Ethanol modulation of the hormonal and nutritional regulation of glucose 6-phosphate dehydrogenase activity in primary cultures of rat hepatocytes

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The hormonal and nutritional regulation of glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) was studied in primary cultures of rat hepatocytes maintained in a chemically defined medium. Inoculation of hepatocytes from starved rats into primary cultures resulted in a 4–5-fold increase in G6PDH activity in 48 h in the absence of hormones. Parallel cultures treated simultaneously with glucocorticoids and insulin exhibited a 12–15-fold increase during the same time. Glucocorticoids by themselves did not elevate G6PDH activity, whereas insulin alone significantly stimulated enzyme activity. Thus the glucocorticoids acted in a 'permissive' role to amplify the insulin stimulation of G6PDH. Elevated concentrations of glucose in the culture medium increased enzyme activity in both the control cultures and those treated with hormones. Ethanol was found to potentiate G6PDH activity in cultures treated with glucocorticoids and insulin. The effect of ethanol was time- and dose-dependent. These results establish that insulin, glucocorticoids, glucose and ethanol interact in some undefined manner to regulate hepatic G6PDH activity.

G6PDH is the key rate-limiting enzyme of the pentose phosphate pathway in glucose metabolism. One of the important roles of this pathway is the generation of NADPH in the cytoplasmic fraction of the cell. The reducing power of NADPH is utilized for various synthetic processes, especially for synthesis of fatty acids. Thus G6PDH participates in the regulation of both lipogenesis and glucose metabolism.

Studies in intact animals have established that liver G6PDH is regulated by hormonal and nutritional factors. G6PDH activity is decreased during starvation or refeeding of a fat-rich diet, and is markedly increased during refeeding of carbohydrate-rich diets to starved animals (Tepperman & Tepperman, 1958; Fitch & Chaikoff, 1960; Potter & Ono, 1961; McDonald & Johnson, 1965; Yugari & Matsuda, 1967; Johnson & Sassoon, 1967; Sassoon *et al.*, 1968; Dror *et al.*, 1973; Yagil *et al.*, 1974; Mack *et al.*, 1974; Kelley *et al.*, 1975; Dao *et al.*, 1982). Enzyme activity is increased by injection of insulin (Freedland *et al.*, 1966; Rudack

et al., 1971; Szepesi *et al.*, 1972), glucocorticoids (Berdanier *et al.*, 1976; Berdanier & Shubeck, 1979; Bouillon & Berdanier, 1980; Wurdeman *et al.*, 1978) and thyroid hormone (Glock & McLean, 1955; Huggins & Yao, 1959; Miksicek & Towle, 1982), and decreased by injection of glucagon (Rudack & Holten, 1974). Since studies of intact animals fail to distinguish between the direct and indirect effects of hormones or nutrients, studies with isolated hepatocytes in primary culture have been performed. The findings reported with cultured hepatocytes are not consistent with those in the intact animals, and furthermore the results of the two different groups that have studied G6PDH in cultured hepatocytes are not in agreement. Kurtz & Wells (1981) reported that glucose and thyroid hormone had no effect on enzyme activity, whereas insulin and dexamethasone each caused a 2–3-fold stimulation. However, Nakamura *et al.* (1982) reported that the glucocorticoids had no effect on basal or insulin-stimulated G6PDH activity. Both of these groups either used calf serum to coat the culture dishes or maintained cells in medium containing serum.

In view of the above disagreements and the fact that serum may be interfering with the precise

Abbreviation used: G6PDH, glucose 6-phosphate dehydrogenase (EC 1.1.1.49).

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assessment of hormonal involvement in G6PDH regulation, we have examined the regulation of the enzyme in cultured hepatocytes attached to collagen-coated dishes maintained in a completely defined chemical medium. Our results establish that the glucocorticoids by themselves did not increase enzyme activity, but that they amplified the insulin stimulation of G6PDH. Glucose was found to enhance both the basal and the hormonally stimulated G6PDH activity. An unexpected finding during the course of these studies was that ethanol strongly potentiated the increase in G6PDH activity caused by the combination of glucocorticoids and insulin in cells maintained in a high-glucose medium. Since liver plays a central role in lipid metabolism, and accumulation of fat in the liver is the most common disturbance of lipid metabolism produced by ethanol (Baraona & Lieber, 1979), the enhancement of G6PDH activity by ethanol may be a contributing factor to alcoholic fatty liver, because more NADPH would be available for fatty acid synthesis.

Experimental

Materials

Male Sprague-Dawley rats were obtained from Hilltop Farms (Scottsdale, PA, U.S.A.). Swim's S-77 and Waymouth's 752 media were purchased from GIBCO. Crystalline pig insulin (lot no. 615-07J-256) was generously given by Dr. W. W. Bromer of Eli Lilly and Co. Dexamethasone acetate, NADP⁺, glucose 6-phosphate, 6-phosphogluconic acid (grade III), 6-phosphogluconate dehydrogenase (type V) and collagenase (type I) were supplied by Sigma Chemical Co. Dexamethasone sodium phosphate was kindly given by Dr. K. Bohra of Organon.

Methods

Animal preparation. Rats were maintained in individual cages with free access to laboratory chow and water. The animal quarters were maintained at 25°C with an alternating light and dark cycle of 12h each. The animals weighed 200–300g at the time of death and were kept under the controlled conditions for at least 1 week before hepatocyte isolation. Food was withheld for 24h before the isolation of liver cells, which was done 2–3h after the onset of light cycle.

Hepatocyte isolation and culture. Hepatocytes were isolated by the method of Berry & Friend (1969) as modified by Bonney *et al.* (1974), with the following additional changes. Recirculating liver perfusion *in situ* was performed with Ca²⁺-free EGTA (0.5mM)-containing Krebs-Ringer bicarbonate medium, pH7.4, at 37°C. After a 15min

perfusion with the above medium, the perfusion medium was changed to one that lacked EGTA but contained Ca²⁺ (1.0mM), collagenase (type I; 0.5mg/ml) and soya-bean trypsin inhibitor (1mg/100ml). The collagenase-containing medium was recirculated through the liver, but the Ca²⁺-free pre-perfusion medium was not recirculated. The liver cells were dispersed after a 10–12min perfusion with collagenase-containing medium. Liver cells were collected in Ca²⁺-free Swim's S-77 medium, and hepatocytes were isolated by filtration and centrifugation as previously reported (Kletzien *et al.*, 1982). Hepatocytes suspended in Waymouth's 752 medium were cultured in 100-mm collagen-coated culture dishes as described by Kletzien *et al.* (1982). In the present studies oleate, bovine serum albumin and insulin were not added to the Waymouth's medium. Gentamycin (20µg/ml) was added to both the Swim's and Waymouth's media. The glucose concentration in the Waymouth medium was 28mM and that of S-77 medium was 5.5mM in all experiments unless specified otherwise.

The culture medium was changed at 2h after plating and then every 24h. As indicated in the Figure legends, Waymouth's medium was used for the entire period of cell culture in some experiments, but in others cells were transferred to S-77 medium 2h after plating. In these latter experiments, cultures were rinsed twice with Swim's S-77 medium at 2h, and the same medium was used to maintain the cultures, and for subsequent changes. Insulin (0.5mg/ml) was dissolved in a solution containing 0.2% bovine serum albumin (fraction V) adjusted to pH2.5 with 0.1M-HCl. Dexamethasone acetate in 10% (v/v) ethanol or dexamethasone sodium phosphate in water were dissolved to a concentration of 0.1mM. Alternatively, dexamethasone acetate or cortisol was dissolved in dimethyl sulphoxide and diluted to a final concentration of 0.1mM in S-77 medium (final concentration of dimethyl sulphoxide was 0.01%). Then 60µl of the hormone solution or diluting solutions was added to each dish containing 6ml of the medium. Unless otherwise specified, insulin (140munits/ml) and dexamethasone (1µM) were added at 2h after plating and were present until the time of cell harvest.

Cell harvest. Cells in each culture dish were rinsed with 3 × 6ml of 0.9% NaCl solution. The culture dishes were then immediately frozen at –20°C, and kept frozen until the cells were scraped into 1.0ml of a pH8.0 buffer containing 0.15M-KCl, 10mM-Tris, 1mM-EDTA, 0.5mM-NADP⁺ and 2mM-β-mercaptoethanol. The cell suspension was homogenized for 2min in a Potter-Elvehjem homogenizer surrounded by an ice slurry. The cell homogenate was centrifuged at 1000g for 1h. The supernatant from this centri-

fugation was used for enzyme and protein determinations. All the operations of cell harvest, homogenization and centrifugation were performed at a temperature of 0–4°C. The supernatants were stored at –20°C until the enzyme activity was determined.

Determination and expression of enzyme activity. The activity of G6PDH in the supernatants was determined by the method of Dror *et al.* (1970) by using 50–100 μ l samples of the enzyme supernatant in a final reaction volume of 1 ml. The reaction mixture was at pH 7.6, and it contained 0.16 M-Tris base, 0.03 M-MgCl₂, 0.0033 M-glucose 6-phosphate, 0.0012 M-NADP⁺ and 0.5 unit of 6-phosphogluconate dehydrogenase/ml. One unit of G6PDH activity represents the production of 1 μ mol of NADPH/min at 25°C. Protein in the supernatant was determined by the method of Lowry *et al.* (1951). Enzyme activity is expressed as munits/mg of protein.

Results

G6PDH activity in cultured hepatocytes

To find the time required for the maximum stimulation of G6PDH activity by the hormones or the medium alone, we determined the enzyme activity in cultures harvested at several different times after the initial plating. Comparisons of such activities with that found in the freshly isolated hepatocytes are shown in Fig. 1. The maximum activity owing to the addition of dexamethasone acetate and insulin or the diluting solutions for these hormones (control) was attained within about 48 h after plating. At this time there was about 4–5-fold increase in enzyme activity in the control group and about 12–15-fold in the hormonal group when compared with that in the freshly isolated hepatocytes. In subsequent experiments the cultures were treated with hormones for 48 h.

Effect of different hormone concentrations on G6PDH activity

The effect of six different insulin concentrations (0.0014–140 munits/ml) individually or in the presence of dexamethasone (1 μ M) in cultures maintained in Waymouth medium was tested and is shown in Fig. 2(a). Insulin concentrations of 0.014 munits/ml or lower did not affect the enzyme activity, and the maximum effect was obtained with an insulin concentration of 14 munits/ml. The presence of dexamethasone increased the enzyme activity obtained in response to insulin, but it did not affect the concentration of insulin needed for maximum or half-maximum stimulation of en-

Fig. 1. Time course for G6PDH activity of cultured hepatocytes

Hepatocytes were maintained in Waymouth's medium throughout the culture period, with fresh medium provided at 2, 24 and 48 h after the initial plating. The hormones were first added after the 2 h change of medium and then after every change of medium. The control group (○) received the diluting solutions for the hormones, whereas the hormone-treated group (□) received insulin (140 munits/ml) and dexamethasone acetate (Dex; 1 μ M). Enzyme activity of the freshly isolated hepatocytes is represented by the triangle. Data shown are the means $\pm$ S.E.M. from three different experiments, except for the 36, 60 and 72 h time points, where they represent two experiments. Where the data points are without standard errors, either they represent two experiments or the standard errors are within the symbols.

zyme activity. In subsequent experiments insulin concentrations of 140 munits/ml were used.

In the experiment shown in Fig. 2(b), the effects of five different concentrations of dexamethasone acetate added individually or concomitantly with insulin (140 munits/ml) were tested. Individually, none of the dexamethasone doses had any effect on G6PDH activity. When added with insulin, dexamethasone concentrations of 0.1 nM did not affect the insulin stimulation of the enzyme activity. Potentiation of insulin's effect on G6PDH activity by dexamethasone was observed at the latter's concentration of 1 nM, and the maximum enhancement was obtained at 0.1 μ M-dexamethasone. Dexamethasone at a concentration of 1 μ M was used in subsequent experiments.

Effect of glucose on G6PDH activity

Feeding of carbohydrate-rich diet to starved animals causes a 10–20-fold increase in liver G6PDH activity; however, glucose was reported

Fig. 2. Effect of insulin and dexamethasone alone and in combination on G6PDH activity

Cells were maintained in Waymouth medium, with fresh medium being provided at 2 and 24 h after plating. Different concentrations of insulin (a) alone (○) or with 1 μ M-dexamethasone acetate (Dex; □) were added after the changes of media at 2 and 24 h. In (b), different concentrations of dexamethasone acetate alone (○) or in the presence of insulin (140 munits/ml; □) were added after the changes of media at 2 and 24 h. Cells were harvested at 48 h after initial plating and prepared for the determination of enzyme activity. The enzyme activity of freshly isolated hepatocytes is shown by Δ . Data shown are from a representative of the two experiments.

to have no effect on this enzyme in cultured rat hepatocytes (Kurtz & Wells, 1981). We therefore examined the effect of glucose on G6PDH activity in hepatocytes maintained under our culture conditions. Fig. 3 shows that increasing the glucose concentration in the medium from 5.5 to 45.5 mM approximately doubled the enzyme activity in both the control and dexamethasone + insulin-treated groups. In both groups the maximum enzyme activity was obtained with a glucose concentration of about 25 mM. In both the low (5.5 mM)- and high (45.5 mM)-glucose media, dexamethasone + insulin caused about a 3-fold increase compared with the corresponding control groups with no hormones added. If the enzyme activities of different groups are compared with that in the freshly isolated

Fig. 3. Effect of glucose on G6PDH activity
Cells plated in Waymouth medium were transferred to S-77 medium after 2 h in culture, and fresh S-77 medium was again provided at 24 h. Glucose concentrations in the S-77 medium were adjusted by adding glucose dissolved in water to the basal medium. The concentration of glucose in the medium without supplementation was 5.5 mM. Dexamethasone acetate (Dex; 1 μ M) and insulin (140 munits/ml) were added to the hormone-treated group (□), and the diluting solutions were added to the group not treated with hormones (○) after the 2 h change of medium and then again after the 24 h change. Enzyme activity of freshly isolated hepatocytes is shown by Δ . Data shown are the means $\pm$ S.E.M. for three different experiments.

hepatocytes, high glucose alone caused a 4–5-fold increase, whereas high glucose plus both the hormones caused about 12-fold increase in enzyme activity. These increases in enzyme activity in S-77 medium containing high glucose are comparable with those in Waymouth medium, which contains 28 mM-glucose (Fig. 1). Low glucose alone caused only 2–3-fold increase in enzyme activity, whereas low glucose plus hormones caused a 6–7-fold increase.

Effect of ethanol on G6PDH activity

Results presented in Figs. 1–3 were obtained by using dexamethasone acetate [dissolved in ethanol, with a final concentration of 17 mM (0.1%)]. Since this was a considerable concentration of ethanol, we next examined if ethanol itself could affect the enzyme activity. Data presented in Fig. 4 show that ethanol caused a more than 2-fold increase in enzyme activity in cultures treated with insulin and dexamethasone phosphate (dissolved in water). The maximum ethanol effect was obtained with a concentration of 17 mM. Addition of 17 mM-ethanol to cells maintained in S-77 medium containing

5.5 mM-glucose and both the hormones, or 45.5 mM-glucose in the absence of hormones, caused less than a 20% increase in enzyme activity. Ethanol did not cause a significant increase in enzyme activity in cultures maintained in S-77 medium with 5.5 mM-glucose and no hormones (results not shown). The concentration of ethanol required to cause maximum stimulation of G6PDH activity for cells maintained in Waymouth medium was again about 17 mM; this concentration caused about a 50% increase in enzyme activity in both the control and hormone-treated groups (results not shown).

Relationship between time and G6PDH activity after ethanol addition to cultures

The time-dependence of the ethanol effect on G6PDH activity was examined to try to distinguish between the activation of pre-existing enzyme versus the induction of new enzyme synthesis by ethanol. Results presented in Fig. 5 show that ethanol had no effect on enzyme activity up to 12 h after its addition. The effect was noticeable at 24 h, and there was almost a linear increase in enzyme activity between 24 and 48 h after ethanol addition. These results suggest that the effect of ethanol on G6PDH activity does not involve activation, but more likely results from the synthesis of new enzyme.

Fig. 4. *Effect of ethanol on G6PDH activity*

At 2 h after plating in the Waymouth medium, cells were maintained in S-77 medium containing 45.5 mM-glucose and different concentrations of ethanol as indicated. Insulin (140 munits/ml) and dexamethasone phosphate (1 μ M) were also included in the S-77 medium. Fresh S-77 medium containing the same concentrations of ethanol, glucose and hormones as at 2 h was provided at 24 h after plating. Cells were harvested to assay the enzyme activity at 48 h after plating. Data shown are the means $\pm$ S.E.M. for three culture dishes from a single experiment. Similar results were obtained in several experiments.

Fig. 5. *Time course of ethanol effect on G6PDH activity*

Hepatocytes maintained in S-77 medium containing glucose and hormone concentrations indicated in the legend to Fig. 4 were provided with 17 mM-ethanol after the changes of media at 2 and 24 h. Cells were harvested at the indicated times after the addition of ethanol, and the enzyme activity was determined. The data shown are from a representative of the two experiments.

Discussion

Our results show that G6PDH activity of the cultured hepatocytes is regulated by glucose, insulin, glucocorticoids and ethanol. The detectable enzyme activity seems to result from the interaction of these and perhaps some other factors. The culture medium alone without any hormones caused an increase in enzyme activity when the activity of cultured hepatocytes was compared with that found in freshly isolated hepatocytes. Although other factors might contribute to this increase, our data indicate that glucose in the medium is at least partially responsible for it. When cells were maintained in S-77 medium containing only 5.5 mM-glucose, there was only 2–3-fold increase in enzyme activity, but there was a 4–5-fold increase in enzyme activity when cells were maintained in S-77 medium containing 45.5 mM-glucose or in Waymouth medium containing 28 mM-glucose. The hypothesis that glucose regulates enzyme activity is further strengthened by the positive correlation between enzyme activity and glucose concentration in the medium (Fig. 3). Results in Fig. 3 show that glucose influences both the basal and hormonally regulated G6PDH activity. These findings are consistent with studies *in vivo*, where carbohydrate-rich diets caused a several-fold increase in liver G6PDH activity (McDonald & Johnson, 1965; Tepperman & Tepperman, 1958; Fitch & Chaikoff, 1960; Potter & Ono, 1961), but are not in agreement with the results obtained with cultured hepatocytes, where glucose was reported to have no effect on G6PDH activity (Kurtz & Wells, 1981). We are not sure of the reasons for the discrepancy between our results and those reported previously (Kurtz & Wells, 1981); perhaps the differences in culture conditions or animal preparation might be responsible for this variation.

Excessive ethanol ingestion results in profound derangements of lipid and lipoprotein metabolism, leading to fatty liver and subsequent liver failure. The contribution of enhanced fatty acid synthesis to alcoholic fatty liver is not clear. Results from experiments conducted *in vivo* showed no stimulation of fatty acid synthesis by ethanol from precursors other than ethanol, although the experiments conducted *in vitro* showed the incorporation of a large fraction of the carbon skeleton of ethanol into fatty acids (Baraona & Lieber, 1979). To the best of our knowledge none of the previous studies have viewed the role of G6PDH in the development of alcoholic fatty liver. In one study *in vivo*, chronic feeding of ethanol to rats decreased G6PDH activity in the livers of male, but not of female, rats (Winston & Reitz, 1980). Such results obtained with intact animals do not distinguish between the direct and indirect effects of ethanol. G6PDH is

one of the key enzymes affecting the reduction of NADP^+ , which in turn regulates the activity of the enzyme fatty acid synthetase (Baraona & Lieber, 1979). In view of our findings about the effect of ethanol on G6PDH activity, it appears that the induction of this enzyme by ethanol may be at least partially responsible for the development of alcoholic fatty liver.

In monolayer cultures of rat hepatocytes, we have found that ethanol doubled the enzyme activity induced by the combined influence of high glucose concentration and hormones (Fig. 4). The ethanol effect on G6PDH activity could not be observed in hepatocytes maintained in S-77 medium containing 5.5 mM-glucose and devoid of hormones. Furthermore, ethanol caused less than a 20% increase in G6PDH activity in cells maintained in S-77 medium with 45.5 mM-glucose but no hormones, or when hormones were included in the medium containing 5.5 mM-glucose (results not shown). These findings show that a combination of glucocorticoid, insulin and high concentration of glucose are required for the maximum stimulation of G6PDH activity by ethanol.

Our results do not distinguish between the specific sites at which insulin, glucocorticoids, glucose and ethanol affect G6PDH activity. The effect of all these modulators required several hours before it could be noted, thus suggesting that synthesis of new protein(s) is involved. Ethanol or some of its metabolites could also affect G6PDH activity and/or synthesis by changing the $\text{NADP}^+/\text{NADPH}$ ratio. Several different mechanisms involving transcription, translation or post-translation modification could explain the effects observed in the present study.

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References

- Baraona, E. & Lieber, C. S. (1979) *J. Lipid Res.* **20**, 289–315
- Berdanier, C. D. & Shubeck, D. (1979) *J. Nutr.* **108**, 1766–1771
- Berdanier, C. D., Wurdeman, R. & Tobin, R. B. (1976) *J. Nutr.* **106**, 1791–1800
- Berry, M. N. & Friend, D. S. (1969) *J. Cell Biol.* **43**, 506–520
- Bonney, R. J., Becker, J. E., Walker, P. R. & Potter, V. R. (1974) *In Vitro* **9**, 399–433
- Bouillon, D. J. & Berdanier, C. D. (1980) *J. Nutr.* **110**, 286–297
- Dao, M. L., Johnson, B. C. & Hartman, P. E. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2860–2864
- Dror, Y., Sassoon, H. F., Watson, J. J. & Johnson, B. C. (1970) *Clin. Chim. Acta* **28**, 291–298

- Dror, Y., Sassoon, H. F., Watson, J. J., Mack, D. D. & Johnson, B. C. (1973) *J. Nutr.* **103**, 342–346
- Fitch, W. M. & Chaikoff, J. L. (1960) *J. Biol. Chem.* **235**, 554–557
- Freedland, R. A., Cunliffe, T. L. & Zinkl, J. G. (1966) *J. Biol. Chem.* **241**, 5448–5451
- Glock, G. E. & McLean, P. (1955) *Biochem. J.* **61**, 390–397
- Huggins, C. & Yao, F. O. (1959) *J. Exp. Med.* **110**, 899–919
- Johnson, B. C. & Sassoon, H. F. (1967) *Adv. Enzyme Regul.* **5**, 93–106
- Kelley, D. S., Watson, J. J., Mack, D. O. & Johnson, B. C. (1975) *Nutr. Rep. Int.* **12**, 121–135
- Kletzien, R. F., Weber, C. A., Butcher, F. R. & Stumpo, D. J. (1982) *J. Cell. Physiol.* **110**, 304–310
- Kurtz, J. W. & Wells, W. W. (1981) *J. Biol. Chem.* **256**, 10870–10875
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mack, D. D., Watson, J. J., Chandler, A. M. & Johnson, B. C. (1974) *J. Nutr.* **104**, 12–17
- McDonald, B. E. & Johnson, B. C. (1965) *J. Nutr.* **87**, 161–167
- Miksicek, R. J. & Towle, H. C. (1982) *J. Biol. Chem.* **257**, 11829–11835
- Nakamura, T., Yoshimoto, K., Aoyama, K. & Ichihara, A. (1982) *J. Biochem. (Tokyo)* **91**, 681–693
- Potter, V. R. & Ono, T. (1961) *Cold Spring Harbor Symp. Quant. Biol.* **26**, 355–362
- Rudack, D. G. & Holten, D. (1974) *J. Biol. Chem.* **250**, 3960–3965
- Rudack, D. G., Chrisholm, E. M. & Holten, D. (1971) *J. Biol. Chem.* **246**, 1249–1254
- Sassoon, H. F., Watson, J. J. & Johnson, B. C. (1968) *J. Nutr.* **94**, 52–56
- Szepesi, B., Vegors, R. & Demouy, J. M. (1972) *Nutr. Rep. Int.* **5**, 281–286
- Tepperman, H. M. & Tepperman, J. (1958) *Diabetes* **7**, 478–485
- Winston, G. W. & Reitz, R. C. (1980) *Life Sci.* **26**, 201–209
- Wurdeman, R., Berdanier, C. D. & Tobin, R. B. (1978) *J. Nutr.* **108**, 1457–1461
- Yagil, G., Shimron, F. & Hizi, A. (1974) *Eur. J. Biochem.* **45**, 189–200
- Yugari, Y. & Matsuda, T. (1967) *J. Biochem. (Tokyo)* **61**, 541–549