Changes in glutathione status and 3,5,3′-triiodothyronine action in livers of rats given cysteine-deficient diets

By H. GARCIN, C. SUBERVILLE and P. HIGUERET

Laboratoire Physiologie de la Nutrition, Département Alimentation et Nutrition, Université de Bordeaux I, Avenue des Facultés, 33405 Talence–Cédex, France

and D. HIGUERET

Laboratoire Biochimie Médicale, Hôpital Pellegrin, Place Raba Léon, 33000 Bordeaux, France

(Received 4 July 1988 – Accepted 14 October 1988)

1. For a period of 32 d young rats were given a diet containing (g/kg) 220 casein, 120 casein + 1·93 L-cysteine (Cys), or 120 casein.

2. The formation of 3,5,3′-triiodothyronine (T_3)–nuclear protein complexes was reduced in rats fed on the Cys-deficient diet.

3. Scatchard analysis showed that decreased formation of T_3–nuclear protein complexes was due to a decreased affinity of T_3 receptors; this decrease was induced, at least in part, by a reduced glutathione content.

4. In rats fed on the Cys-deficient diet there was an expected decrease in growth but an unexpected increase in the activities of glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49) and malate dehydrogenase (oxaloacetate-decarboxylating) ($NADP^+$) (*EC* 1.1.1.40). It is suggested that this increase is related to an increased oxidized glutathione: reduced glutathione ratio.

As previously described dietary cysteine (Cys) deprivation in rats induces changes in the peripheral metabolism of thyroid hormones (Suberville *et al.* 1988). It has been shown that there is a decrease in thyroxine (T_4) turnover (resulting from reduced deiodination in liver), and decreases in 3,5,3′-triiodothyronine (T_3) distribution space, the extrathyroidal pool of T_3, and T_3-disposal rate. However, there is no information about cellular response to T_3 stimulation in Cys-deficient rats. It is now well known that, in target cells, the binding of T_3 to specific nuclear T_3-receptor sites is a point of initiation of thyroid hormone actions and that the biological responses are mediated by changes in the levels of specific mRNA, and particularly those of growth hormone (GH) and lipogenic enzymes (for review, see Oppenheimer *et al.* 1987). The aim of the present work was to study, in rats fed on a Cys-deficient diet, the formation of T_3–nuclear receptor complexes and some biological variables relating to thyroid status (i.e. growth and some relevant enzyme activities).

METHODS

Animals and diets

Male wistar rats were obtained from IFFA Credo (l'Arbresle, France), having an initial weight of 100–110 g. They were randomized into three groups (ten or eleven rats in each group) and maintained for 32 d on one of the following semi-synthetic diets (g/kg): casein 220 (diet 1), casein 120 + L-Cys 1·93 (diet 2), casein 120 (diet 3); the diets were isoenergetic. Diets 2 and 3 have been described in detail previously (Suberville *et al.* 1987); in diet 1 increasing protein level was compensated by reducing carbohydrate. The rats were housed in an air-conditioned room with a mean temperature of 21 ± 1° and were weighed daily. Water and diets were offered *ad lib*. At the end of the experimental period the rats were killed by decapitation at 09.00 hours and the livers rapidly excised. A portion was

https://doi.org/10.1079/BJN19890118 Published online by Cambridge University Press

immediately used for glutathione analysis and nuclei preparation; the remainder was frozen in liquid nitrogen and stored at −80° for subsequent analysis.

In vitro binding of T_3 in rat-liver nuclei

Isolation of liver nuclei. All tissue fractionations were carried out at 0°. Nuclei were prepared according to De Groot & Torresani (1975). A portion of the liver was homogenized in 0·32 M-sucrose + 1 mM-magnesium chloride (0·32 SM), filtered through cheesecloth and centrifuged at 1000 **g** for 10 min. The crude pellet was washed once and centrifuged through a 2·2 M-sucrose (2·2 M-sucrose + 1 mM-$MgCl_2$) layer at 100000 **g** for 60 min. The nuclear pellet was gently resuspended in 0·32 SM + Triton X-100 (2·5 ml/l), centrifuged at 1000 **g** for 10 min and washed once with 0·32 SM. Then the nuclei were frozen as a pellet at −23° for only a few days.

Extraction of nuclear proteins. The final nuclear pellet derived from 2 g liver was gently resuspended in 1 ml TKEM (20 mM-Tris hydrochloride, 0·4 M-potassium chloride, 2 mM-EDTA, 1 mM-$MgCl_2$, pH 7·9 at 25°). After 30 min at 0° with frequent pipetting of the suspension, the nuclear residue was pelleted by centrifugation at 100000 **g** for 30 min. The supernatant fraction containing nuclear proteins was used for the T_3-binding assay (Torresani & De Groot, 1975).

Incubation conditions. Incubations of nuclear proteins were performed in 0·2 ml TKEM containing 30 μg protein (24 μg DNA), 0·006–0·12 pmol $[^{125}I]T_3$ (IM321, specific activity > 1·2 mCi/μg, Amersham, France) with or without 0·5 mM-dithiothreitol (DTT) for 3 h at 20°. The binding reaction was stopped by the addition of 1·8 ml of an ice-cold suspension of Dowex 1X8-400 resin in TKEM (67 mg/ml). After mixing, the resin was sedimented by centrifugation (1000 **g** for 5 min).

Protein-bound T_3 was estimated by measuring radioactivity in a portion of the supernatant fraction. Non-specific T_3 binding was determined by incubation in the presence of a 1000-fold excess of unlabelled T_3. Specifically-bound T_3 was determined by subtraction of the non-specifically-bound T_3 from the total T_3 bound. All incubations were performed in duplicate. Saturation curves and Scatchard analysis (Scatchard, 1949) were performed using final concentrations of $[^{125}I]T_3$ in the incubation medium ranging from 0·03 to 0·6 nM.

Scatchard curves were drawn using a linear regression analysis of the data. The slope of the straight line gave the affinity constant k_a and the intercept of the slope with the abscissa represented the maximum binding capacity, i.e. the binding site concentration.

Liver enzyme activities

Glucose-6-phosphate dehydrogenase (EC *1.1.1.49*; *G6P-DH*). G6P-DH and phosphogluconate dehydrogenase (decarboxylating) (*EC* 1.1.1.44; PG-DH) were assayed according to Löhr & Waller (1965) and King (1965) respectively.

L-Malate dehydrogenase (*oxaloacetate-decarboxylating*) (*$NADP^+$*) (EC *1.1.1.40*; *MDH*). MDH was determined by the method described by Wise & Ball (1964).

Analytical procedures

Protein assay. Proteins were measured according to Bradford (1976) using the Bio-Rad protein assay (Bio-Rad Laboratories, West Germany).

Glutathione assay. Glutathione, oxidized from (GSSG) and reduced from (GSH), was measured enzymically according to Tietze (1969) using glutathione reductase (*EC* 1.6.4.2) and 5,5′-dithiobis(2-nitrobenzoïc)acid. Sample GSH was preserved with vinyl-2-pyridine (Griffith, 1980).

DNA determination. DNA was estimated according to the method of Labarca & Paigen (1980) with calf thymus as the standard.

https://doi.org/10.1079/BJN19890118 Published online by Cambridge University Press

https://doi.org/10.1079/BJN19890118 Published online by Cambridge University Press

Statistical procedure

Statistical analysis of multiple group means was performed using an analysis of variance program (ANOVA) and, when F was associated to a probability $P < 0{\cdot}05$, inter-group comparison was achieved using Student's t test.

RESULTS

Binding of [^{125}I]T_3 with nuclear extracts

Fig. 1 represents binding, after 3 h of incubation at 20°, of increasing amounts of [^{125}I]T_3 with solubilized nuclear proteins isolated from livers of rats fed on 220 g casein/kg (diet 1), 120 g casein/kg + L-Cys (diet 2) or 120 g casein/kg (diet 3). The addition of DTT (the most common reducing agent used in vitro) to the incubation medium slightly increased the formation of complexes, even when the concentration of DTT (0·5 mM) was optimal for the experimental conditions used (nuclei stored at −23° for only a few days). Both with and without DTT, there was reduced formation of complexes with nuclear extracts from rats fed on 120 g casein/kg (diet 3). With nuclei from rats fed on the Cys-supplemented diet (diet 2) the formation of complexes was improved but did not reach the level of the control rats (diet 1).

Reduced formation of complexes could be explained as follows. Complex formation depends on both the capacity and the affinity of receptor proteins, which can be deduced from Scatchard analysis (Fig. 1). Under basal conditions (without DTT) the capacity, expressed as pmol T_3 bound/mg protein, was similar for the three groups and this value is in agreement with that previously described by Torresani & De Groot (1975). Expressed on a per mg DNA basis the average binding capcity of receptor protein is 750 fmol T_3/mg DNA, according to Oppenheimer *et al.* (1974) and Barsano (1983). The determination of

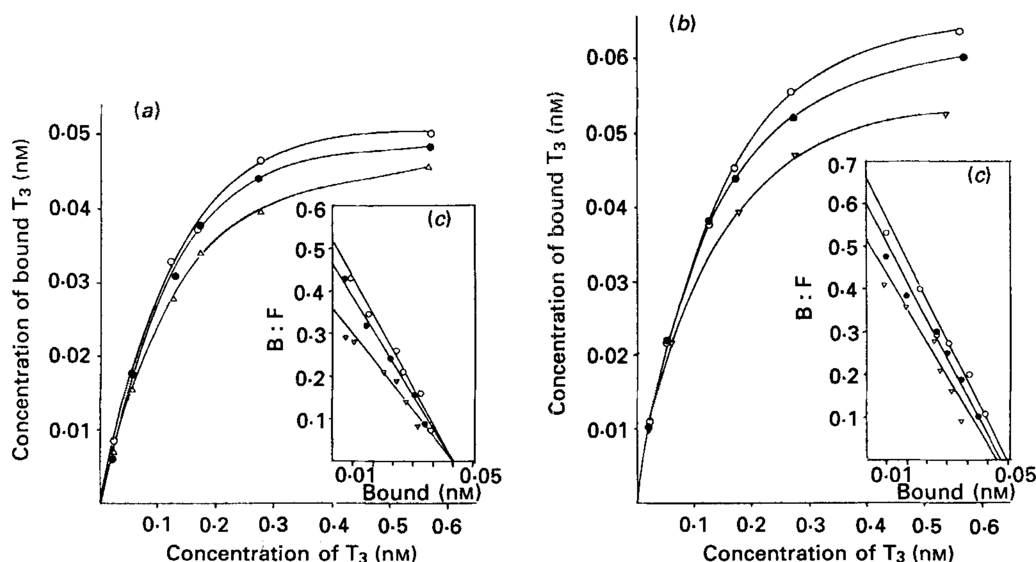

Fig. 1. Formation of receptor–3,5,3′-triiodothyronine (T_3) complexes at increasing concentrations of T_3 in the incubation medium (*a*) without and (*b*) with dithiothreitol (DTT). (○—○), rats fed on 220 g casein/kg; (●—●), rats fed on 120 g casein + 1·93 g L-cysteine/kg; (△—△), rats fed on 120 g casein/kg. (*c*) Scatchard analyses of T_3 binding to nuclear proteins: results are plotted as the bound (B): free (F) hormone (B:F) *v*. B concentrations.

Table 1. *Binding characteristics of hepatic nuclear 3,5,3′-triiodothyronine (T_3) receptors, measured with or without dithiotreitol (DTT), in nuclear extracts obtained from rats fed on three semi-synthetic diets*

(Mean values, with their standard errors, for five soluble nuclear receptor preparations. Each preparation was derived from livers pooled from two rats)

			Basal				+0·5 mM-DTT			
	Protein: DNA		Capacity (pmol T_3 bound/ mg protein)		Affinity $k_d \times 10^{-10}$M		Capacity (pmol T_3 bound/ mg protein)		Affinity $k_d \times 10^{-10}$M	
Diet (g/kg)	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Casein 220	1·77[a]	0·01	0·42[a]	0·03	0·84[a]	0·02	0·49[a]	0·03	0·91[a]	0·03
Casein 120+L-cysteine 1·93	1·82[a]	0·08	0·43[a]	0·02	0·72[b]	0·03	0·47[a]	0·01	0·87[ab]	0·05
Casein 120	1·84[a]	0·06	0·42[a]	0·04	0·58[c]	0·03	0·46[a]	0·02	0·80[b]	0·03

k_d, Dissociation constant.
[a,b,c] Within columns, mean values with different superscript letters were significantly different (ANOVA): $P < 0.05$.

the dissociation constant (k_d), however, showed (Table 1) significant differences in affinity between the three groups ($F_{(2,12)} = 22.25$; $P < 0.001$).

The diet containing 120 g casein/kg induced a 31 % decrease in affinity compared with control animals fed on the 220 g casein/kg diet ($F_{(1,8)} = 59$; $P < 0.001$). Compared with diet 3 (120 g casein/kg), Cys supplementation (diet 2) improved affinity (+19 %) but the value was lower (−12 %) than that of control rats (diet 1) ($F_{(1,8)} = 9.8$; $P < 0.001$).

Addition of 0·5 mM-DTT increased nuclear protein affinity for T_3. The effect of DTT on the different groups of rats was not similar: the more the liver glutathione content was deficient, the higher the increase in affinity (+21 % with extracts of rats fed on diet 2, +38 % with extracts of rats fed on diet 3). However, after DTT addition the affinity measured in rats fed on diet 3 did not reach the control value (diet 1) ($F_{(1,8)} = 10$; $P < 0.05$). DTT had no effect on the binding capacity in any experimental group.

Liver glutathione and liver enzyme activities

Hepatic glutathione. Table 2 shows hepatic GSH and GSSG values for the three dietary groups. In rats fed on diet 3 (120 g casein/kg) total glutathione was lower than that in rats fed on diet 1 ($F_{(1,18)} = 274.5$; $P < 0.001$) or diet 2 ($F_{(1,19)} = 47.3$; $P < 0.001$). Reduced liver glutathione values in rats fed on a low-protein diet, using casein, have already been described (Suberville *et al.* 1987; Hosokawa *et al.* 1988). Cysteine supplementation (diet 2) increased total glutathione but not to control levels. GSSG:GSH was higher in rats fed on the 120 g casein/kg (diet 3) than that for the other groups.

Activities of three hepatic lipogenic enzymes. The activity of hexose monophosphate shunt enzymes was measured for the three dietary groups. G6P-DH activity was apparently stimulated in the 120 g casein/kg diet group *v.* control ($F_{(1,19)} = 8.6$; $P < 0.01$). The pattern was the same for MDH activity but more marked ($F_{(1,19)} = 5.05$; $P < 0.05$) (Table 2).

https://doi.org/10.1079/BJN19890118 Published online by Cambridge University Press

Table 2. *Hepatic glutathione and lipogenic enzyme activities measured in livers of rats fed on three semi-synthetic diets*

(Mean values with their standard error)

		Liver glutathione						Liver enzyme activities					
		Reduced form (GSH) (μmol/g wet wt)		Oxidized form (GSSG) (μmol/g wet wt)		GSSG:GSH (×100)		G6P-DH (IU*/g protein)		PG-DH (IU*/g protein)		MDH (IU†/mg protein)	
Diet (g/kg)	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Casein 220	10	7·05[a]	0·23	0·048[a]	0·002	0·68[a]	0·21	54·8[a]	7·3	65·7[a]	4·3	87·5[a]	10·6
Casein 120 + L-cysteine 1·93	10	4·45[b]	0·26	0·044[a]	0·009	0·97[a]	0·17	62·6[a]	6·1	67·2[a]	3·6	68·6[a]	9·5
Casein 120	11	2·39[c]	0·16	0·060[a]	0·010	2·48[b]	0·43	78·3[b]	3·5	71·0[a]	2·7	115·2[b]	7·8

G6P-DH, glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49); PG-DH, phosphogluconate dehydrogenase (decarboxylating) (*EC* 1.1.1.44); MDH, malate dehydrogenase (oxaloacetate decarboxylating) ($NADP^+$) (*EC* 1.1.1.40).

[a,b] Within columns, mean values with different superscript letters were significantly different (ANOVA); $P < 0.05$.

*One unit of enzyme is the amount of enzyme which reduces 1 μmol NADP/min at 25°.

†One unit of enzyme is the amount of enzyme which reduces 1 nmol NADP/min at 37°.

https://doi.org/10.1079/BJN19890118 Published online by Cambridge University Press

DISCUSSION

Cysteine content of tissue plays a critical part in the action of T_3 at the cellular level because (i) the T_3 receptor is a Cys-rich thiol protein (Weinberger *et al.* 1986), and (ii) Cys is a component of GSH whose SH groups play an essential role in maintaining nuclear T_3 binding proteins (NTBP) and their binding site in a proper conformation (Torresani *et al.* 1978).

Scatchard analysis under basal conditions (without DTT) showed that binding capacities were similar in the three groups of rats. On the contrary, the analysis showed that the affinity of nuclear receptors was lower with diet 3 (120 g casein/kg) than with diet 2 (120 g casein/kg + Cys) and lower with diet 2 than with diet 1 (220 g casein/kg). Thus the Cys-deficient diet used principally induces functional disorders. The increase in receptor affinity found in the presence of DTT confirms the deficiency of an SH group source (probably GSH). This experiment also shows that with DTT, the affinity was increased but did not reach the control level (diet 1). Since the concentration of DTT was optimal in our experiments (see previously) this result suggests a further interaction.

Reduced affinity associated with unchanged binding capacity leads to reduced binding of T_3 to its nuclear receptor. In the cell the major proportion of T_3 is located in the nucleus (Balsam, 1974), so reduced binding of T_3 to nuclear proteins can be related to a reduced cell T_3 content, a finding in agreement with the reduced T_3 distribution space previously noted (Suberville *et al.* 1988) since the liver is the organ principally involved in T_3 uptake.

As binding of hormone to its receptor initiates cellular responses, reduced biological responses would be expected in cysteine-deficient groups. Indeed, reduced growth was noted (weight increase (g/d): diet 1, 6·8; diet 2, 4·6; diet 3, 3·8), confirming previous results (Suberville *et al.* 1987). This finding can be related to the fact that the growth hormone gene is under thyroid hormone control (Nyborg *et al.* 1984) and to the likelihood that various organs, among them the pituitary gland, are affected similarly to the liver.

Surprisingly increased lipogenic enzyme activity was found with the 120 g casein/kg group (diet 3), even though thyroid hormones also regulate the mRNA of these enzymes, and subsequently enzyme activities (Oppenheimer *et al.* 1987). This finding suggests that factors other than hormonal control at the transcriptional level are responsible for the increase in MDH and G6P-DH activities. It is known that MDH and enzymes of the hexose monophosphate shunt generate reducing power in the form of NADPH, and that NADPH allows generation of GSH from GSSG through glutathione reductase (Meister, 1982). NADPH-consuming pathways produce activation of the pentose phosphate cycle (Fabregat *et al.* 1985). Also GSSG induces activation by overcoming inhibition produced by NADPH (Nogueira *et al.* 1986). With rats fed on 120 g casein/kg (diet 3) there was a decrease in liver glutathione, but also a significant increase in GSSG:GSH. It can be suggested that the measured increase in lipogenic enzyme activity is the result of a stimulation of these enzymes by the reduced intracellular compounds. The oxidation–reduction of GSH status may act as a third messenger in various biological processes (Kaplowitz *et al.* 1985).

Recently, it has been reported that the cellular c-erb-A gene encodes a thyroid hormone receptor (Weinberger *et al.* 1986). Structural determination of this product exhibits a strong peptide homology to those of receptors for glucocorticoids, progesterone, oestradiol, vitamin D and aldosterone (for review, see Oppenheimer *et al.* 1987), particularly in the Cys-rich region (the C region), which is the putative DNA-binding site. It is possible that there exist hormonal and metabolic changes, besides thyroid disorders, in animals fed on a Cys-deficient diet.

https://doi.org/10.1079/BJN19890118 Published online by Cambridge University Press

This work was supported in part by La Fondation Française pour la Nutrition (Paris). The authors express sincere thanks to Dr J. Torresani for her expert help in liver T_3-receptor extraction.

REFERENCES

Balsam, A. (1974). Augmentation of the peripheral metabolism of L-triiodothyronine and L-thyroxine after acclimatation to cold. *Journal of Clinical Investigation* **53**, 1145–1156.

Barsano, C. P. (1983). Quantitation of 3,5,3′-triiodothyronine (T_3) receptors by a microcentrifuge exchange assay: evidence for the existence of a nucleoplasmic pool of rat liver T_3 receptors. *Endocrinology* **112**, 1479–1489.

Bradford, M. M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

De Groot, L. J. & Torresani, J. (1975). Triiodothyronine binding to isolated liver cell nuclei. *Endocrinology* **96**, 357–369.

Fabregat, I., Vitorica, J., Satrustegui, J. & Machado, A. (1985). The pentose phosphate cycle is regulated by NADPH/NADP ratio in rat liver. *Archives of Biochemistry and Biophysics* **236**, 110–118.

Griffith, O. W. (1980). Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinyl pyridine. *Analytical biochemistry* **106**, 207–212.

Hosokawa, Y., Niizeki, S., Tojo, H., Sato, I. & Yamaguchi, K. (1988). Hepatic cysteine dioxygenase activity and sulfur amino acid metabolism in rats: possible indicators in the evaluation of protein quality. *Journal of Nutrition* **118**, 456–461.

Kaplowitz, N., Aw, T. Y. & Ookhtens, M. (1985). The regulation of hepatic glutathione. *Annual Review of Pharmacology and Toxicology* **25**, 715–744.

King, J. (1965). 6-Phosphogluconate dehydrogenase. In *Methods in Enzymatic Analysis*, pp. 632–635 [H. Bergmayer, editor]. New York: Academic Press.

Labarca, C. & Paigen, K. (1980). A simple and sensitive DNA assay procedure. *Analytical Biochemistry* **102**, 344–352.

Löhr, G. W. & Waller, H. D. (1965). Glucose 6-phosphate dehydrogenase. In *Methods in Enzymatic Analysis* pp. 636–643. [H. Bergmayer, editor]. New York: Academic Press.

Meister, A. (1982). Glutathione. In *The Liver: Biology and Pathobiology*, pp. 297–306 [I. Arias, H. Popper, D. Schachter and D. A. Shafritz, editors]. New York: Raven Press.

Nogueira, M., Garcia, G., Mejuto, C. & Freire, M. (1986). Regulation of the pentose phosphate cycle. *Biochemical Journal* **239**, 553–558.

Nyborg, J. K., Nguyen, A. P. & Spindler, S. R. (1984). Relationship between thyroid and glucocorticoid hormone receptor occupancy, growth hormone gene transcription, and mRNA accumulation. *Journal of Biological Chemistry* **259**, 12377–12382.

Oppenheimer, J. H., Schwartz, H. L., Mariash, C. N., Kinlaw, W. B., Wong, N. C. W. & Freake, H. C. (1987). Advances in our understanding of thryoid hormone action at the cellular level. *Endocrine Reviews* **8**, 288–308.

Oppenheimer, J. H., Schwartz, H. L. & Surks, M. I. (1974). Tissue differences in the concentration of triiodothyronine nuclear binding sites in the rat liver, kidney, pituitary, heart, brain, spleen and testis. *Endocrinology* **95**, 897–903.

Scatchard, G. (1949). The attractions of proteins for small molecules and ions. *Annals of the New York Academy of Sciences* **51**, 660–672.

Suberville, C., Higueret, P., Taruoura, D., Higueret, D. & Garcin, H. (1987). Relative contribution of cysteine and methionine to glutathione content and thyroid hormone levels in the rat. *British Journal of Nutrition* **58**, 105–111.

Suberville, C., Higueret, P., Taruoura, D., Higueret, D. & Garcin, H. (1988). Glutathione deficiency and peripheral metabolism of thyroid hormones during dietary cysteine deprivation in rats. *British Journal of Nutrition* **59**, 451–456.

Tietze, F. (1969). Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione. Application to mammalian blood and other tissues. *Analytical Biochemistry* **27**, 502–522.

Torresani, J., Anselmet, A. & Wahl, R. (1978). Properties of solubilized nuclear triiodothyronine binding proteins. *Molecular and Cellular Endocrinology* **9**, 321–333.

Torresani, J. & De Groot, L. J. (1975). Triiodothyronine binding to liver nuclear solubilized proteins in vitro. *Endocrinology* **96**, 1201–1209.

Weinberger, C., Thompson, C. C., Ong, E. S., Lebo, R., Gruol, D. J. & Evans, R. M. (1986). The C-erb-A gene encodes a thyroid hormone receptor. *Nature* **324**, 641–646.

Wise, E. M. Jr. & Ball, E. G. (1964). Malic enzyme and lipogenesis. *Proceedings of the National Academy of Sciences, USA* **52**, 1255–1263.

Printed in Great Britain

https://doi.org/10.1079/BJN19890118 Published online by Cambridge University Press