Identification of a liver growth factor as an albumin–bilirubin complex

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We have reported the purification and characterization of a protein that behaves as a liver growth factor, showing activity either *in vivo* or *in vitro* [Díaz-Gil *et al.* (1986) *Biochem. J.* **235**, 49–55]. In the present paper, we identify this liver growth factor (LGF) as an albumin–bilirubin complex. This conclusion is supported by the results of chemical and spectroscopic characterization of this protein as well as by experiments *in vivo*. Incubation of albumin isolated from normal rats with bilirubin at several bilirubin/albumin molar ratios (r) resulted (when $r = 1$ or 2) in a complex with liver DNA synthesis promoter activity identical with that of LGF. The exact amount of bilirubin bound to albumin was assessed by fluorescence and c.d. spectra. This albumin–bilirubin complex showed the same dose-dependence profile as LGF either at low or high dose of protein injected per mouse. Both LGF and albumin–bilirubin complex produced similar increases in the mitotic index of mouse hepatocytes *in vivo*. A new mechanism for the onset of the hepatic regenerative process is proposed.

INTRODUCTION

We recently reported the purification of a 64000- M_r protein from plasma of partially hepatectomized rats which exhibits hepatic promoter activity both *in vivo* and *in vitro* (Díaz-Gil *et al.*, 1986a). The injection of this protein into mice at nanogram doses (up to 150 ng/mouse) increases [^3H]thymidine uptake by liver DNA, producing an increase in the mitotic index of hepatocytes. In primary liver cell cultures, it produces an increase in the uptake of [^3H]thymidine into DNA in the range of 1–10 ng/ml, as well as an increase, immediately upon addition, in the uptake of $^{22}\text{Na}^+$. In view of the activity exhibited by this protein and to avoid confusing its context with other areas of research, we will refer to this protein hereafter as ‘liver growth factor’ (LGF).

The present paper deals with the characterization of this LGF.

MATERIALS AND METHODS

Materials

Chemicals were purchased from Sigma, Bio-Rad, Culti, Merck and Pharmacia. [^3H]Thymidine (specific radioactivity 20 Ci/mmol) was from New England Nuclear.

Wistar rats (90–110 g body wt.) were 70% hepatectomized by the method of Higgins & Anderson (1931). Plasma was obtained from heparinized blood of normal or hepatectomized rats by cardiac puncture. Plasma samples were freeze-dried and kept at 4 °C until use.

LGF purification and analysis of activity

The purification of LGF was carried out as previously

described (Díaz-Gil *et al.*, 1986a). Commercial rat serum albumin (fraction V from Sigma) was purified by a three-step procedure very similar to that used for LGF purification: chromatography on Sephadex G-75, DEAE-cellulose and hydroxyapatite. We refer to this as purified serum albumin. Following the same strategy, we purified albumin from plasma of either normal or hepatectomized rats. We call these fractions F 121(N) and F 121(PH), respectively.

The determination of the activity in terms of DNA synthesis promoter was carried out in mice, as previously indicated (Díaz-Gil *et al.*, 1986a). For the extraction of DNA, the method of MacManus *et al.* (1972) was used; total DNA was determined by the method of Burton (1968). Specific incorporation of [^3H]thymidine into DNA was expressed as d.p.m./ μg of DNA. Protein determination was performed by the method of Lowry *et al.* (1951).

For experiments *in vitro*, hepatocytes from 150 g body wt. Wistar rats were isolated by a collagenase perfusion technique (Bonney *et al.*, 1974). More details regarding the procedure followed to evaluate LGF activity are given in a previous publication (Díaz-Gil *et al.*, 1986a).

Spectroscopic measurements

Absorbance was determined on a Cary 118 spectrophotometer at a 0.2 nm/s scanning speed on an auto slit program. C.d. spectra were measured in a Jobin Yvon Mark III dichrograph, fitted with a 250 W xenon lamp, at a 0.2 nm/s scanning speed; 0.05 cm optical path cells were used in the far-u.v. region whereas 1 cm cells were employed in the near-u.v. C.d. results are the average of at least five determinations. The mean residue weight for

Abbreviations used: F 121(N), albumin from plasma of normal rats; F 121(PH), albumin from plasma of partially hepatectomized rats; LGF, liver growth factor; r , bilirubin/albumin molar ratio.

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calculations was 111.5, based on the amino acid composition of the protein (see below). Fluorescence spectra were obtained on a Perkin-Elmer MPF 44E spectrofluorimeter. Spectra were corrected for detector response.

The protein solutions were filtered through a Millipore filter (0.5 μ m pore diameter) prior to the spectroscopic analysis, which was performed at 20 °C. The protein concentration for the spectroscopic calculations was determined by amino acid analysis.

Other analytical procedures

SDS/polyacrylamide-gel electrophoresis was performed according to Laemmli (1980). For mitotic index determination, the procedure of Morley & Kingdon (1973) was employed. The Ouchterlony immunodiffusion and immunoelectrophoresis experiments were carried out on agarose (1.5% solution with 35 mM-barbital buffer), using 65 mM-barbital, pH 8.6, as running buffer.

Performic acid oxidized proteins (Hirs, 1956) were digested with trypsin at 100:1 protein:enzyme weight ratio for 4 h at 37 °C in 0.2 M-NH₄HCO₃, pH 8.0. The resulting peptides were separated by using an Ultrasphere ODS column (4.5 mm $\times$ 250 mm; 5 μ m particle size).

The protein samples were hydrolysed at 108 °C (for 24, 48 and 96 h) with constant-boiling HCl containing 0.1% (w/v) phenol, in evacuated and sealed tubes. Hydrolysates were analysed on a Durrum model D-500 amino acid analyser.

Albumin-bilirubin complex formation

Bilirubin was purchased from Sigma. Its purity was checked by the procedure of McDonagh & Assisi (1971).

Table 1. Amino acid composition of the liver growth factor; values for rat serum albumin are also included for comparison

Values are expressed as the nearest integer value based on an M_r of 65000 for both proteins. For each hydrolysis time, three separate determinations were performed. Serine and threonine contents were corrected to zero hydrolysis time. Valine and isoleucine contents were determined from the 96-h hydrolysates. Half-cystine was determined as cysteic acid after oxidation of the protein samples with performic acid at -10 °C (Hirs, 1956).

Amino acid	Composition (residues/ M_r 65000)	
	LGF	Purified albumin
Cys	32	31
Asx	47	46
Thr	33	35
Ser	27	28
Glx	75	76
Pro	29	28
Gly	27	23
Ala	61	63
Val	35	36
Met	6	6
Ile	16	15
Leu	54	54
Tyr	21	21
Phe	27	27
His	16	16
Lys	49	49
Arg	29	29

Fig. 1. Reversed phase h.p.l.c. of tryptic digests of performic-acid-oxidized LGF (a) and purified serum albumin (b)

Samples (100 μ g in 20 μ l) were applied in 0.2 M-NH₄HCO₃, pH 8.0. The column was eluted at 60 ml/h: 10 mM-ammonium acetate, pH 6.5, for 8 min; a linear gradient of 0–2% acetonitrile in the acetate buffer for 10 min; a linear gradient of 2–9% acetonitrile in the same buffer for 28 min; 9% acetonitrile in acetate for 10 min; a linear gradient of 9–15% acetonitrile in the acetate buffer for 24 min; and a linear gradient of 15–35% acetonitrile in acetate buffer for 60 min. Detection of the peptides was performed by measurement of A_{214} . See the Materials and methods section for more details.

Binding of bilirubin to albumin was carried out by the method of Wooley & Hunter (1970). In experiments where bilirubin was injected alone, it was previously incubated in the same conditions described above for bilirubin-albumin samples.

RESULTS

Protein-chemical similarities between LGF and serum albumin

The amino acid compositions of albumin and LGF are very similar (Table 1). The similarity between the two proteins is also observed by comparing their tryptic maps (Fig. 1). The u.v.-absorbance spectra of purified serum albumin and LGF are coincident. The fluorescence emission spectra for excitation at 295 nm are almost coincident. Only slight differences are observed in the c.d. spectra of the two proteins in the near-u.v. region, although no differences are observed at the level of the secondary structure (Fig. 2). The secondary structure of LGF would be composed of 59% α -helix, 17% β -structure and 24% aperiodic conformation, with an average number of 10 residues per helical segment, based on the far-u.v. c.d. spectrum and using the reference parameters of Cheng *et al.* (1974). The immunodiffusion and immunoelectrophoresis experiments indicate that purified serum albumin and LGF have the same antigenic determinants [a commercial preparation of anti-(rat serum albumin) serum from rabbit (Nordic Immunological Reagents) was used]. All of these results strongly suggest that this liver growth factor contains, or is a form of, albumin. Thus, we studied the potential effect of both commercial and purified rat serum albumin, F 121(N), as liver growth factors, finding no such activity in these proteins. However, albumin purified from partially hepatectomized rats, F 121(PH),

exhibits liver growth factor activity both *in vivo* and *in vitro*. The chemical analysis of F 121(PH) revealed the identity between this protein and LGF. Thus, we will refer to both of them simply as LGF. All of these observations corroborate the supposition that LGF is a special kind of albumin present in plasma of partially hepatectomized rats.

Serum albumin-bilirubin complex prepared *in vitro* is a liver growth factor

Another feasible explanation is that the LGF isolated could simply be albumin with a very-low- M_r ligand, X. As a further development of this hypothesis, the hypothetical X ligand could be the true liver growth factor, appearing in situations of partial hepatectomy, and absent from normal rats. We considered the possibility that bilirubin be responsible for the liver growth factor activity of albumin from plasma of partially hepatectomized rats, thus implying that bilirubin would be a component of LGF.

To check this possibility, we performed an experiment incubating albumin from normal rats, F 121(N), with increasing quantities of bilirubin. We adjusted the concentrations of F 121(N) and bilirubin to reach bilirubin/albumin molar ratios (r) of 0.1, 0.5, 0.8, 1, 2, 3, 4 and 10. After the incubation period (see the Materials and methods section), we checked their activity *in vivo* by injection into mice at doses of 150 ng of protein per mouse in every case. Samples with $r = 1$ and 2 showed DNA synthesis promoting activity (Table 2). The increase in [^3H]thymidine uptake was very similar to that corresponding to LGF alone at the same amount of protein injected per mouse. When r was increased to values of 3, 4 or 10, or decreased to 0.8, 0.5 or 0.1, a lower DNA synthesis stimulation was detected.

As controls for this experiment, F 121(N) or bilirubin alone were injected, revealing a lack of any stimulatory

Fig. 2. C.d. spectra of LGF (—) and purified serum albumin (---)

(a) Far-u.v. region; (b) near-u.v. region. Mean residue ellipticities are expressed in units of degrees·cm²·dmol⁻¹. See the Materials and methods section for more details.

Table 2. Formation *in vitro* of the LGF

Albumin, F 121(N), was incubated with bilirubin at the indicated molar ratios (*r*), and injected into mice (*n* mice for each condition) to measure specific incorporation of [³H]thymidine in liver DNA (see the Materials and methods section).

	<i>n</i>	Specific incorporation	
		(d.p.m./μg of DNA)	(% of control)
Control (saline-injected)	10	38.2 ± 16.5	100 ± 43
LGF (150 ng/mouse)	3	108.7 ± 11.6	286 ± 31*
F 121(N) (150 ng/mouse)	3	40.5 ± 8.8	106 ± 23†
+ bilirubin (<i>r</i> = 0.1)	7	47.7 ± 17.3	125 ± 45†
+ bilirubin (<i>r</i> = 0.5)	9	60.0 ± 16.4	157 ± 43‡
+ bilirubin (<i>r</i> = 0.8)	15	64.0 ± 18.4	168 ± 48§
+ bilirubin (<i>r</i> = 1.0)	19	101.7 ± 38.0	266 ± 99*
+ bilirubin (<i>r</i> = 2.0)	8	101.1 ± 32.2	265 ± 85*
+ bilirubin (<i>r</i> = 3.0)	8	77.9 ± 37.8	204 ± 102‡
+ bilirubin (<i>r</i> = 4.0)	8	73.0 ± 18.7	191 ± 49*
+ bilirubin (<i>r</i> = 10.0)	6	35.8 ± 18.0	94 ± 47†
Bilirubin (same amount as <i>r</i> = 1.0)	11	48.3 ± 18.9	127 ± 50†
Bilirubin (same amount as <i>r</i> = 2.0)	10	46.1 ± 14.3	121 ± 37†

* $P < 0.001$.

† Statistically not significant.

‡ $0.01 < P < 0.02$.

§ $0.001 < P < 0.002$.

effect. In the case of bilirubin alone, the same total quantity was injected as in experiments with bilirubin/albumin ratios of 1 and 2 (1.57 or 3.15 ng/mouse, respectively), and no increases were observed (Table 2). Furthermore, bilirubin was injected alone at doses of

from 5 to 214 μg/mouse (eight intermediate doses), and in all cases totally lacked DNA synthesis activity (results not shown). With this experiment, we discarded the possibility of considering bilirubin alone as the trigger to hepatocyte proliferation, as only the albumin-bilirubin complex proved active.

In order to determine the amount of bilirubin bound to F 121(N), fluorescence and c.d. measurements were performed. The results are given in Table 3.

When both ellipticity values at 400 and 452 nm are plotted versus protein/bilirubin molar ratio, a saturation curve is obtained; however, two inflection points can be observed (results not shown). These factors could be interpreted in terms of two different contributions to the c.d. signal arising from bound bilirubin. It is known that albumin possesses two kinds of binding sites for bilirubin, one of high affinity and one or two of low affinity (Berde *et al.*, 1979; Lavie & Blauer, 1979). Although the percentage of bound bilirubin was estimated from the c.d. measurements (Table 3), these data have not been considered in Fig. 3. This Figure was designed only on the basis of the values obtained from the fluorescence determinations. In fact, the potential existence of two different contributions to the c.d. signal upon bilirubin binding could mask the calculation of the value obtained by extrapolation to a (protein/bilirubin)⁻¹ zero value.

Fig. 3 shows the liver growth factor activity of bilirubin-albumin complexes (at different bilirubin/albumin ratios) versus the percentage of bound bilirubin. A plateau of maximal activity is reached and the specific incorporation detected on both sides of the plateau decreases. This result indicates that when bilirubin binds roughly between 26 and 33% with respect to the albumin present, in terms of molar ratios, a maximum activity of the complex is attained.

The dose-dependence curve of the albumin-bilirubin complex was compared with that of LGF. F 121(N) plus

Table 3. Bilirubin bound to albumin determined by fluorescence and c.d.

The percentage of bound bilirubin was calculated by considering that the fluorescence of bound bilirubin may be estimated by extrapolation to zero in a double-reciprocal plot of the fluorescence increase at 510 nm versus albumin/bilirubin molar concentration. When ellipticity versus albumin/bilirubin double-reciprocal plots are constructed, the ellipticity of bound bilirubin can be determined by extrapolation to (albumin/bilirubin)⁻¹ zero value. This value would be that corresponding to the first type of contribution. This value can be used to calculate the bound bilirubin. Abbreviation: NC, not calculated.

Bilirubin/ albumin* molar ratio	Bound bilirubin determined by:			
	Fluorescence		C.d.	
	(μmol)	(%)	(μmol)	(%)
0.1	0.088	88	NC	NC
0.5	0.21	42	NC	NC
0.8	0.29	36	NC	NC
1	0.33	33	0.45	45
2	0.52	26	0.60	30
3	0.51	17	0.69	23
4	0.44	11	0.68	17

* Reference, 1 μmol of albumin.

Fig. 3. Percentage of bilirubin bound to albumin necessary to form the liver growth factor

Percentage of bound bilirubin (over total bilirubin incubated at several bilirubin/albumin molar ratios) was obtained from fluorescence spectra (Table 3) and specific incorporation from Table 2 (see the Results section for details).

Fig. 4. Dose-dependence curve of LGF and albumin-bilirubin preparations *in vivo* (low doses)

—, Specific incorporation of [3 H]thymidine in mouse liver DNA by injection of LGF preparations; ----, the corresponding values for albumin-bilirubin complex ($r = 1$). Points are the means for nine injected mice, $\pm$ S.D.

bilirubin ($r = 1$) was injected into mouse and the [3 H]thymidine uptake measured. The profile obtained (Fig. 4) was identical with that of LGF. When LGF and albumin-bilirubin complex were injected together (150 ng of each protein), the observed stimulation of the

Fig. 5. Dose-dependence curve of LGF and albumin-bilirubin preparations *in vivo* (higher doses)

—, Specific incorporation of [3 H]thymidine in mouse liver DNA by injection of LGF preparations; ----, the corresponding values for albumin-bilirubin complex ($r = 1$). Points are the means for nine injected mice, $\pm$ S.D.

Table 4. Effect of either LGF or F 121(N) plus bilirubin ($r = 1$) injection on mitotic index in mouse liver

10^4 cells were examined in different fields, for three mice per group. See the Materials and methods section for more details.

Group of mice	No. of mitoses
Control (saline-injected)	2
150 ng of LGF/mouse	82
150 ng of F 121(N) + bilirubin/mouse	77
0.15 mg of LGF/mouse	88
0.15 mg of F 121(N) + bilirubin/mouse	73

DNA synthesis was similar to that produced by 300 ng of LGF or bilirubin complex alone (Fig. 4). This experiment provides additional evidence for the identity of LGF and albumin-bilirubin complex.

Previous results in our laboratory (results not shown) indicated that LGF also exhibits a peak of activity at milligram doses. In order to verify this behaviour for the albumin-bilirubin complex, we have performed the experiments in Fig. 5, at $r = 1.0$. The results in this Figure also corroborate that LGF and albumin-bilirubin complex are identical entities.

Finally, we have also measured the effect of albumin-bilirubin complex ($r = 1$) on the mitotic index in mouse liver, comparing it with the corresponding values obtained with LGF (Table 4). The albumin-bilirubin complex mimics the previously reported increase in mitotic index provoked by injection of LGF (Díaz-Gil *et al.*, 1986a).

DISCUSSION

The appearance of liver growth factor activity in albumin after its binding to bilirubin could be the result of a conformational change that exposed to the exterior an active site of the albumin, sequestered in normal conditions (without a significant amount of bound bilirubin). In this respect, Taylor *et al.* (1975) demonstrated that the 'enzyme-like activity' of bovine serum albumin in catalysing the decomposition of the Meisenheimer complex is exquisitely sensitive to the conformational integrity of the protein. Conformational changes in albumin by binding of ligands are widely documented in the literature (Beaven *et al.*, 1973; Lavie & Blauer, 1979). We suggest this possibility in this case owing to the slight differences detected between the c.d. spectra of LGF and albumin.

The existence of two peaks of LGF activity, separated by a factor of 1000, is an unexplained result. In spite of that, some hypotheses can be considered. Both peaks could represent different mitotic signals: the lowest would switch on at very low concentrations of albumin-bilirubin complex, while the highest would act at much higher ones; the two situations could be attained at different degrees of hepatic loss. On the other hand, the two peaks might indicate two different populations of albumin, able to act with different specific activities. Several authors have detected different subpopulations (microheterogeneity) in the pool of albumin in plasma (Foster *et al.*, 1965; McMenamy & Lee, 1967). Finally, the hypothetical LGF receptor on the

hepatocyte could have multiple binding sites, in a similar fashion to that described for oestrogen receptors on human prostate (Ekman *et al.*, 1983).

The identification of LGF as an albumin–bilirubin complex suggests a new concept of the onset of the hepatic regeneration process. Immediately after partial hepatectomy, the albumin–bilirubin complex would be formed, producing a wave of liver regeneration. This albumin–bilirubin complex would disappear in the course of regeneration and, upon reaching normal liver size, would be undetectable (we were not able to detect LGF activity in normal rats). The biological role of this liver growth factor would be operative not only in situations of partial hepatectomy, but in cases of liver injury, when hepatocyte proliferation was required. Some facts seem to support this proposed model of the regeneration process. First, when ethanol is administered to rats at high doses (acute toxicity), there is no change in the synthesis of total liver protein, but a retention of newly synthesized albumin in the liver is detected (Morland, 1975; Baraona *et al.*, 1980). These authors feel that it could be due to an impairment of albumin secretion by hepatocytes, but an alternative explanation could lie in the uptake of newly synthesized albumin by hepatocytes in the form of LGF molecules. Second, in rats in which cirrhosis has been induced by injection of carbon tetrachloride/phenobarbitone (Courtoy *et al.*, 1981), a decreased serum albumin concentration is observed. These authors detected indirect evidence demonstrating that the rate of albumin synthesis per hepatocyte could even be increased. One possible explanation could be the uptake of part of the serum albumin by hepatocytes as LGF, producing a wave of regeneration. Third, in our laboratory, we have detected the presence of LGF in rat plasma after injection of dimethylnitrosamine or thioacetamide, and we have observed that higher levels of LGF in plasma coincide with greater hepatic necrosis (J. J. Díaz-Gil *et al.*, unpublished work). This could be interpreted as the regeneration wave that follows liver necrosis to repair the damage.

This model may be applicable to other species. In this context, we have detected a liver DNA synthesis promoter activity in human plasma from patients with hepatitis, with apparently the same characteristics as LGF (Díaz-Gil *et al.*, 1986b). On the other hand, some authors (Weiss *et al.*, 1983; Seligson *et al.*, 1985) described a special kind of albumin–bilirubin complex, called biliprotein, only detected in pathological states of the liver. This abnormal product of bilirubin metabolism could be the counterpart, in humans, of rat LGF.

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REFERENCES

- Baraona, E., Pikkarainen, P., Salaspuro, M., Finkelman, F. & Lieber, C. S. (1980) *Gastroenterology* **79**, 104–111
- Beaven, G. H., d'Albis, A. & Gratzner, W. B. (1973) *Eur. J. Biochem.* **33**, 500–510
- Berde, C. B., Hudson, B. S., Simoni, R. D. & Sklar, L. A. (1979) *J. Biol. Chem.* **254**, 391–400
- Bonney, R. J., Becker, J. E., Walker, P. R. & Potter, U. R. (1974) *In Vitro* **9**, 399–413
- Burton, K. (1968) *Methods Enzymol.* **12**, 163–166
- Cheng, Y. H., Yong, J. T. & Chaw, K. H. (1974) *Biochemistry* **13**, 3350–3359
- Courtoy, P. J., Feldman, G., Rogier, E. & Moguelevsky, N. (1981) *Lab. Invest.* **45**, 67–76
- Díaz-Gil, J. J., Escartín, P., García-Cañero, R., Trilla, C., Veloso, J. J., Sánchez, G., Moreno-Caparrós, A., Enrique de Salamanca, C., Lozano, R., Gavilanes, J. G. & García-Segura, J. M. (1986a) *Biochem. J.* **235**, 49–55
- Díaz-Gil, J. J., Sánchez, G., Santamaría, L., Trilla, C., Esteban, P. & Escartín, P. (1986b) *Hepatology* **6**, 658–661
- Ekman, P., Barrack, E. R., Greene, G. L., Jensen, E. V. & Walsh, D. C. (1983) *J. Clin. Endocrinol. Metab.* **57**, 166–176
- Foster, J. F., Sogami, M., Petersen, H. H. & Leonard, W. J., Jr. (1965) *J. Biol. Chem.* **240**, 2495–2507
- Higgins, G. M. & Anderson, R. M. (1931) *Arch. Pathol. (Chicago)* **12**, 186–202
- Hirs, C. H. W. (1956) *J. Biol. Chem.* **219**, 611–621
- Laemmli, U. K. (1980) *Nature (London)* **227**, 680–685
- Lavie, E. & Blauer, G. (1979) *Arch. Biochem. Biophys.* **193**, 191–203
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- MacManus, J. P., Franks, D. J., Youdale, T. & Braceland, B. M. (1972) *Biochem. Biophys. Res. Commun.* **49**, 1201–1207
- Majumdar, C., Tsukada, K. & Lieberman, I. (1967) *J. Biol. Chem.* **242**, 700–704
- McDonagh, A. F. & Assisi, F. (1971) *FEBS Lett.* **18**, 315–317
- McMenamy, R. H. & Lee, Y. (1967) *Arch. Biochem. Biophys.* **122**, 635–643
- Morland, J. (1975) *J. Biochem. Pharmacol.* **24**, 439–442
- Morley, C. G. D. & Kingdon, H. S. (1973) *Biochim. Biophys. Acta* **308**, 260–275
- Seligson, D., Seligson, H. & Wu, T.-W. (1985) *Clin. Chem.* **31**, 1317–1321
- Taylor, R. P., Berga, S., Chau, V. & Bryner, C. (1975) *J. Am. Chem. Soc.* **97**, 1943–1948
- Weiss, J. S., Gautam, A., Lauff, J. J., Sundberg, M. W., Jatlow, P., Boyer, J. L. & Seligson, D. (1983) *New Engl. J. Med.* **309**, 147–150
- Woolley, P. V., III & Hunter, M. J. (1970) *Arch. Biochem. Biophys.* **140**, 197–209