PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 47-51 (1976)

Effect of Fasting and Insulin on the Glucagon-Induced Orthophosphate Incorporation to the Isolated Perfused Rat Liver[1] (39324)

F. DE VENANZI, F. PEÑA, O. JIMÉNEZ V., AND H. DE ALVARADO

Cátedra de Patología General y Fisiopatología, Instituto de Medicina Experimental, Universidad Central de Venezuela, Apartado Postal 50587, Sabana Grande, Caracas, Venezuela

In previous experiments (1) it was shown that glucagon, epinephrine, cAMP, and dB-cAMP were all able to increase the incorporation of orthophosphate to the isolated perfused fed rat liver. This phenomenon may explain, in part, the well-known hypophosphatemic effect exhibited by these glycogenolytic factors (2–6). The functional meaning of the increased orthophosphate incorporation to the liver has not been clarified yet. It has been postulated that such incorporation may be related to an increment of phosphate requirements during glycogenolysis (1); on the other hand, in the last few years several investigators have pointed out that glucagon and cAMP may activate protein phosphorylations in the liver (7–12), and this effect could also have some relationship with the above mentioned phenomenon.

Our previous experiments (1) were performed in fed rat livers in which a clear increase in spontaneous or glucagon-induced glycogenolysis was evidenced; therefore, it was considered of interest to study the effect of glucagon in two experimental conditions which inhibit glycogenolysis through different mechanisms: fasting and insulin administration.

Material and methods. Male Sprague-Dawley rats (300–350 g body weight) bred in our Institute were used in the experiments. Livers were perfused by means of a previously described technique (1, 13) using 70 ml of perfusate which consisted of Krebs-Ringer-bicarbonate containing 3 g/100 ml of bovine serum albumin (Armour Fraction V), 80 mg/100 ml of glucose, 1.7 mg/100 ml of buffered sodium orthophosphate, and 20–22 vol % of washed rat erythrocytes. Heparin (500 U) was added at the beginning of the experiments. The perfusate was equilibrated with a 95% O_2–5% CO_2 gas mixture, which was also used in the oxygenator. pH was adjusted to 7.40 and the temperature was kept at 37°. Gross and ultramicroscopic observation as well as adequate bile flow were indicative of good liver preservation.

After 15-min perfusion, 70 μCi of high-activity [^{32}P]orthophosphoric acid (Amersham/Searle, PBS-1200 mCi/mmol) was added to the perfusate (0 time). Thereafter samples were withdrawn every 15 min, up to 120 min, and collected in ice-chilled test tubes. The radioactivity of the samples was determined in duplicate 0.1-ml perfusate aliquots plated in plastic cups and counted up to 10,000 in a Nuclear Chicago gas flow counter. The number of counts registered in the 45-min samples was taken as 100% for the expression of the total radioactivity of the subsequent samples. The percentage of ^{32}P incorporated into the liver was estimated by substraction and expressed per 10 g of fresh tissue.

Perfusate glucose concentrations were determined in duplicate by a commercial glucoxidase procedure (Glucostat, Worthington). The figures were corrected for changes found in blank experiments performed in absence of the liver. Results were expressed in term of differences from 45-min values, mg/100 ml/10 g of fresh liver.

Perfusate plasma Pi was determined in duplicate with the method of Martin and Doty (14). Figures were corrected for blank values and expressed in terms of differences with respect to the 45-min values (mg/100 ml). In the absence of the liver there is some decrease of perfusate plasma Pi due to incorporation to red cells; the differences against the 45-min values were: at 60 min, -0.12 ± 0.14; at 75 min, -0.28 ± 0.09; at 90 min, -0.41 ± 0.15; and at 120 min, -0.41 ± 0.17 ($N = 12$).

[1] Supported by Grants No. 44 of Council for Scientific Development of Universidad Central de Venezuela, and No. DF SI-040 of National Research Council of Venezuela.

The radioactivity of the final colored solutions obtained after the Pi determinations were measured in order to estimate the specific activities of the samples. For this purpose, 0.5-ml aliquots were plated in plastic cups and counted to 10,000 counts in a Nuclear Chicago gas flow counter. The specific activities were expressed in counts per milligram of Pi. The specific activity obtained at 45 min was taken as 100%, and the changes of the following samples were calculated as the percentage of this figure.

The pH was determined at 0, 60, and 120 min; some degree of alkalinization was observed at 0 min, but thereafter it was maintained close to 7.40.

Fasted animals were deprived of food during the 24 hr preceding the experiments. Fed animals had free access to food up to the time of the experiments. Water was *ad libitum* in both experimental groups. Glucagon, when administered, was used at the dose of 2 μg and was infused continuously to the perfusate from 45 to 90 min.

Experiments on the effect of insulin were carried out on fed rat livers and the hormone (250 or 500 mU) was continuously infused to the perfusate from 0 to 90 min. Glucagon was used as previously described for the fasting experiments. Insulin-free glucagon, generously provided by Ely Lilly Co., was dissolved in a 0.2 *M* glycine–NaOH buffer, pH 9, containing 1 g/100 ml of bovine serum albumin; working solutions were prepared in saline containing 1 g/100 ml of bovine serum albumin. Glucagon-free insulin, also furnished as a gift by Ely Lilly Co. as a 40 U/ml solution, was dissolved in saline containing 0.1 g of bovine serum albumin, pH 3.5. The selected doses of both hormones were made up in 1-ml vol, and the same amount of saline was injected in the control experiments. The solutions were introduced in the overflow tube of the perfusion apparatus in order to allow for mixture with the bulk of the perfusate before reaching the liver.

The Student's *t* test was used to appraise the statistical significance of the results, and $P = 0.05$ was taken as the limit of significance.

Results. The effect of fasting on the studied parameters is shown in Fig. 1. It is interesting, in the first place, to compare control fasted rat livers with control fed rat livers; it can be seen that the spontaneous glucose and orthophosphate liberation which took place from fed rat livers did not occur in the fasted rat livers. With respect to the effect of glucagon, it can be observed that in the fasted rat livers no significative liberation of

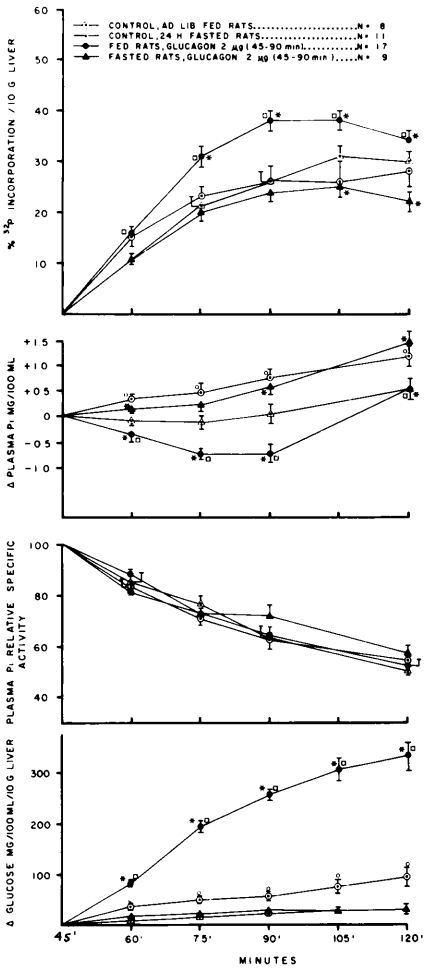

FIG. 1. Effect of glucagon (2 μg) on [^{32}P]orthophosphate incorporation into the isolated perfused rat liver, perfusate plasma inorganic orthophosphate, relative specific activity of the same, and glucose release from the organ. Glucagon was continuously infused into the perfusate for 45 min beginning at 45 min. Comparison between livers from 24-hr fasted rats and *ad libitum* fed rats.

Statistically significant differences: *, Glucagon-fed rat livers vs control fed rat livers; ○, control fed rat livers vs control fasted rat livers; □, glucagon-fed rat livers vs glucagon-fasted rat livers.

glucose or orthophosphate incorporation takes place under the action of the hormone.

Figures 2 and 3 show the effect of insulin and of glucagon plus insulin. Figure 2 pertains to the 250-mU insulin dose: as is well known, insulin, if given since the beginning of the perfusion reduces the glucose output

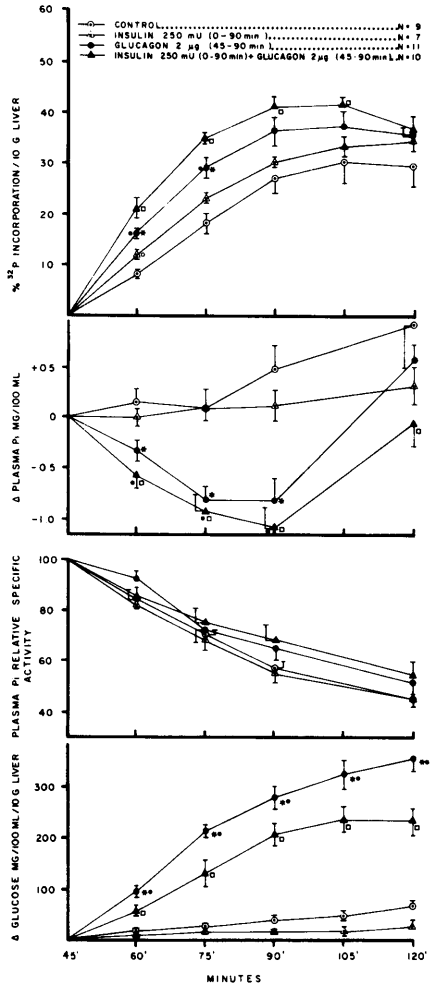

FIG. 2. Effect of insulin (250 mU), continuously infused from 0 to 90 min, glucagon (2 μg) continuously infused from 45 to 90 min, and both types of treatments administered simultaneously to perfusate, on [^{32}P]orthophosphate incorporation into the isolated perfused rat liver, perfusate plasma inorganic orthophosphate, relative specific activity of the same, and glucose release from the organ.

Statistically significant differences: *, Glucagon vs control; ○, insulin vs control; □, glucagon + insulin vs control; ★, glucagon vs glucagon + insulin.

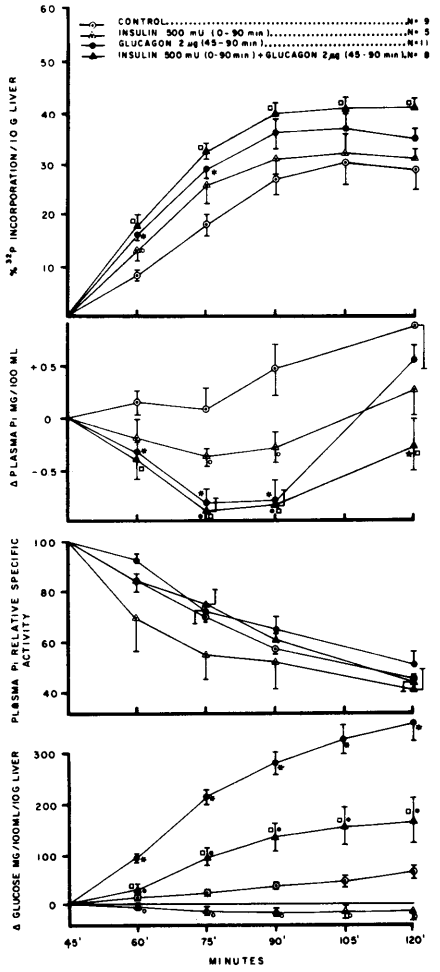

FIG. 3. Effect of insulin (500 mU) continuously infused from 0 to 90 min, glucagon (2 μg) continuously infused from 45 to 90 min, and both types of treatments administered simultaneously to perfusate, on [^{32}P]orthophosphate incorporation into the isolated perfused rat liver, perfusate plasma inorganic orthophosphate, relative specific activity of the same, and glucose release from the organ.

Statistically significant differences: *, Glucagon vs control; ○, insulin vs control, □, glucagon + insulin vs control; ★ glucagon vs glucagon + insulin.

(15, 16); it also inhibits the spontaneous orthophosphate efflux (16, 17). The effect of glucagon on glucose liberation is also reduced by insulin (16, 18); however, it is possible to observe that glucagon-induced orthophosphate incorporation to the liver is not inhibited by insulin. No significant difference was registered among the groups in relation to Pi specific activities. Figure 3

refers to the 500-mU dose of insulin. The results are similar to the ones described with the 250-mU dose, but the changes observed are more evident.

Discussion. It is well known that the reduction of the liver glycogen stores, determined by fasting in the absence of gluconeogenic substrates, inhibits the spontaneous or glucagon-induced glucose release from the perfused rat liver. The spontaneous glucose liberation that takes place from the fed perfused liver has been attributed to anoxia, surgical manipulations (19), and sympathetic discharges originated by surgery (20). However, it could also be possible that insulin lack may play some role, since the phenomenon may be inhibited by the administration of this hormone (15, 16) and is increased by the administration of anti-insulin serum (16). The mechanism of the spontaneous orthophosphate release is not known, but the work of Jefferson *et al.* (16) and our own experiments (17) have shown that it can be inhibited by insulin administration. A similar situation has been mentioned in connection with the perfused rat heart (21, 22). It is interesting that fasted rat perfused livers do not show a significant spontaneous orthophosphate release into the perfusate. The facts that in the fed rat perfused liver there is a concomitant output of glucose and orthophosphate and that both phenomena may be inhibited by insulin administration suggest the possibility that both effects may be functionally related. In the fed rat perfused liver, however, glucagon induces glycogenolysis and more glucose is released, while, at the same time, orthophosphate is incorporated into the organ. In the fasted rat perfused liver, both phenomena are inhibited. Hence, regarding orthophosphate exchange, in the fed perfused rat liver, spontaneous glycogenolysis is accompanied by orthophosphate release, and glucagon-induced glycogenolysis is accompanied by orthophosphate incorporation. This discrepancy could be explained by differences in the action of *in situ* liberated catecholamines and glucagon; however, it should be mentioned in this regard, that in previous experiments (1), we have shown that at least one of the catecholamines, epinephrine, is also able to induce orthophosphate incorporation into the perfused fed rat liver. It could be possible that the main factor in spontaneous glucose and orthophosphate liberation from fed rat liver is the lack of insulin, since this hormone acts on the orthophosphate liver exchange producing retention of the anion (1); however, this mechanism is not easily understandable in terms of cAMP levels regulated by the glucagon–insulin interplay.

In the experiments on insulin-inhibition of the glucagon-induced glucose release, the incorporation of orthophosphate to the liver was not affected, a result which dissociates both phenomena and also suggests a different mechanism of regulation. The inhibition of glucose liberation falls in the above mentioned interplay, but the same cannot be said for orthophosphate incorporation. A similar situation has been mentioned in connection with other glucagon actions (23, 24). Effects of insulin, independent of the cAMP levels, have been also detected (23, 25).

More experimental work is required to further our understanding of the regulation of liver orthophosphate exchange in connection with the action of glucagon and insulin. Studies in course in our laboratory concerning the role of modifiers of the adenylate system and adrenergic blockers, may provide additional information on this matter.

Summary. Isolated perfused fed rat livers spontaneously liberated glucose and orthophosphate to the medium; 24-hr fasted rat livers did not exhibit these phenomena. In perfused fed rat livers, glucagon (2 μg) increased glucose output and promoted orthophosphate incorporation. In perfused fed rat livers, insulin (250 or 500 mU) inhibited the spontaneous liberation of glucose and orthophosphate. Comparable doses of insulin significantly reduced the glucagon (2 μg)-induced increase in glucose output from perfused fed rat liver, but did not affect orthophosphate uptake by the organ.

The authors are indebted to Mr. Mario Martínez, Miss Emilia Pérez Ayuso, Mrs. Violeta de Herrera, Mrs. Erlinda de Quintero, and Mrs. Blanca Sleem for the various technical or secretarial contributions, and also acknowledge the revision of the manuscript by Dr. J. F. Pérez González.

1. De Venanzi, F., Peña, F., Jiménez V., O., and de Alvarado, H., Endocrinology **95,** 741 (1974).

2. Perlzweig, W. A., Latham, E., and Keefer, C. S., Proc. Soc. Exp. Biol. Med. **21,** 33 (1923).
3. De Venanzi, F., Proc. Soc. Exp. Biol. Med. **90,** 112 (1955).
4. Carnevalli, T., and Peña, F., Acta Physiol. Latinoam. **9,** 184 (1961).
5. Rasmussen, H., Pechet, M., and Fast, D., J. Clin. Invest. **47,** 18 (1968).
6. Wells, H., and Lloyd, W., Endocrinology **84,** 861 (1969).
7. Langan, T. A., Proc. Nat. Acad. Sci. USA **64,** 1276 (1969).
8. Langan, T. A., Ann. N.Y. Acad. Sci. **185,** 786 (1972).
9. Blat, C., and Loeb, J. E., FEBS Lett. **18,** 124 (1971).
10. Shlatz, L., and Marinetti, G. V., Biochem. Biophys. Res. Commun. **45,** 51 (1971).
11. Zahlten, R. N., Hochberg, A. A., Stratman, F. W., and Lardy, H. A., Proc. Nat. Acad. Sci. USA **69,** 800 (1972).
12. Johnson, E. M., and Allfrey, V. G., Arch. Biochem. Biophys. **152,** 786 (1972).
13. De Venanzi, F., Pfister, E., and Jiménez V., O., Acta Cient. Venezolana **22,** 37 (1971).
14. Martin, J. B., and Doty, D. M., Anal. Chem. **21,** 965 (1949).
15. Mortimore, G. E., Amer. J. Physiol. **204,** 699 (1963).
16. Jefferson, L. S., Exton, J. H., Butcher, R. W., Sutherland, E. W., and Park, C. R., J. Biol. Chem. **243,** 1031 (1968).
17. De Venanzi, F., Acta Physiol Latinoam **23,** 221 (1973).
18. Glinsmann, W. H., and Mortimore, G. E., Amer. J. Physiol. **215,** 553 (1968).
19. Kaji, H., and Park, C. R., Fed. Proc. **20,** 190 (1961).
20. Sarkar, A. K., and Ottaway, H., Biochem. J. **84,** 578 (1962).
21. Burton, S. D., and Ishida, T., Amer. J. Physiol. **209,** 1145 (1965).
22. Williams, T. F., Exton, J. H., Friedman, M., and Park, C. R., Amer. J. Physiol. **221,** 1645 (1971).
23. Park, C. R., Lewis, S. B., and Exton, J. H., Diabetes **21,** 439 (1972).
24. Mallette, L. E., and Exton, J. H., Endocrinology **93,** 640 (1973).
25. Walaas, O., Walaas, E., and Grønnerød, O., Acta Endocrinológica **77,** 93 (1973).

Received September 2, 1975. P.S.E.B.M. 1976, Vol. 152.