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ON THE POSSIBLE ROLE OF BILIVERDIN STIMULATION OF CYCLIC AMP LEVELS AS A TRIGGER FOR LIVER REGENERATION IN THE RAT

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Abstract—Contrary to a recent report by Okazaki *et al.* (*Biochem. biophys. Res. Commun.*, 1978, **81**, 512–520) we show that high concentrations of biliverdin (100 mg/kg, i.p.) slightly reduce the mitotic rate in rat liver. Adenylate cyclase and both the "high K_m" and "low K_m" phosphodiesterase of rat liver plasma membranes are inhibited by high concentrations of biliverdin.

Biliverdin has no effect on cyclic AMP production in isolated hepatocytes. An intracellular binding protein (glutathione-*S*-transferase B) is shown to bind biliverdin providing a mechanism for maintaining a low free intracellular concentration of the tetrapyrrole analogous to that of albumin in plasma.

In conclusion, these results are not consistent with a role for biliverdin in stimulating liver regeneration in the rat via a mechanism involving elevated cyclic AMP levels.

The capacity of mammalian liver to regenerate after partial hepatectomy is well-documented (for review see [1]). Although there is a considerable body of evidence to indicate that this phenomenon is humorally-mediated [2, 3] the nature of the factor(s) is far from clear [4]. Indeed, there is a controversy as to whether the mechanism of action is stimulatory [3] or inhibitory [2, 4]. Recently Okazaki *et al.* [5] have suggested that biliverdin§ initiates liver regeneration in the rat by a stimulatory mechanism. Support for this hypothesis has been presented by Matsui *et al.* [6] who have described a biliverdin-dependent increase in ornithine decarboxylase activity. In addition these workers observed a biliverdin-mediated increase in cAMP levels and cAMP-dependent protein kinase activity prior to the increase in ornithine decarboxylase activity. This sequence of events is exhibited in many systems prior to cell proliferation [7–9]. Unfortunately, both Okazaki *et al.* [5] and Matsui *et al.* [6] used commercial biliverdin which is notoriously impure [14]. Since their results are potentially important we have examined the effect of purified biliverdin on mitotic rate, adenylate cyclase and 'cyclic AMP' phosphodiesterase activity. Our results suggest that biliverdin is unlikely to play a role in liver regeneration in the rat.

MATERIALS AND METHODS

Materials. Biliverdin was prepared as in ref. [15]. Ox kidney biliverdin reductase was purified to homogeneity as previously described [10]. Glutathione-*S*-transferase B was purified as previously described [16] except that the enzyme levels were induced by phenobarbital treatment [17]. Rat liver plasma membranes were prepared by a modified [18] method of Pilkis *et al.* [19].

Enzyme assays. Biliverdin reductase activity was measured spectrophotometrically as in ref. [10]. Assays were conducted at 30° in 0.1 M sodium phosphate, pH 7.2 containing 700 μM NADH and 20 μM biliverdin. For routine column monitoring 37 μM bovine serum albumin was added, although on occasions this was omitted or replaced by 55 μM glutathione-*S*-transferase B (see text for details). The extinction coefficient employed for bilirubin, bilirubin bovine serum albumin and bilirubin–glutathione-*S*-transferase B were 30 $mM^{-1}\,cm^{-1}$ [20], 57 $mM^{-1}\,cm^{-1}$ [10] and 62 $mM^{-1}\,cm^{-1}$ [21]. Adenylate cyclase activity was assayed as described in ref. [18]. Biliverdin did not affect the binding assay used to detect cAMP. Phosphodiesterase activity of purified plasma membranes was measured by a modification [22] of the method of Thompson and Appleman [23]. Care was taken to correct for binding of [^{3}H]-adenosine to the Dowex 1 resin (see [22]).

The phosphodiestrerase assay was conducted at final cAMP concentrations of 1 μM and 1 mM. At 1 mM cAMP the activity of the "high K_m" integral enzyme is monitored whilst at 1 μM cAMP the activity of the "low K_m" peripheral enzyme is monitored (see [22]).

Determination of mitotic rate. Male Albino rats of a Wistar strain were maintained under a standardized regime (artificial light from 0800 to 2000 hr alternating with darkness; 22–23°; restricted access with handling only between 1100 and 1130 hr daily) for 1–4 weeks before experiments.

The animals were killed and small pieces of the median lobe of the liver sectioned at 5 μm and stained

§ In accordance with IUPAC recommendations biliverdin IXα and bilirubin IXα are referred to as biliverdin and bilirubin respectively.

with haematoxylin and eosin. Mitotic activity was assessed using the metaphase-arrest agent vincristine sulphate (Onconvin, Eli Lilly, Ltd.) which was given three hours before killing the animals. The number of metaphases present in fifty randomly selected fields viewed at a magnification of ×400 were determined for each animal. The number of nuclei per field was derived from sample counts. These gave similar results for control and biliverdin-treated samples indicating that biliverdin treatment does not alter cell size. The mitotic activity could therefore be expressed as the mitotic index (I_m, the number of metaphases per 10^4 cells).

A subsidiary experiment was performed to determine the time–course of the accumulation of metaphases after administration of vincristine sulphate. Groups of 4–6 rats were given vincristine sulphate (1 mg/kg i.p.) between 1400 and 1645 hr and killed 0.5–6 hr after the administration of vincristine. The arrangement of times chosen was such that the mid-point of each collection time was 1700 hr. The use of this design was intended to minimize the effects of circadian variation on mitotic activity (see [24]).

To test the effect of biliverdin on the mitotic rate groups of six rats (body weights 260–420 g) were injected with biliverdin (100 mg/kg i.p.) between 1030 and 1130 hr. The biliverdin was dissolved in 0.2 M sodium bicarbonate. The rats were killed 24, 30 and 36 hr after injection and for determination of the mitotic rate. Each biliverdin injected group had a time-matched control group of 4–6 rats injected with carrier.

Determination of intracellular cyclic AMP production in hepatocytes. Hepatocytes were prepared and incubated as previously described [25]. Hepatocyte viability was checked by determining ATP levels, which were 8.6 nmoles per mg dry weight of cells. Stock biliverdin solutions were made up and diluted with incubation buffer and gassed with O_2/CO_2 (19:1) prior to addition to the hepatocyte suspension. The cells were preincubated with biliverdin for 10 min prior to the addition of glucagon (10 nM). After five minutes incubation the reaction was terminated by centrifuging the cells through bromododecane into perchloric acid and the cyclic AMP determined as described previously [25].

Treatment of data. Initial rate data for the kinetics of biliverdin reductase were fitted by a least squares method to equations for total and partial substrate inhibition (see e.g. [10]).

RESULTS AND DISCUSSION

The mitotic index of normal adult liver is very low and values of 0.5–1, 1.4 and 1 have been reported [26–28]. Using vincristine to block spindle formation causes proliferating cells to accumulate in the metaphase stage of mitosis as shown in Fig. 1. A collection time of three hours was chosen for further experiments since significant degeneration of blocked metaphases occurred six hours after injection of vincristine (see [29] for a discussion of this problem). It should be noted that a significant proportion of the metaphases counted were in an "exploded" configuration. A similar observation has recently been reported for rat liver using the metaphase-arrest agent colchicine (Alison, personal communication). Table 1 shows the arrested mitotic index of rat liver 24, 30 and 36 hr after injection of purified biliverdin (100 mg/kg). This dose is the same as that employed by Okazaki *et al.* [5] although their preparation was not 100% biliverdin [14]. In contrast to the time-matched controls there is a statistically significant drop in the arrested mitotic index particularly 30 hr after treatment (see Table 1). The control values for the mitotic index exhibit a possible circadian rhythm peaking 30 hr after injection with carrier (i.e. at 1700 hr), which might be anticipated from previous work with the rat [24]. It should be noted that the control group for the 30 hr time point gave a mitotic index that is reduced three-fold when compared to the vincristine time course experiment shown in Fig. 1, both experiments had a collection time at 1700 hr. We have no explanation for this difference although it may be due to long term "carrier" effects.

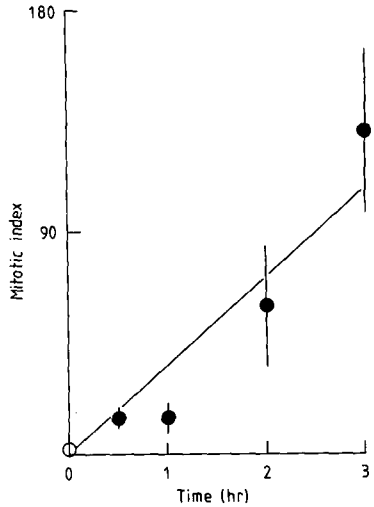

Fig. 1. Accumulation of metaphases in rat liver after administration of vincristine sulphate. The open symbol at zero time is taken from data in [27]. For other details see text.

Table 1. Effect of biliverdin on mitotic activity in rat liver

Time after administration (hr)	Number of mitoses per 10,000 cells[a]	
	Control	Biliverdin[c] (100 mg/kg)
24	16.6 ± 9.81 (5)	4.0 ± 3.74 (4)*
30	47.7 ± 11.83 (6)	4.3 ± 3.27 (6)***
36	11.8 ± 3.66 (6)	3.8 ± 3.22 (6)**
30[b]	49.75 ± 22.60 (4)	14.8 ± 5.74 (6)*

[a] Values are given as means ± standard deviation. Values in parentheses indicate the number of animals per group. Vincristine sulphate was administered 3 hr before killing the animals.

[b] Experiments performed with commercial (Sigma) biliverdin.

[c] Probability of significant difference (Student's *t*-test) between biliverdin-treated and controls: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

To check whether the discrepancy between our data and that of Okazaki *et al.* [5] was due to some impurity in the commercial biliverdin we repeated the experiment using biliverdin obtained from the Sigma Chemical Co. In this experiment we obtained a mitotic index of 14.8 ± 5.74(4) for the biliverdin-treated group (30 hr after injection) and 49.8 ± 22.68(4) for a time-matched control group. The good agreement between the 30 hr time-matched controls in the "purified" and "commercial" biliverdin experiments demonstrates the reproducibility of the metaphase arrest technique.

We have conducted preliminary experiments omitting vincristine sulphate and have obtained no evidence for any biliverdin-dependent stimulation of the mitotic rate (Phillips and Mantle, unpublished work).

Effect of biliverdin on adenylate cyclase and phosphodiesterase activity. Although Matsui *et al.* [6] have presented evidence that administration of biliverdin caused an increase in the level of cyclic AMP they did not suggest whether this was due to a stimulation of adenylate cyclase and/or an inhibition of phosphodiesterase. Figure 2 shows the effect of various concentrations of biliverdin on the basal, fluoride stimulated, glucagon stimulated and glucagon plus 0.1 mM GTP stimulated activity of adenylate cyclase. Biliverdin had no effect on basal or glucagon-stimulated adenylate cyclase activity at a final concentration of 10 μM. At higher concentrations biliverdin inhibited both glucagon and glucagon plus GTP stimulated adenylate cyclase activity with an I_{50} of 250 μM. Interestingly, there was no inhibition of fluoride-stimulated adenylate cyclase activity by any of the concentrations of biliverdin used (Fig. 2), which is consistent with the view that fluoride and hormones activate adenylate cyclase by distinct mechanisms [30, 31].

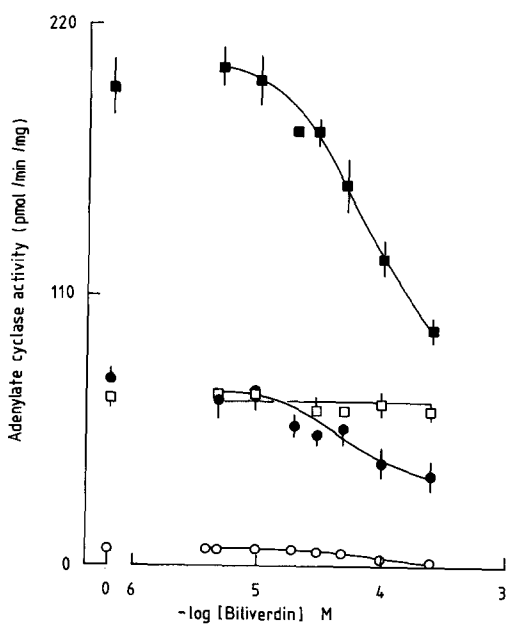

Fig. 2. Effect of biliverdin on the activity of adenylate cyclase. Basal (○), 15 mM fluoride stimulated (□), 1 μM glucagon stimulated (●) and 1 μM glucagon plus 100 μM GTP stimulated (■) activity of adenylate cyclase was assayed as described in text.

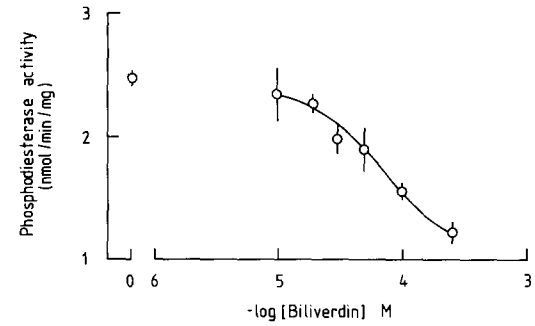

Fig. 3. Effect of biliverdin on phosphodiesterase activity at high substrate concentration (1 mM cyclic AMP). For details see text.

Biliverdin, at a final concentration of 10 μM, had no effect on the activity of the integral "high K_m" phosphodiesterase (Fig. 3), although under these conditions the peripheral "low K_m" phosphodiesterase was inhibited 13% (Fig. 4). The activity of both the "low K_m" and "high K_m" phosphodiesterase activity of plasma membranes was inhibited at higher concentrations of biliverdin. The "high K_m" phosphodiesterase showed an I_{50} of 250 μM, while the "low K_m" phosphodiesterase was maximally inhibited by 30% at approximately 25 μM biliverdin. As cyclic AMP levels in the hepatocyte range (see [32]) from 0.2 μM (basal) to 5 μM (glucagon stimulated) only the "low K_m" phosphodiesterase is kinetically relevant [33, 34]. Reynolds [34] has recently shown that the steady state level of cyclic AMP is particularly sensitive to changes in the maximal velocity of the "low K_m" phosphodiesterase, so that the weak inhibition of this enzyme by low levels of biliverdin (10–25 μM), which are without effect on the activity of adenylate cyclase might be predicted to elevate cyclic AMP levels. However, this is not found experimentally and may reflect a depletion of the intracellular levels of biliverdin by GST-B and other binding proteins (see below). At higher concentrations of biliverdin the effect on cyclic AMP levels is difficult to predict since adenylate cyclase (both basal and hormone-stimulated activities) and the "high K_m" phosphodiesterase activities are inhibited. The experimental observation (see below)

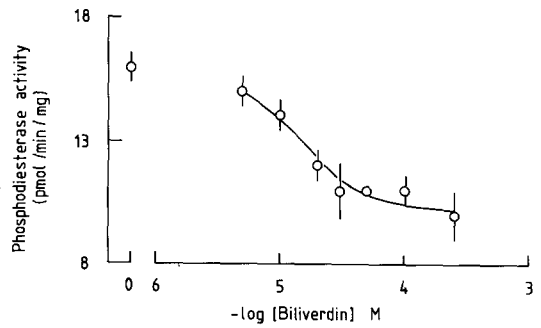

Fig. 4. Effect of biliverdin on phosphodiesterase activity at low substrate concentration (1 μM cyclic AMP). For details see text.

is that even at high concentrations (200 μM) biliverdin is without effect on the intra-cellular production of cyclic AMP.

Effect of biliverdin on intracellular cyclic AMP production in hepatocytes. Figure 5 shows the effect of various concentrations of biliverdin on glucagon-stimulated intracellular cyclic AMP production in hepatocytes. It can be seen that biliverdin has no effect at any concentration tested, either in the presence or absence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine. Matsui *et al.* [6] observed a 30% increase in cyclic AMP levels in rat liver *in vivo*, 2 hr after administering biliverdin (i.p.; 100 mg/kg). This dose results in peak plasma levels of biliverdin of 30 μM after 1 hr which are maintained for several hours (T. J. Mantle, unpublished work). This concentration is well within the range we used in the present study with isolated hepatocytes. We have no explanation for the apparent discrepancy between our results and those in ref. [6] unless additional long-term effects of biliverdin administration occur *in vivo* to increase the level of cyclic AMP. It should be noted that the hepatocyte medium contains a physiological concentration of albumin so that the free biliverdin should be the same in the experiments reported here and those in ref. [6].

Role of intracellular binding proteins. Although the "low K_m" and "high K_m" phosphodiesterases and adenylate cyclase are associated with the plasma membrane their substrates (and presumably inhibitors) are cytosolic, so that the presence of intracellular biliverdin-binding proteins will play an analogous role to that of serum albumin in maintaining low free levels of biliverdin and thus modulating against any inhibitory action. The glutathione-*S*-transferases are a family of proteins that are believed to play a significant role as intracellular binding proteins [11]. The effect of a physiological concentration of glutathione-*S*-transferase B (a major form, see e.g. [11]) on the activity of biliverdin reductase is shown in Fig. 6.

The results are consistent (see [10] for details of this method) with glutathione-*S*-transferase B binding biliverdin, with a K_d of 8 μM. As the concentration of glutathione-*S*-transferase B *in vivo* has been estimated to be 55 μM, this protein will play a major role in modulating against any inhibitory action of biliverdin. The glutathione-*S*-transferases are induced by a variety of stimuli (see e.g. [12, 13]) including partial hepatectomy [13] which will enhance the buffering effect.

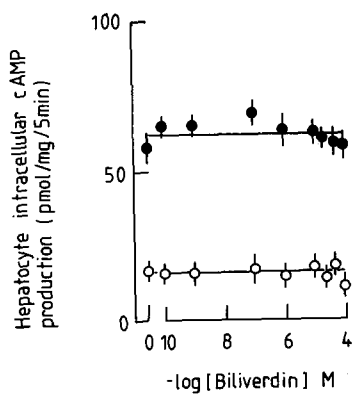

Fig. 5. Effect of biliverdin on intracellular cyclic AMP production in hepatocytes. The experiments were conducted in the presence (●) or absence (○) of 3-isobutyl-1-methylxanthine (1 mM).

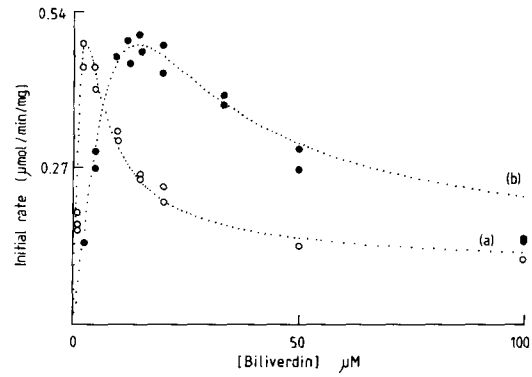

Fig. 6. Effect of glutathione-*S*-transferase B on the kinetics of biliverdin reductase. The initial rate data points obtained for the control (○) and in the presence of 55 μM glutathione-*S*-transferase B (●) were fitted by a least squares method to the equations for partial and total substrate inhibition. By the criteria described in [35] better fits were obtained to partial substrate inhibition.

We have not extended these studies to include other forms of glutathione-*S*-transferase, but these might also be expected to bind biliverdin (see e.g. [11]). As the studies on biliverdin inhibition of adenylate cyclase and the "low" and "high" K_m forms of phosphodiesterase were conducted *in vitro* in the absence of any binding protein the inhibition observed is an overestimate of the potency *in vivo* since these binding proteins will significantly deplete the free concentration of biliverdin. As the inhibition *in vitro* was not particularly potent this factor would also mediate against biliverdin playing any physiological role in liver regeneration. Indeed, even in pathological situations (e.g. 90% hepatectomy) the biliverdin concentration in plasma is unlikely to play a role in modulating cyclic AMP levels or stimulating mitosis (see also [36]).

GENERAL CONCLUSIONS

We have been unable to confirm the results of Okazaki *et al.* [5] that biliverdin triggers mitosis in rat liver. While this manuscript was in preparation Castell and Gomez-Lechon [36] reported a similar conclusion. These workers found no evidence for an increased mitotic index when biliverdin (at levels observed after 90% hepatectomy) was infused intravenously. However, they noted a moderate increase in mitotic index when biliverdin was administered intraperitoneally. This effect was shown to result from a non-specific stimulation of the peritoneal cavity by fine particulate suspensions (including the high concentration of biliverdin used). In an elegant series of experiments Castell and Gomez-Lechon [36] showed that these mitotic figures did not incorporate tritiated thymidine and concluded that these cells were derived from a G_2 blocked quiescent cell

population in liver. Our biliverdin was made up in an alkaline buffer so that we did not experience major problems with solubility. This probably explains why we observed no increase in mitotic index using an intraperitoneal route.

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REFERENCES

1. N. L. Bucher, *Int. Rev. Cytol.* **15**, 245 (1963).
2. A. Sakai, *Nature* **228**, 1186 (1970).
3. B. Fisher, P. Szuch, M. Levine and E. R. Fisher, *Science* **171**, 575 (1971).
4. L. M. Patt and J. C. Houck, *FEBS Lett.* **120**, 163 (1980).
5. K. Okazaki, H. Nishimura, H. Arizono, N. Nishimura and Y. Suzuki, *Biochem. biophys. Res. commun.* **81**, 512 (1978).
6. I. Matsui, S. Otani and S. Morisawa, *Life Sci.* **24**, 2231 (1979).
7. S. Thrower and M. G. Ord, *Biochem. J.* **144**, 361 (1974).
8. C. V. Byus and D. H. Russel, *Life Sci.* **15**, 1991 (1974).
9. C. V. Byus, M. K. Haddox and D. H. Russell, *J. Cyclic Nucleotide Res.* **4**, 45 (1978).
10. O. Phillips and T. J. Mantle, *Biochem. Soc. Trans.* **9**, 275 (1981).
11. W. B. Jakoby, *Adv. Enzym.* **46**, 383 (1978).
12. G. Clifton and N. Kaplowitz, *Biochem. Pharmac.* **27**, 1284 (1978).
13. M. Younes, R. Schlichting and C-P. Siegers, *Pharmac. Res. Commun.* **12**, 115 (1980).
14. A. F. McDonagh and L. A. Palma, *Biochem. J.* **189**, 193 (1980).
15. A. F. McDonagh, *The Porphyrins* (Ed. D. Dolphin), Vol. 6, p. 453. Academic Press, New York (1979).
16. N. C. Scully and T. J. Mantle, *Biochem. J.* **193**, 367 (1981).
17. C. Guthenberg, R. Morgenstern, J. W. DePierre and B. Mannervik, *Biochim. biophys. Acta* **631**, 1 (1980).
18. M. D. Houslay, J. C. Metcalfe, G. B. Warren, T. R. Hesketh and G. A. Smith, *Biochim. biophys. Acta* **436**, 489 (1976).
19. S. J. Pilkis, J. H. Exton, R. A. Johnson and C. R. Park, *Biochim. biophys. Acta* **343**, 250 (1974).
20. R. Tenhunen, M. E. Ross, H. S. Marver and R. Schmid, *Biochemistry* **9**, 298 (1970).
21. O. Phillips and T. J. Mantle, unpublished work.
22. R. J. Marchmont and M. D. Houslay, *Biochem. J.* **187**, 381 (1980).
23. W. J. Thompson and M. M. Appleman, *Biochemistry* **10**, 311 (1971).
24. O. Redmond and A. R. Tuffery, *J. Anat.* **129**, 731 (1979).
25. M. D. Houslay and K. R. F. Elliot, *FEBS Lett.* **104**, 359 (1979).
26. A. M. Brues and B. B. Marble, *J. exp. Med.* **65**, 15 (1937).
27. A. Marshak and R. L. Byron, *Proc. Soc. exp. Biol. Med.* **59**, 200 (1945).
28. M. Abercombie and R. D. Harkness, *Proc. R. Soc. B* **138**, 544 (1951).
29. W. A. Aherne and R. S. Camplejohn, *Expl Cell Res.* **74**, 496 (1972).
30. R. A. Johnson, S. J. Pilkis and P. Hamet, *J. biol. Chem.* **250**, 6599 (1975).
31. M. D. Houslay, I. Dipple and K. R. F. Elliott, *Biochem. J.* **186**, 649 (1980).
32. R. J. Marchmont and M. D. Houslay, *Nature* **286**, 904 (1980).
33. D. A. Fell, *J. theor. Biol.* **84**, 361 (1980).
34. C. H. Reynolds, *Biochem. J.* **202**, 125 (1982).
35. B. Mannervik, B. Gorna-Hall and T. Bartfai, *Eur. J. Biochem.* **37**, 270 (1973).
36. J. Castell and M. J. Gomez-Lechon, *Rev. esp. Fisiol.* **39**, 183 (1983).