Stability of hexokinases A, B and C and *N*-acetylglucosamine kinase in liver cells isolated from rats submitted to diabetes and several dietary conditions

Alejandro REYES,* Eliana RABAJILLE, María Luz CÁRDENAS† and Hermann NIEMEYER
Departamento de Biología, Facultad de Ciencias Básicas y Farmacéuticas, Universidad de Chile, Santiago, Chile

(Received 13 December 1983/Accepted 10 April 1984)

The effect of dietary and hormonal variations on the specific activities of hexokinase isoenzymes, *N*-acetylglucosamine kinase and pyruvate kinase isoenzymes in parenchymal and non-parenchymal liver cells was studied. Hexokinase D was markedly decreased in hepatocytes from animals fasted or fed on the carbohydrate-free diet as well as from diabetic rats, attaining a constant low level of about 17% of normal values. Pyruvate kinase L was also diminished in hepatocytes under the same experimental conditions. In contrast, the three high-affinity hexokinase isoenzymes A, B and C remained without variation in total amount or in their relative proportions in hepatocytes and non-parenchymal liver cells isolated from animals under the various conditions studied. *N*-Acetylglucosamine kinase activities also did not change either in parenchymal or in non-parenchymal liver cells under all conditions. The results are discussed in relation to the significance of *N*-acetylglucosamine kinase and the various hexokinase isoenzymes for the phosphorylation of glucose after dietary and hormonal manipulations.

From the work of several groups it is known that hexokinase D, 'glucokinase' (ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2), is affected markedly in the whole liver by dietary and hormonal manipulations [for references see reviews by Niemeyer *et al.* (1975) and by Weinhouse (1976)]. The variations in hexokinase D activities are the result of changes in the amount of enzyme protein, as recognized by immunotitration (Clark-Turri *et al.*, 1974), and are related to the concentrations of the specific RNA (Spence, 1983). Work with isolated hepatocytes generally confirms the results obtained in the whole animal (Katz *et al.*, 1979; Schudt, 1979; Nakamura *et al.*, 1979; Spence & Pitot, 1979; Wakelam & Walker, 1980; Spence *et al.*, 1981).

The study of the effect of diets and hormones on hexokinases A, B and C (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) in liver has, however,

been neglected. Most workers have only measured the combined activity of hexokinases A, B and C by using the spectrophotometric assay method of Viñuela *et al.* (1963), a procedure that has several limitations (Niemeyer & Ureta, 1972; Reyes & Cardenas, 1984). Although there is general agreement on the constancy of total high-affinity hexokinase activity in liver after dietary and hormonal treatments [for reviews see Niemeyer *et al.* (1975) and Weinhouse (1976)], some authors have described variations in certain conditions. Thus Machiya & Hoyosa (1969) reported a decrease in hexokinase B activity in liver from diabetic rats; Pilakis (1970) reported an increase in the total activity of high-affinity hexokinases in the liver of hypophysectomized rats treated with thyroxine; and Wakelam *et al.* (1979) similarly observed an increase in the liver of neonatal rats treated with triiodothyronine.

The fact that *N*-acetylglucosamine kinase (ATP:2-acetamido-2-deoxy-D-glucose 6-phosphotransferase, EC 2.7.1.59) can be mistakenly identified as hexokinase D (Davagnino & Ureta, 1980; Allen *et al.*, 1980; Vera *et al.*, 1984) introduces the question of how much of the residual activity of hexokinase D generally observed can be attributed

* Present address: Instituto de Bioquímica, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile.

† To whom reprint requests and correspondence should be addressed at the Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

to *N*-acetylglucosamine kinase, whose response to dietary and hormonal factors has not been established.

In the present work we investigate the behaviour of each individual isoenzyme and *N*-acetylglucosamine kinase in isolated liver cells, under experimental conditions of the donor animals that promoted large changes on hexokinase D in the whole liver.

The results show that only the specific activities of hexokinase D displayed changes, and that those of the three high-affinity hexokinases and *N*-acetylglucosamine kinase remained without detectable modifications in hepatocytes and in non-parenchymal cells under all conditions used. Preliminary accounts of this work have been given in abstract form (Reyes *et al.*, 1980, 1981).

Experimental

With the exceptions mentioned below, reagents and methods were as in the preceding paper (Reyes & Cárdenas, 1984).

Reagents

Streptozotocin was a product of the Upjohn Co. Casein, salt and vitamin mixtures for the synthetic diets were from General Biochemicals.

Animal treatments

Male albino rats weighing about 200g were used. The animals were kept individually in cages in a room maintained at about 23°C with alternating 12 h periods of light and darkness, and were fed on an equilibrated synthetic diet (see below) for about 6 days before the start of the experiments. During this period the rats were weighed daily to accustom them to handling. This equilibrated synthetic diet and a carbohydrate-free diet, in which fat replaced carbohydrate with the same energy value (Niemeyer *et al.*, 1962), were used as experimental diets. The compositions of diets, as percentage of total energy, were as follows: equilibrated diet, casein 27%, carbohydrates (mixture of maltose and dextrins) 62% and fat (margarine and vegetable oil) 11%; carbohydrate-free diet, casein 26% and fat 74%. Both diets contained the same amount of salts and vitamins per 100 calculated joules of energy value (Niemeyer *et al.*, 1962). Intakes were restricted to be approximately isoenergetic by giving an equivalent to 210 kJ (50 kcal) of diet daily. Water was given *ad libitum*. To induce diabetes animals received an intravenous dose of streptozotocin (65 mg/kg body wt.) dissolved in 10 mM-sodium citrate buffer, pH 4.5. The injected animals were maintained several days on equilibrated diet. The severity of the diabetic state was monitored daily by measuring blood glucose. Animals dis-

playing minimal values of 350 mg of glucose/100 ml of blood after 96 h from the streptozotocin injection were employed.

Analytic procedures

Blood glucose was measured by the Somogyi-Nelson procedure (Nelson, 1944).

Results

Chromatographic quantification of hexokinases, N-acetylglucosamine kinase and pyruvate kinases

Fig. 1 shows chromatographic separation of hexokinase isoenzymes and *N*-acetylglucosamine kinase from hepatocytes and non-parenchymal cells isolated from normal or diabetic rats. As expected, hepatocytes isolated from normal rats (Fig. 1a) displayed a higher specific activity of hexokinase D than did those isolated from diabetic animals (Fig. 1b). However, the amounts of the three high-affinity hexokinases, as well as that of *N*-acetylglucosamine kinase, were apparently unaltered in hepatocytes (Figs. 1a and 1b) and non-parenchymal cells (Figs. 1c and 1d). Similar profiles to those obtained with the diabetic animals were obtained when fasted rats or rats fed on a carbohydrate-free diet for 6 or 12 days were used.

In order to quantify the amount of each enzyme, the areas under the chromatographic peaks of activities were integrated and referred to 1 mg of DNA of the original homogenates (Table 1). This approach assumes that any loss of enzyme activity is the same for each one. Since this presumption cannot be rigorously demonstrated, the data must be considered to be semi-quantitative. Hexokinase D decreased to about 17% of normal values in hepatocytes isolated from normal rats fasted for 4 days or fed on a carbohydrate-free diet for 6 days, as well as from 4-day-diabetic animals (Table 1). Differences in residual values were not significant ($P > 0.10$). The extension of the period on carbohydrate-free diet up to 12 days did not produce a further decrease of hexokinase D. The sum of hexokinases A, B and C for both parenchymal and non-parenchymal liver cells did not vary under the different experimental conditions. Small decreases observed in hepatocytes from fasted normal rats and diabetic animals were not statistically significant ($P > 0.10$) (Table 1). The rather high dispersion of the data may be attributed to either normal variation or experimental error. Not only the total activities, but also the relative proportions, of hexokinases A, B and C were very constant under all circumstances in hepatocytes as well as in non-parenchymal liver cells. Isoenzyme A is the predominant high-affinity hexokinase in both types of cells, and exists in a higher proportion in non-parenchymal cells (about 60%) than in hepatocytes

Fig. 1. DEAE-cellulose chromatography of hexokinase isoenzymes and *N*-acetylglucosamine kinase of hepatocytes and non-parenchymal cells isolated from normal and diabetic rats

Hepatocytes (*a* and *b*; left-hand column) and non-parenchymal liver cells (*c* and *d*) from a normal (*a* and *c*; top row) or from a 4-day-diabetic rat (*b* and *d*) were obtained and processed as described in Reyes & Cárdenas (1984). Diabetes was induced by the intravenous injection of 65 mg of streptozotocin/kg body wt. Samples of supernatants equivalent to 0.4–0.67 mg of cell DNA were chromatographed, and the enzyme activities were normalized to 1 mg of DNA of the original homogenates. Hexokinases were measured at 0.5 mM-glucose (●) by a radioassay or at 100 mM-glucose (○) spectrophotometrically. Activities at 100 mM-glucose are shown as one-tenth of the actual values. *N*-Acetylglucosamine kinase (□) was measured by a radioassay with 0.7 mM-*N*-acetylglucosamine.

(about 40%). The ratio between isoenzymes B and C was, however, the same, roughly 3:5 in both types of cells.

Table 1 also shows that *N*-acetylglucosamine kinase from hepatocytes and non-parenchymal cells did not change under the different experimental conditions studied.

The effect of a carbohydrate-free diet on the cellular distribution of liver pyruvate kinase isoenzymes was also studied, because pyruvate kinase isoenzymes L and K are good cellular markers for hepatocytes and non-parenchymal cells respectively in normal well-fed rats (Reyes & Cárdenas, 1984). Isoenzyme L activity fell to about 20% of normal values in hepatocytes isolated from animals submitted to a carbohydrate-free diet for 6

days (from 4.27 units/mg of DNA to 0.77 unit/mg of DNA). In contrast, the isoenzyme K activity was not affected (from 2.86 units/mg of DNA to 2.66 units/mg of DNA). Under this condition pyruvate kinase isoenzymes are still good cellular markers. As observed with hexokinase D, the extension of the period on carbohydrate-free diet to 12 days gave almost no further decrease in the isoenzyme L activity, the final value being 0.71 unit/mg of DNA, equivalent to 18% of normal values. These results in isolated liver cells from animals on carbohydrate-free diet agree with data from others, demonstrating that adaptive fluctuations of pyruvate kinase in the whole liver correspond only to changes of isoenzyme L (Tanaka *et al.*, 1965, 1967; Kohl & Cottam, 1976).

Table 1. Activities of hexokinase D, high-affinity hexokinases and *N*-acetylglucosamine kinase in hepatocytes and non-parenchymal liver cells isolated from animals submitted to different experimental conditions

Activities were estimated from the corresponding peaks of the chromatographic patterns. Results are means $\pm$ S.E.M., with the numbers of observations in parentheses. (For each individual high-affinity hexokinase the number of experiments was the same as for the total of the three). Significance was tested by using Student's *t* test.

Experimental conditions	Activity (munits/mg of cell DNA)					
	High-affinity hexokinases				Hexokinase D (‘glucokinase’)	<i>N</i> -Acetylglucos- amine kinase
	A	B	C	Total		
Hepatocytes						
Equilibrated diet	9.0±2.2	5.5±1.3	8.4±2.1	22.9±2.8 (6)	572±124 (4)*	36.4±5.3 (4)
Carbohydrate-free diet:						
6 days	9.7±1.4	5.1±0.9	8.6±1.7	23.4±2.0 (7)†	97±30 (3)	34.1±6.0 (3)
12 days				20.3±1.5 (4)†	92±23 (4)	30.2±4.5 (3)
Fasting 4 days	8.3±1.6	3.8±0.5	6.1±0.2	18.2±1.4 (4)†	88±17 (3)	42.8 (1)
Diabetes 4 days	8.2±2.1	3.5±0.7	6.4±1.1	18.0±2.8 (3)†	85±24 (3)	44.8 (1)
Non-parenchymal cells						
Equilibrated diet	16.6±1.4	4.4±0.3	7.4±0.7	26.7±1.0 (6)	0.0	4.8 (2)
Carbohydrate-free diet:						
6 days	16.7±1.2	4.0±0.4	6.6±0.4	27.3±0.7 (5)	0.0	4.0 (2)
Fasting 4 days	15.5	3.5	6.7	25.7 (1)	0.0	
Diabetes 4 days	16.2	4.7	5.7	26.5 (1)	0.0	4.2 (1)

* This activity is about 20% higher than that observed with hepatocytes from animals with a commercial pellet diet [cf. results quoted by Niemeyer *et al.* (1975) for whole liver].

† Not significantly different from the value for the equilibrated diet ($P > 0.1$).

Discussion

This study shows that neither the total amounts nor the relative proportions of the hexokinases A, B and C displayed any significant changes in parenchymal or non-parenchymal liver cells isolated from rats submitted to conditions that markedly affected the amount of hexokinase D. The invariability of the activities of high-affinity hexokinases generally agrees with the observations made by González *et al.* (1964) in whole liver, but contrasts with the work by Machiya & Hosoya (1969), who reported a decrease of hexokinase B in the whole liver of diabetic rats. One possible explanation of the last result is that during the separation procedure inactivation or leakage of hexokinase B could have occurred in the preparation from diabetic animals, since it is known to be unstable (Grossbard & Schimke, 1966; Reyes & Cárdenas, 1984). In our work the constant presence of dithiothreitol and glycerol in all steps of the preparation of extracts and separation of the enzymes, together with strict precautions taken during cell isolation (Reyes & Cárdenas, 1984), would avoid any inactivation or leakage. Our results thus give solid support to the constancy of combined high-affinity hexokinase activity in the liver from diabetic rats and animals submitted to dietary manipulations. Brain and kidney, whose high-affinity hexokinase patterns are similarly characterized by high activities of hexokinase A,

also show stable combined hexokinase activity in diabetes (Grossbard & Schimke, 1966; Katzen, 1967; Katzen *et al.*, 1970). On the other hand hexokinase B activity diminishes as an effect of fasting or diabetes in muscle, adipose tissue and mammary gland, which do not contain hexokinase D but exhibit high activities of isoenzyme B and little or no isoenzyme A and C (Katzen & Schimke, 1965; Katzen, 1967; Walters & McLean, 1967; Hansen *et al.*, 1967; Pilkis, 1970; Katzen *et al.*, 1970).

A piece of information about the adaptive character of hexokinase D that was still missing is whether it disappears completely or attains a low basal level, indicating that a new steady state has been established. This problem was not solved previously, in part because most observations have not been made for periods long enough to let hexokinase D activities decrease to very low values, and also because of the uncertainty of the enzyme assay in crude extracts, especially at low activities of the enzyme (Niemeyer & Ureta, 1972; Reyes & Cárdenas, 1984). Furthermore, a small residual activity, as measured at 100 mM-glucose, could be the result of glucose phosphorylation by *N*-acetylglucosamine kinase (Davagnino & Ureta, 1980; Allen *et al.*, 1980; Vera *et al.*, 1984). Our results indicate very clearly that hexokinase D decreases under several conditions to about 17% of normal values. This residual activity is clearly distinct from the glucose-phosphorylating activity resulting from the presence of *N*-acetylglucosamine

about 2–4-fold higher than the sum of hexokinases A, B and C when physiological concentration of 5–15 mM and the half-saturation glucose concentration values of the isoenzymes are considered for calculations. Both the residual hexokinase D and the high-affinity hexokinases would thus contribute to the low glucose phosphorylation observed by Bontemps *et al.* (1978) in hepatocytes isolated from diabetic adult rats. If each isoenzyme had a specific role in a particular metabolic pathway, as postulated by Ureta (1978), hexokinase D could still accomplish its function in conditions such as diabetes and starvation, but at a diminished rate. The residual hexokinase D is in accord with the residual concentration of translatable mRNA coding for rat liver hexokinase D in starved and diabetic animals, as reported by Spence (1983).

N-Acetylglucosamine kinase activities did not show any significant modification under the various experimental conditions explored, either in hepatocytes or in non-parenchymal liver cells. Residual hexokinase D activity present after prolonged diabetes, starvation or feeding on a carbohydrate-free diet is over 40 times higher than the glucose-phosphorylating activity that *N*-acetylglucosamine kinase would have at physiological glucose concentrations (5–15 mM), and thus a possible subsidiary role of this enzyme under those conditions cannot be significant.

We are grateful to Dr. T. Ureta and Dr. A. J. Cornish-Bowden for help in revising this manuscript. This work was supported by grants from the Departamento de Desarrollo de la Investigación, Universidad de Chile, from the Multinational Program of Biochemistry, Organization of the American States, and from the Regional Program for Graduate Training in Biological Sciences, P.N.U.D.–U.N.E.S.C.O. RLA 78/024.

References

- Allen, M. B., Brockelbank, J. L. & Walker, D. G. (1980) *Biochim. Biophys. Acta* **614**, 357–366
- Bontemps, F., Hue, L. & Hers, H. G. (1978) *Biochem. J.* **174**, 603–611
- Clark-Turri, L., Peñaranda, J., Rabajille, E. & Niemeyer, H. (1974) *FEBS Lett.* **41**, 342–344
- Davagnino, J. & Ureta, T. (1980) *J. Biol. Chem.* **255**, 2633–2636
- González, C., Ureta, T., Sánchez, R. & Niemeyer, H. (1964) *Biochem. Biophys. Res. Commun.* **16**, 347–352
- Grossbard, L. & Schimke, R. T. (1966) *J. Biol. Chem.* **241**, 3546–3560
- Hansen, R., Pilkis, S. & Krah, M. E. (1967) *Endocrinology (Baltimore)* **81**, 1397–1404
- Katz, N. R., Nauck, M. A. & Wilson, P. T. (1979) *Biochem. Biophys. Res. Commun.* **88**, 23–29
- Katzen, H. M. (1967) *Adv. Enzyme Regul.* **5**, 335–356
- Katzen, H. M. & Schimke, R. T. (1965) *Proc. Natl. Acad. Sci. U.S.A.* **54**, 1218–1225
- Katzen, H. M., Soderman, D. D. & Wiley, C. E. (1970) *J. Biol. Chem.* **245**, 4081–4096
- Kohl, E. A. & Cottam, G. L. (1976) *Arch. Biochem. Biophys.* **176**, 671–682
- Machiya, T. & Hosoya, N. (1969) *Endocrinol. Jpn.* **16**, 433–445
- Nakamura, T., Aoyama, K. & Ichihara, A. (1979) *Biochem. Biophys. Res. Commun.* **91**, 515–520
- Nelson, N. A. (1944) *J. Biol. Chem.* **153**, 374–380
- Niemeyer, H. & Ureat, T. (1972) in *Molecular Basis of Biological Activity* (Gaede, K., Horecker, B. L. & Whelan, W. J., eds.), pp. 221–273, Academic Press, New York
- Niemeyer, H., Pérez, N., Radojković, J. & Ureta, T. (1962) *Arch. Biochem. Biophys.* **96**, 662–669
- Niemeyer, H., Ureta, T. & Clark-Turri, L. (1975) *Mol. Cell. Biochem.* **6**, 109–126
- Pilkis, S. (1970) *Biochim. Biophys. Acta* **215**, 461–476
- Reyes, A. & Cárdenas, M. L. (1984) *Biochem. J.* **221**, 303–309
- Reyes, A., Cárdenas, M. L., Rabajille, E. & Niemeyer, H. (1980) *Arch. Biol. Med. Exp.* **13**, 102 (Abstr.)
- Reyes, A., Cárdenas, M. L., Rabajille, E. & Niemeyer, H. (1981) *Arch. Biol. Med. Exp.* **14**, 290 (Abstr.)
- Schudt, C. (1979) *Eur. J. Biochem.* **98**, 77–82
- Spence, J. T. (1983) *J. Biol. Chem.* **258**, 9143–9146
- Spence, J. T. & Pitot, H. C. (1979) *J. Biol. Chem.* **254**, 12331–12336
- Spence, J. T., Merrill, M. & Pitot, H. C. (1981) *J. Biol. Chem.* **256**, 1598–1603
- Tanaka, T., Harano, Y., Morimura, H. & Mori, R. (1965) *Biochem. Biophys. Res. Commun.* **21**, 55–60
- Tanaka, T., Harano, Y., Sue, F. & Morimura, H. (1967) *J. Biochem. (Tokyo)* **62**, 71–91
- Ureta, T. (1978) *Curr. Top. Cell. Regul.* **13**, 233–258
- Vera, M. L., Cárdenas, M. L. & Niemeyer, H. (1984) *Arch. Biochem. Biophys.* **229**, 237–245
- Viñuela, E., Salas, M. & Sols, A. (1963) *J. Biol. Chem.* **238**, PC1175–PC1177
- Wakelam, M. J. & Walker, D. G. (1980) *FEBS Lett.* **111**, 115–119
- Wakelam, M. J., Aragón, C., Giménez, C., Allen, M. B. & Walker, D. G. (1979) *Eur. J. Biochem.* **100**, 467–475
- Walters, E. & McLean, P. (1967) *Biochem. J.* **104**, 778–783
- Weinhouse, S. (1976) *Curr. Top. Cell. Regul.* **11**, 1–50