PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE, **147** 581-584 (1974)

Studies on the Effect of Collagenase and Hyaluronidase on Glycogen Content of Isolated Rat Liver Parenchymal Cells[1] (38392)

S. R. WAGLE AND W. R. INGEBRETSEN, JR.[2]

Department of Pharmacology, Indiana University School of Medicine, Indianapolis, Indiana 46202

Recently we reported an improved method (1) for the isolation of rat liver parenchymal cells. In this procedure the liver is exposed to low concentrations of collagenase for a very short period and no hyaluronidase is needed; these isolated cells retain hormone sensitive receptors (2–4). Although this method has now been accepted by a number of workers (5–9), others (10–19) have continued to use the combination of high concentrations of collagenase and hyaluronidase for the cell preparation. In this communication, we demonstrate significant differences in glycogen content in liver cells prepared with the addition of hyaluronidase to the perfusion medium in the presence of collagenase. We also report studies on ^{14}C amino acid incorporation into protein under various physiological conditions.

Materials and Methods. Animals. Fed (180–200 g or 350–400 g) or 8–10 and 18–24 hr fasted male Cox rats were used for these studies. All rats were maintained on Purina Laboratory Chow and tap water fed *ad libitum*. Two methods of feeding were used in the present studies as it was observed that liver glycogen was significantly increased in animals that had easy access to food when it was placed in a dish on the floor as compared to those rats that had to obtain their food from suspended wire baskets.

Preparation of rat liver parenchymal cells. Normal and fasted rats of various age groups were anesthetized with Na-pentobarbital and the liver was rapidly removed and placed in a Miller perfusion apparatus and perfused for 15 min with 100 ml of Hanks calcium free buffer containing 1.5 g albumin (Sigma, Fraction V) and 10 mg each of streptomycin and penicillin. Following this initial perfusion, either collagenase alone (20 mg, Worthington, 199 units/mg) or both collagenase and hyaluronidase (80 mg, Sigma Type I, 360 units/mg) were added and the perfusion continued for 10–15 min. The liver was removed, finely minced and bubbled gently with 95% O_2 and 5% CO_2 for 1 min. Cells were isolated as described previously (1) and were brought to a final volume of 40–50 ml (3–4 g of cells) with Umbreit–Ringer buffer (1).

Incubation of cells. A 1 ml aliquot of the final cell suspension (50–70 mg) was incubated in 3 ml of Umbreit–Ringer bicarbonate buffer (25 m*M*) in stoppered 1 oz plastic vials (Nalgene 202) with various amino acids at 37° and 90 oscillations/min for 1 hr. The vials were gassed for 5 min with 95% O_2 and 5% CO_2 before the start of the incubation. At the end of the incubation period the reactions were stopped by the addition of trichloroacetic acid and radioactivity in isolated protein was assayed as described previously (20). Glycogen was determined by the method of Good *et al.* (21); protein was estimated by the method of Lowry *et al.* (22) after the incubation mixture was sonicated.

Results and Discussion. The results on glycogen content of the whole liver, whole liver perfused for 30 min with no enzymes, and isolated cells with collagenase and with collagenase and hyaluronidase are presented in Table I. The initial glycogen varies a great deal depending upon the condition of feeding. Rats which had easy access to their food on the floor showed twice as much glycogen content as compared to rats which had to make a special effort to obtain the food. When the liver was perfused for 30 min

[1] Supported by a grant from Eli Lilly and Company and the Human Growth Hormone Foundation.

[2] Present address: Department of Pharmacology and Medicine, University of California, San Diego, La Jolla, CA.

TABLE I. Effect of Collagenase and Hyaluronidase on Glycogen Content of Isolated Rat Liver Parenchymal Cells.[a]

	Glycogen expressed as μmole glucose/g		
Whole liver	340 ± 80[b] (6)	220 ± 56[c](†) (6)	80 ± 32[d](††) (4)
Perfused liver for 30 min with no enzymes	228 ± 52 (4)	134 ± 35 (†) (4)	—
Perfused liver with 80 mg hyaluronidase	240 ± 45 (4)	144 ± 32 (†) (4)	22 ± 8(††) (4)
Isolated cells with 20 mg collagenase	220 ± 60 (6)	130 ± 30 (†) (6)	18 ± 8(††) (4)
Isolated cells with 20 mg collagenase and 80 mg hyaluronidase	130 ± 40[e] (6)	60 ± 15 (†) (6)	12 ± 6(††) (4)

[a] Isolated livers were perfused for 15 min with 100 ml of Hanks buffer (1) and following this perfusion period, 20 mg of collagenase or 20 mg collagenase with 80 mg hyaluronidase was added and perfusion was continued for 10–15 min and cells were isolated as described previously (1).

[b] Food was placed on the floor in a dish in these cages. The rat obtained the food very easily without any extra effort.

[c] Food was suspended in a wire basket and rats had to obtain the food with some extra effort.

[d] Rats were starved for 8–10 hr. These rats had received food in a dish placed on the floor.

(†) Significantly different from Column I ($P < 0.05$) as determined by Student's t test.

(††) Significantly different from Columns I and II ($P < 0.05$) as determined by Student's t test.

[e] Significantly different from above values ($P < 0.05$) as determined by Student's t test. All experimental animals were matched for weight and sex in these groups.

with Ca and glucose free Hanks medium, approximately 80–100 μmole of glucose is released in the perfusion medium due to glycogenolysis. Addition of collagenase or hyaluronidase alone did not significantly alter glycogen content of the isolated cells. However, addition of both collagenase and hyaluronidase significantly reduced the glycogen content of the isolated liver cells. This is seen in both the groups of animals which had initial glycogen content in the range of 260–400 μmole/g and 170–250 μmole/g. Furthermore, the reduction in glycogen content with hyaluronidase and collagenase was observed only in cell preparations which had high initial glycogen content. There was a reasonable agreement in glycogen content of the isolated cells and glucose that was released in the medium. Glycogen content of the cells is quite significant if one wants to prepare cells from fed rats containing high glycogen to study differential hormonal responses (2–4) but is of little significance in fasted cells as they contain no glycogen. These results clearly demonstrated that hyaluronidase is not essential, and its addition to the perfusion medium significantly reduced the glycogen content of the isolated liver cells although the cell yields are similar. In preliminary studies, we have observed that in isolated hepatocytes with high content of glycogen gluconeogenesis is stimulated by glucagon at the concentration of 10^{-12}–10^{-10} M and a small change in cyclic AMP levels was observed under this condition. However, in cells containing low or no glycogen 10^{-10}–10^{-8} M concentrations of glucagon was needed to stimulate gluconeogenesis and under these conditions 10 to 20-fold increases in cyclic AMP levels were observed (23). These results once again suggest that glycogen content helps maintain the *in vivo* metabolic characteristics of these isolated cells which respond to hormones at physiological concentrations.

Another observation made in the present studies is that when isolated cells from fasted rats were allowed to stand for 30 min or longer with no substrate (such as glucose, lactate or pyruvate) and in the absence of O_2 showed signs of membrane damage (cytoplasmic protrusion or pseudopoding; Fig. 1) and hence, cells should be used immediately for metabolic studies with substrates. Membrane damage was not observed in cell preparations obtained from fed rats, indicating that stored glycogen may serve as an energy source and prevent intracellular damage. We have shown previously that these isolated cells retain much of their intracellular structure even at the end of 6 hr of incubation in the presence of glucose, lactate and amino acids as

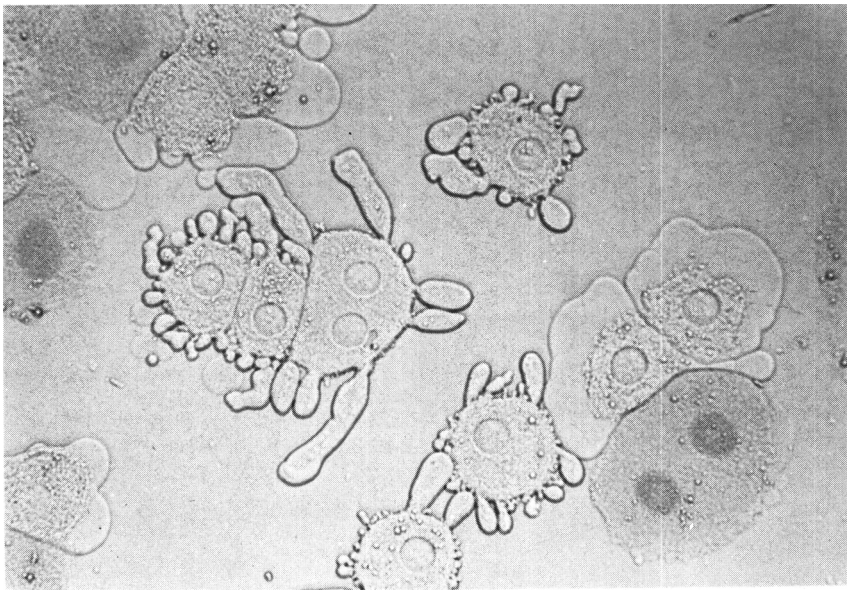

FIG. 1. Light micrograph (magnification 800 ×) of isolated liver cells obtained from fasted rat exhibiting "Pseudopoding" when allowed to stand without shaking for 30 min or longer in Umbreit Ringer bicarbonate medium and with no substrate and absence of oxygen.

substrates mixture and in the presence of insulin (4).

The studies on amino acid incorporation into protein are summarized in Table II. The incorporation of ^{14}C isoleucine, ^{14}C-valine and ^{14}C-phenylalanine into protein by hepatocytes isolated with collagenase alone from younger rats (180–200 g, 6–8 weeks) was significantly higher ($P < 0.05$) than in cells from older rats (350–400 g, 20–25 weeks). Similarly, incorporation of these amino acids was lower in cells from fasted rats than that from fed rats. It is generally assumed that protein synthesis diminishes with age and with fasting. This can be demonstrated when rates of amino acid incorporation into protein in neonatal and adult rats are compared. Recently, it has been demonstrated that there is a decrease in amino acid incorporation into protein of livers from 400 g rats when compared to livers from 200 g rats (24). Present studies with iso-

TABLE II. Incorporation of ^{14}C-Amino Acids into Protein in Liver Cells Isolated with Collagenase only from Fed or Fasted Rats.*

(dpm/mg protein)

	Fed rats Liver cells		24 hr starved rats Liver cells
Rat wt	180–200	350–400	180–200
Isoleucine	1460 ± 238 (12)	890 ± 58** (10)	880 ± 106** (10)
Valine	1530 ± 206 (12)	1012 ± 85** (10)	1060 ± 103** (10)
Phenylalanine	1090 ± 132 (12)	780 ± 75** (10)	802 ± 123** (10)

*Isolated liver cells (with collagenase only approximately 50–70 mg) were incubated for 1 hr in the presence of 5.5 m*M* glucose and 10 m*M* amino acids (0.5 μCi/30 μmoles). Values are mean ± SEM of (*N*) observations.

**Significantly different from column 1 ($P < 0.05$) as determined by Student *t* test.

lated cells agree with this observation. The ability of the isolated cells to retain high amounts of glycogen and to retain much of their *in vivo* metabolic characteristics for prolonged periods (4) suggest that these cells are very useful in studying not only hormonal regulation but also other physiological conditions.

Summary. Rat liver parenchymal cells were isolated with collagenase and hyaluronidase from fed and fasted rats. Addition of hyaluronidase significantly decreased intracellular glycogen content of cells in fed rats, although there was no effect on cell yields. The incorporation of ^{14}C isoleucine, ^{14}C-valine, and ^{14}C-phenylalanine into proteins was significantly higher in younger as compared to older rats. The incorporation of these various ^{14}C amino acids was lower in cells from fasted rats when compared to those from fed rats.

We are grateful to Mrs. Deborah Hutton and Mrs. Vickie Phelps for the preparation of the cells and for their expert technical assistance.

1. Ingebretsen, W. R., Jr., and Wagle, S. R., Biochem. Biophys. Res. Commun. **47,** 403 (1972).

2. Ingebretsen, W. R., Jr., Moxley, M. A., Allen, D. O., and Wagle, S. R., Biochem. Biophys. Res. Commun. **49,** 601 (1972).

3. Wagle, S. R., and Ingebretsen, W. R., Jr., Biochem. Biophys. Res. Commun. **52,** 125 (1972).

4. Wagle, S. R., Ingebretsen, W. R., Jr., and Sampson, L., Biochem. Biophys. Res. Commun. **53,** 937 (1973).

5. Zahlten, R. N., Stratman, F. W., and Lardy, H. A., Proc. Nat. Acad. Sci. **70,** 3213 (1973).

6. Zahlten, R. N., Kneer, N. M., Stratman, F. W., and Lardy, H. A., Arch. Biochem. Biophys. **161,** 528 (1974).

7. Beg, Z. H., Allmann, D. W., and Gibson, D. M., Biochem. Biophys. Res. Commun. **54,** 1362 (1973).

8. Dubinsky, W. P., and Cockrell, R. S., Biochem. Biophys. Res. Commun. **56,** 415 (1974).

9. Christoffersen, T., and Berg, T., Biochem. Biophys. Acta **338,** 408 (1974).

10. Garrison, J. C., and Haynes, R. C., Jr., J. Biol. Chem. **248,** 5333 (1973).

11. Tolbert, M. E. M., Butcher, F. R., and Fain, J. N., J. Biol. Chem. **248,** 5686 (1973).

12. Werner, H. V., Bartley, J. C., and Berry, M. N., Biochem. J. **130,** 1153 (1972).

13. Muller, M., Schreiber, M., Karlenback, J., and Schreiber, G., Cancer Res. **32,** 2568 (1972).

14. Cornell, R. E., and Filkins, J. P., Proc. Soc. Exp. Biol. Med. **145,** 203 (1974).

15. Cornell, N. W., Lund, P., Hem, S. R., and Krebs, H. A., Biochem. J. **134,** 671 (1973).

16. Howard, R. B., Lee, J. C., and Pesch, R. A., J. Cell. Res. **57,** 642 (1973).

17. Quistorff, B., Bondesen, S., and Grunnet, N., Biochem. Biophys. Acta **320,** 503 (1973).

18. Capuzzi, D. M., Rothman, V., and Margolis, S., J. Biol. Chem. **249,** 1287 (1974).

19. Tolbert, M. E. M., and Fain, J. N., J. Biol. Chem. **249,** 1162 (1974).

20. Monier, D., Santhanam, K., and Wagle, S. R., Biochem. Biophys. Res. Commun. **46,** 1881 (1972).

21. Good, R. A., Kramer, H., and Somogi, M., J. Biol. Chem. **100,** 485 (1933).

22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193,** 265 (1951).

23. Wagle, S. R., Biochem. Biophys. Res. Commun. **59,** 1366 (1974).

24. Peters, T., Jr., and Peters, J. C., J. Biol. Chem. **247,** 3858 (1973).

Received May 20, 1974. P.S.E.B.M., 1974, Vol. 147.