150000 per ml, then incubated in 500 ml Pyrex glass flasks, being maintained in suspension through magnetic stirring using a Pyrex glass coated magnet at 150 r.p.m. under a gass phase with 5 % CO_2, 95 % air and at 37°C.

Growth curves were obtained by regulating the system so that cell were removed at the same time as they were produced and the same volume of fresh medium were added. The rate of removal was of 5 % of the total volume each 48 hours, which means a doubling of the cell population each 40 days.

The cell number was counted using a haemacytometer after staining the cells with 0.1 % Crystal Violet in 0.1 M citric acid, the DNA was measured by a modification of the method of Fleck and Munro (1962). The cell number decreased until the 12th day when it became stable at approximately 40 % of the original number. The DNA per ml decreased proportionally more than the cell number per ml. The amount of DNA per 10^6 cells at the beginning was 7.5 μg, similar to the results obtained by other authors (Campbell et al., 1952; Fukuda et al., 1953) and decreased to an average of 6.0 μg, which probably means a transition from a more poliploid cell population to one less so.

Experiments to study the effect of insulin and glucagon on the metabolism of glycogen by hepatic cells which had spent different times in culture, were performed.

The cells were taken from a suspension-culture, spun down at 150 x g, room temperature, during 10 minutes, the

supernatant was poured off and the pellet resuspended in Krebs-Ringer-Phosphate (Krebs, 1940). The centrifugation was repeated and the pellet resuspended in the same salt solution and incubated 3 hours in the case of the insulin addition; when glucagon was added to these cells they were incubated for an additional 1 hour. The reaction was stopped by immersion of the vessels in crushed ice. Then the samples were centrifuged at 900 x g, during 10 minutes at 0°C and the pellets were resuspended in Krebs-Ringer-Phosphate and the centrifugation was repeated. The cells were then analyzed for glycogen by the method of Good et al. (1933), glucose was determined by the glucose oxidase method (Glucostat, Worthington Biochemical Corp.) and protein by the procedure of Lowry et al. (1951).

The glycogen concentration of 25 rat livers used for the cultures was 29.2 $\pm$ 3.0 micromoles of glucose equivalents per 100 mg of protein; 6 hours after the treatment with the D.S. was over and before the culture was started the concentration fell down 10 to 20 times; it started to rise during the culture period until day 7 or 10 when it reached a concentration very close to the one of the liver, the glycogen level then did not change too much until the last day of the experiment here shown (Table 1). The addition of Insulin for 3 hours to cells of young and old cultures increased the glycogen concentration and the addition of glucagon to cells pretreated with insulin decreased it; all theses results are statistically significant (Table 1).

These effects of insulin and glucagon have already been described for the intact animal and might be explained through the control of the glycogen synthetase and the phosphorylase system, since insulin appeared to increase the activity of liver glycogen synthetase and to decrease the activity of phosphorylase, while glucagon had the oppo-

Table 1. Glycogen concentration in cells incubated with insulin and glucagon (expressed as micromoles of glucose equivalents per 100 mg of protein; mean ± standard error). Three observations were done in each experiment. Two series of experiments a and b are shown.*

Days in culture	None	Addition Insulin	Glucagon
a - 0	2.6 ± 0.2	6.0 ± 0.2**	2.0 ± 0.2**
b - 0	4.2 ± 0.2	6.4 ± 0.2**	2.8 ± 0.4**
a - 5	12.0 ± 0.2	20.8 ± 0.8**	10.4 ± 0.4**
b - 15	17.4 ± 0.6	24.8 ± 1.0**	15.2 ± 0.6**
a - 15	19.2 ± 0.6	40.4 ± 1.6**	20.2 ± 1.2**
b - 16	22.8 ± 0.8	30.6 ± 1.2**	19.6 ± 0.8**
a - 30	16.6 ± 1.0	34.6 ± 1.4**	22.2 ± 0.8**
b - 28	25.2 ± 1.4	42.6 ± 0.8**	28.4 ± 1.6**

*The final volume of each vessel was 5 ml, To each one it was added 4 U of Insulin or 1 μg of Glucagon (E.Lilly). Glucose concentration was 10 mM. The length of the incubation period for the Control and Insulin groups was 3 hours. The Glucagon group was incubated another hour after the addition of the hormone.
**$p < 0.02$ p notations applies to annotated value and value in the immediately preceding column to the left.

site effects (Steiner, 1964; Sutherland et al., 1960).

The initial decrease of the concentration of glycogen, comparing the tissue to the dispersed cells, before culturing them, might be explained as a result of the treatment with D.S., but further studies are needed to answer this question as well as the increase of glycogen from 0 time to days 7 or 10.

The effect of the studied hormones, showing a striking similarity with the "in vivo" situation, even in the one month old cells; the slow growth rate as well as the predo-

minance of hepatocytes; and the uniform condition of the suspension cultures permitting accurate sampling, show this system of rat liver cells in culture to be an ideal tool for biochemical and hormonal studies.

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