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# Effect of inhibition of glutathione synthesis on the metabolism and protein conjugation of [$^{14}$C]captopril in the rat

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Captopril (CP)* is an orally active angiotensin-converting enzyme inhibitor used in the treatment of hypertension [1, 2]. The major *in vivo* biotransformations of CP involve mixed disulphide formation with endogenous thiols derived from cysteine [3]. A number of adverse reactions associated with CP are thought to have an underlying immunological mechanism [4], and it has been suggested that CP with its free sulphydryl moiety, or one of its metabolites, may act as a hapten. However, metabolic studies have shown that CP covalently bound to plasma proteins can be dissociated by a thiol disulphide interaction both *in vitro* and *in vivo* [3, 5], thus affecting the ability of CP to act as a hapten. Therefore, we have investigated the effect of glutathione (GSH) depletion on the formation of CP–plasma protein conjugates *in vivo*, in relation to the metabolism of CP. Depletion of GSH was by pre-treatment with DL-buthionine sulfoximine (BSO) which is a potent and specific inhibitor or γ-glutamylcysteine synthetase, the enzyme which catalyses the initial step of glutathione synthesis [6]. In this study, the covalent binding of [$^{14}$C]CP to tissue proteins was also determined.

## *Materials*

[$^{14}$C]Captopril (4.66 μCi/mg) labelled in the amide carbonyl group, and authentic standards of captopril, captopril disulphide, captopril–cysteine mixed disulphide and captopril–glutathione mixed disulphide were supplied by the Squibb Institute (New Brunswick, NJ). DL-Buthionine sulfoximine was obtained from Chemical Dynamics Corporation (NJ, U.S.A.). Silica gel ($60\,F_{254}$) thin-layer chromatography plates (Merck, 20 × 20 × 0.02 cm) were obtained from British Drug Houses (Poole, U.K.). *N*-Ethylmaleimide, glyoxalase I (Grade IV), methylglyoxal, morpholino-propanesulfonic acid and other general reagents were obtained from Sigma Chemical Company (Poole, U.K.). Dialysis tubing was obtained from Medicell Ltd (London, U.K.). Scintillation fluid (NE260) was obtained from Nuclear Enterprises, (Edinburgh, U.K.). NCS tissue solubilizer solution was obtained from Amersham (Bucks, U.K.). All solvents were redistilled before use.

## *Methods*

*Depletion of glutathione by inhibition of synthesis.* Male Wistar rats (250–300 g) were given free access to food and water. Glutathione synthesis was inhibited by the method of Griffith and Meister [6]. DL-Buthionine sulfoximine was administered intraperitoneally (8 mmol/kg), one half of the dose was given initially, and the remainder given 1.5 hr later. The control rats received saline only.

*Determination of glutathione concentrations.* The glutathione concentrations of the liver samples were determined on the same day by the glyoxalase I method [5]. This method is specific for reduced GSH and is not affected by the presence of sulphydryl compounds such as captopril.

*Effect of glutathione depletion on the metabolism of* [$^{14}$C]*captopril in the rat.* The metabolism of [$^{14}$C]CP was studied 3 hr after the initial buthionine sulfoximine (BSO) injection. The rats were anaesthetised with urethane (14% w/v in saline, 10 ml/kg, i.p.) and the trachea, carotid artery and jugular vein cannulated with polythene tubing of the appropriate sizes. [$^{14}$C]CP (4.66 μCi/mg, 4 mg/kg) in saline was administered via the jugular vein. Blood samples (0.5 ml) were collected at 5, 30, 60, 120 and 180 min. Plasma was obtained immediately by centrifugation (2000 *g*) in a Microfuge and *N*-ethylmaleimide (NEM, 4 mg/ml) was added to derivatise any free captopril present. The volume of blood taken out was replaced by an equal volume of saline. [$^{14}$C]captopril covalently bound to plasma proteins and [$^{14}$C]CP metabolites were determined by SDS-equilibrium dialysis and thin-layer radiochromatography, respectively, by methods previously described [3].

After 3 hr, the rat was killed and the liver, lungs, kidneys and spleen were removed and weighed. A section of the liver was taken for the determination of glutathione concentration by the glyoxalase I method. The tissues were

* Abbreviations used: BSO, buthionine sulfoximine; CP, captopril; CP-cys, captopril cysteine mixed disulphide; CPD, captopril disulphide; EDTA, ethylenediamine tetreacetic acid; GSH, reduced glutathione; NEM, *N*-ethylamaleimide; SDS, sodium dodecyl sulphate.

then immediately frozen in liquid nitrogen and stored at −60° until use.

*Tissue distribution of [$^{14}$C]CP metabolites.* The total radioactive content of the tissues was determined by liquid scintillation spectrometry [3]. Tissue distribution of [$^{14}$C]CP metabolites was determined by exhaustive solvent extraction and thin-layer radiochromatography. A 25% w/v homogenate of each tissue was prepared in 0.1 M sodium phosphate buffer (pH 7.4) containing 1 mM EDTA and 1% NEM. Aliquots (200 μl) of the homogenate were incubated with NCS tissue solubiliser (1 ml) at 50° overnight, neutralized with glacial acetic acid (30 μl) and then mixed with liquid scintillation fluid (12 ml) to quantitate the total radioactivity. [$^{14}$C]CP metabolites were determined by thin-layer radiochromatography after exhaustive solvent extraction with three vol. of various solvents, in the order: 3% trichloroacetic acid, absolute ethanol, 70% ethanol, 80% methanol in water, and sodium phosphate buffer (0.01 M, pH 7.4). The total extractable radioactivity was also determined with the pooled solvents which were concentrated *in vacuo*.

The amount of [$^{14}$C]CP covalently bound to tissue proteins was determined by dissolving the protein pellet (after exhaustive solvent extraction) in sodium hydroxide (1 M, 10 ml) and aliquots (0.5 ml) of the solution were neutralized with glacial acetic acid (50 μl) and then mixed with 10 ml of scintillation fluid. The amount of radioactivity was quantified by liquid scintillation spectrometry and the protein concentration of the solution was determined [7].

*Urinary excretion of [$^{14}$C]CP metabolites.* To study the effect of inhibition of GSH synthesis on the urinary excretion of [$^{14}$C]CP, male Wistar rats (250–300 g) were housed in metabolic cages and given free access to food and water. BSO was administered as previously described and control rats received saline only. Urine was collected into tubes containing NEM (2 mg/ml) after [$^{14}$C]CP (9.96 μCi/mg, 4 mg/kg) was administered intraperitoneally. Total radioactivity and [$^{14}$C]CP metabolites in urine at 24 hr and 48 hr were determined by liquid scintillation spectrometry after separation by thin-layer chromatography.

## Results

*Depletion of glutathione by inhibition of synthesis.* As shown in Table 1, glutathione (GSH) synthesis was inhibited by DL-buthionine sulfoximine (BSO). Hepatic GSH was depleted by 86% after BSO pre-treatment.

*Effect of glutathione (GSH) depletion on the metabolism of [$^{14}$C]CP.* [$^{14}$C]captopril (CP) became extensively and covalently bound to plasma proteins (Fig. 1) in the control rats, to a similar degree to that reported previously [3]. At 3 hr, 36% of the radioactivity in plasma was covalently bound. The amount of [$^{14}$C]CP covalently bound to plasma proteins was significantly increased by GSH depletion, with 55% of radioactivity in plasma covalently bound and thus representing a 53% increase after 3 hr.

The plasma concentration of total radioactivity showed significant difference only after 120 min (Fig. 2). At 180 min, the total plasma concentration was 0.51 ± 0.08% of dose per ml plasma and 0.83 ± 0.09% of dose per ml plasma for BSO-treated and control rats, respectively. However, there was no significant difference in the plasma concentration–time curves of unchanged [$^{14}$C]CP and [$^{14}$C]CP-cys between the control and BSO-treated rats.

*Tissue distribution of [$^{14}$C]CP metabolites.* There was no significant difference in the amounts of radioactivity in the kidney, lung and spleen between control and BSO-treated rats 3 hr after [$^{14}$C]CP administration (Fig. 3). The total radioactivity present in the liver was, however, significantly decreased after GSH depletion, with 1.8 ± 0.5% of the dose in the BSO-treated rats compared to 4.8 ± 0.8% of the dose in the controls. Exhaustive solvent extraction of the liver homogenates showed that about 70% of the radioactivity present was extractable in both sets of animals, even though the amount of total radioactivity present was significantly different.

Thin-layer radiochromatographic studies of the tissue homogenates, after exhaustive solvent extraction, showed

Table 1. Depletion of glutathione (GSH) in whole blood and in the liver 6 hr after i.p. administration of 8 mmol/kg of buthionine sulfoximine (BSO) in the rat

| Treatment | Hepatic GSH (μmol/g liver) | GSH in whole blood (μmol/ml) |
|---|---|---|
| Control | 5.65 ± 0.67 | 1.02 ± 0.16 |
| BSO-treated | 0.77 ± 0.13* | 0.02* |

Results are mean ± SD of five animals. * $P < 0.001$ using Student's *t*-test.

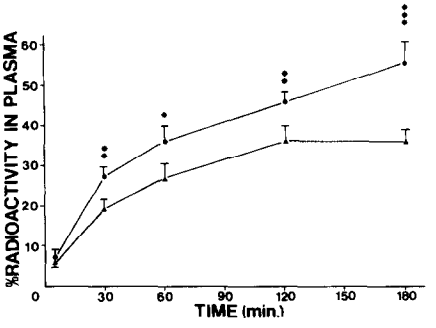


Fig. 1. Percentage in plasma of [$^{14}$C]captopril (CP) covalently bound to plasma proteins after i.v. administration of [$^{14}$C]CP to control rats (▲) and rats pre-treated with BSO (●). Results are mean ± SD of five animals. *** $P < 0.001$, ** $P < 0.005$ and * $P < 0.01$ using Student's *t*-test.

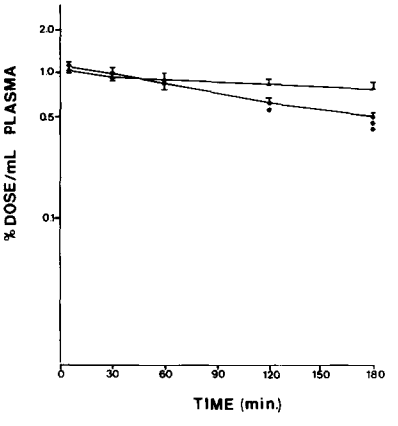


Fig. 2. Concentrations in plasma of total [$^{14}$C]radioactivity after i.v. administration of [$^{14}$C]captopril to control rats (▲) and rats pretreated with BSO (●). Results are mean ± SD of five animals. ** $P < 0.005$, * $P < 0.05$ using Student's *t*-test.

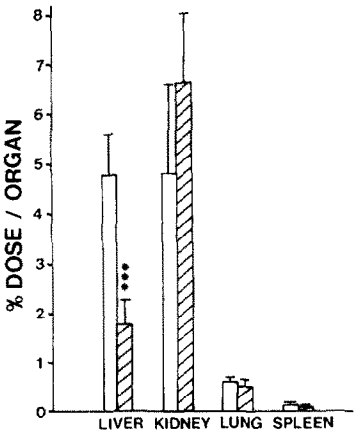


Fig. 3. Tissue distribution of radioactivity 3 hr after administration of [$^{14}$C]captopril (CP) to control rats (□) and rats pretreated with BSO (■). Results are mean ± SD of five animals. $P < 0.001$ using Student's *t*-test.

that [$^{14}$C]CP-cys were the major radioactive components (59.1 ± 3.9% in the controls and 45.6 ± 6.1% in the BSO-treated rats). There is relatively little covalent binding of [$^{14}$C]CP to liver proteins. In the kidney, the amount of [$^{14}$C]CP covalently bound was significantly higher ($P < 0.005$) after GSH depletion (13%) compared to the controls (8%). A large proportion of the radioactivity present in both the lung and spleen was covalently bound (approximately 50%) and this was unaffected by GSH depletion.

*Effect of GSH depletion on the urinary excretion of* [$^{14}$C]*CP metabolites.* The extraction of total radioactivity in urine over 48 hr (Table 2) was not affected after GSH depletion, with about 86% of the dose excreted in 48 hr (74% in 24 hr). The major urinary metabolite was unchanged [$^{14}$C]CP in both the control and BSO-treated rats. However, the relative distribution of [$^{14}$C]CP metabolites was significantly different in the BSO treated rats. The amount of unchanged [$^{14}$C]CP excreted was decreased by 20% at 24 hr and 25% at 48 hr after GSH depletion. The amount of [$^{14}$C]CP-cys excreted was much higher in the BSO-treated rats (two-fold increase at 24 hr and four-fold increase at 48 hr).

## *Discussion*

Glutathione (GSH) is known to function both directly and indirectly in many biological processes including the synthesis of proteins, transport of amino acids, enzyme activity, metabolism and protection of cells [8–10]. The importance of GSH in the detoxification of chemically reactive chemicals or metabolites has also been extensively documented, with numerous examples of drug-induced toxicity after GSH depletion [11–15]. Perturbation of the GSH status can occur as a consequence of genetic defects associated with deficiencies of specific enzymes of GSH metabolism [16]; certain physiological conditions such as diabetes and renal diseases [17–22]; and chemical reactions with xenobiotics. In this study, depletion of GSH was achieved by pretreatment with BSO to inhibit GSH synthesis. The hepatic GSH level was depleted to 14% of the control level 6 hr after BSO administration. This is consistent with the

Table 2. Metabolites of [$^{14}$C]captopril present in urine after administration to control rats and rats pre-treated with DL-buthionine-*S*,*R*-sulfoximine (BSO)

| Time | Treatment | % Dose in urine | % Urinary metabolites as CP | CPD | CP-Cys |
|---|---|---|---|---|---|
| 0–24 | Control | 75.0 ± 8.8 | 77.5 ± 6.8 | 7.5 ± 4.4 | 6.5 ± 0.5 |
| 0–24 hr | BSO-treated | 74.2 ± 16.3 | 62.6 ± 9.9* | 14.6 ± 4.1* | 15.9 ± 6.8* |
| 24–48 hr | Control | 12.7 ± 4.8 | 74.4 ± 3.6 | 13.4 ± 2.1 | 4.6 ± 0.4 |
| 24–48 hr | BSO-treated | 12.6 ± 8.8 | 55.5 ± 2.2† | 16.5 ± 2.6 | 21.1 ± 2.3† |

Results are mean ± SD of five animals. CP = captopril, CPD = captopril disulphide and CP-Cys = captopril cysteine mixed disulphide.
* $P < 0.05$, † $P < 0.001$ using Student's *t*-test.

Table 3. Tissue distribution of [$^{14}$C]captopril (CP) metabolites 3 hr after i.v. administration of [$^{14}$C]CP to rats pre-treated with BSO, compared to controls

| Tissue | Treatment | Relative distribution of radioactivity (%) Covalently bound | Unchanged CP | CP disulphide | Mixed disulphide |
|---|---|---|---|---|---|
| Kidney | Control | 8.2 ± 0.7 | 27.9 ± 2.5 | 6.3 ± 0.7 | 34.2 ± 2.6 |
| | BSO-treated | 12.6 ± 0.8† | 24.9 ± 12.4 | 17.6 ± 3.8* | 22.9 ± 9.5 |
| Liver | Control | 2.7 ± 1.0 | — | 8.6 ± 1.4 | 59.1 ± 3.9 |
| | BSO-treated | 4.9 ± 0.8* | — | 19.5 ± 6.5* | 45.6 ± 6.1 |
| Lung | Control | 52.6 ± 10.7 | — | 1.4 ± 0.7 | 35.1 ± 10.5 |
| | BSO-treated | 47.0 ± 18.2 | — | 0.8 ± 0.1 | 44.7 ± 17.4 |
| Spleen | Control | 48.1 ± 8.9 | 2.3 ± 0.9 | 5.0 ± 1.8 | 31.0 ± 6.5 |
| | BSO-treated | 51.7 ± 5.0 | 1.5 ± 0.3 | 4.0 ± 2.4 | 22.2 ± 0.5 |

Results are mean ± SD of five animals.
* $P < 0.05$, ** $P < 0.005$ using Student's *t*-test.

findings by Griffith and Meister [6], who showed a depletion of 81% in hepatic GSH 3 hr after BSO injection. Furthermore, the dose of BSO employed in this study would have no effect on metabolizing enzyme activity in the liver [23].

The urinary excretion of [$^{14}$C]CP total radioactivity was not reduced in the BSO treated rats compared to controls but the relative amounts of urinary metabolites was significantly altered. The major urinary metabolite in both cases was unchanged [$^{14}$C]CP after 24 hr and 48 hr. The percentage of radioactivity excreted as [$^{14}$C]CP-cys was increased upon GSH depletion, and the radioactivity excreted as unchanged [$^{14}$C]CP in the 24–48 hr urine was decreased by 20%. The change in the relative distribution of urinary metabolites is similar to that observed after infusion of [$^{14}$C]CP–plasma protein conjugates [3].

The amount of [$^{14}$C]CP covalently bound to plasma proteins was significantly increased in the BSO treated rats. This is probably due to the decrease in endogenous GSH concentration from both intravascular and extravascular sites (e.g. the liver). The dissociation of CP–plasma protein conjugates involves both thiol-disulphide interchange reactions, and reduction of disulphide bonds by thiol reductases [3, 24]. Thus, depletion of GSH by BSO pretreatment may lead to a slower rate of dissociation and therefore the observed accumulation of CP–plasma protein conjugates *in vivo*. Although cysteine has been shown to effect the dissociation of CP–plasma protein conjugates *in vitro*, it is very much less effective than GSH [5].

In previous studies, with the use of CP disulphide-linked to heterologous proteins, we have established that a disulphide bond is sufficiently stable to carry CP through the various biochemical and cellular processes, which lead to recognition by B and T lymphocytes in humoral and cell-mediated immunity, respectively [25–27]. The anti-CP antibody is highly specific for disulphide-linked forms of CP in both the rabbit and human [25–28], which have been established as major metabolites of CP *in vivo* [3, 29]. Thus, extensive covalent binding of CP to plasma proteins may provide a metabolic basis for CP to act as a hapten. In the absence of a sufficiently high concentration of GSH, these CP–plasma protein conjugates could accumulate and increase the potential of CP to act as a hapten. Furthermore, it has been found that CP–plasma protein conjugates may provide a reservoir from which pharmacologically active drugs can be regenerated. Thus accumulation of mixed-disulphide metabolites in patients with renal failure is associated with a prolonged haemodynamic effect [30].

Tissue distribution studies showed that only a small amount of [$^{14}$C]CP was covalently bound in the liver, which was significantly increased after GSH depletion. The amount of covalent binding of [$^{14}$C]CP to the kidney was also increased after GSH depletion, giving further evidence that GSH is essential in the metabolism of CP–protein conjugates in tissues. It is also of interest that both the lung and the spleen, which have the capacity to take up "foreign" particles such as drug-protein conjugates by phagocytosis, had relatively high percentages of [$^{14}$C]CP covalently bound to the tissue proteins. However, it is not possible, in the present study, to distinguish whether [$^{14}$C]CP was taken up into the tissues as the free drug and subsequently bound covalently to the tissue proteins or that [$^{14}$C]CP was taken up in the form of [$^{14}$C]CP-protein conjugates by macrophage which are present in high concentrations in these tissues (lung and spleen).

In conclusion, depletion of GSH by BSO pretreatment caused a significant increase in the covalent binding of [$^{14}$C]CP to plasma proteins. Since the metabolism of CP and CP–plasma protein conjugates requires cysteine and GSH, GSH status should be considered as an important determinant of both the pharmacological and the toxicological response to CP.

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# Phorbol ester-induced attenuation of tissue DNA synthesis: Antagonism by prolactin in liver and thymus

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Recent evidence suggests that prolactin (PRL) may play a central role in mammalian growth regulation. Administration of PRL to rats stimulates entry of cells into cell cycle. Ornithine decarboxylase (ODC, EC 4.1.1.17) is induced rapidly in a dose-dependent manner in liver, thymus, kidney, heart, spleen, and adrenal gland of rats within 6 hr of intraperitoneal PRL administration [1–3]. Plasminogen activator (PA, EC 3.4.21.31) is similarly induced in these tissues by PRL treatment [4]. Since the inductions of ODC and PA occur early during the $G_1$ phase of cell cycle, these observations suggest that PRL may regulate cell cycle progression in these tissues. Moreover, it has been demonstrated recently that PRL administration stimulates cell replication in rat liver as assessed by [$^3$H]thymidine ([$^3$H]TdR) incorporation into hepatocyte nuclei [5, 6]. Further, chronic PRL treatment causes hepatomegaly and increases the hepatic mitotic index [7]. Its administration subsequent to a hepatocarcinogen increases the expression of biochemical as well as histochemical markers characteristic of hepatic preneoplasia [7], suggesting that increases in PRL concentration may promote liver carcinogenesis as a direct result of its hepatotrophic effects. In addition, the role of PRL as an immunomodulatory hormone may be related to its ability to serve as a co-mitogen for lymphocytes [8].

The phorbol ester tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which directly activates protein kinase C (PKC, EC 2.7.1.37), stimulates *de novo* synthesis of ODC and PA activities in the liver [9–11] in a manner strikingly similar to the response produced by PRL. In the T51B liver cell line, activation of protein kinase C is positively coupled to cell cycle progression [12]. Recent evidence from our laboratory links the mitogenic action of PRL to the activation of PKC as an early event in liver replication in response to PRL or partial hepatectomy [13]. However, Tsukamoto and Kojo [14] have shown that phorbol ester treatment 8 hr after partial hepatectomy inhibits liver regeneration. Here, we report that TPA decreased DNA synthesis in liver, thymus, kidney, and heart. Further, co-administration of PRL abrogated the effects of TPA in liver and thymus, but not in kidney and heart.

## *Materials and Methods*

Male weanling Sprague–Dawley rats (26- to 28-days-old, 60–75 g) were housed in a controlled environment at 23° with a 12-hr light/dark cycle. Food (LM485, Tekland, Madison, WI) and water were available *ad lib*. Rats received ovine PRL (5.5 mg/kg, i.p., NIDDK, Bethesda, MD), TPA (0.6 mg/kg, i.p.), or the combined regimen at 12-hr intervals for 48 hr. At 46 hr, 1.0 $\mu$Ci/g of [$^3$H]TdR (70–75 Ci/mmol) was administered. No overt toxicity was caused by any of the treatment regimens. Treated and control animals continued to gain weight at similar rates over the 48-hr experimental period. Tissue DNA synthesis, assessed as the amount of [$^3$H]TdR incorporated into trichloroacetic acid precipitated DNA, was determined, as previously described [5], at 48 hr. This method for estimating DNA synthesis has been well characterized *in vivo* and *in vitro* and has been shown to positively correlate with other methods for determining cell proliferation responses [15–17]. We have demonstrated previously that PRL-stimulated [$^3$H]TdR incorporation in liver is time- and dose-dependent and specific for hepatic parenchymal cells [5, 7].

## *Results and Discussion*

Data presented in Fig. 1 demonstrate the effects of PRL and TPA administration on [$^3$H]TdR incorporation in various PRL-responsive tissues. Administration of PRL significantly increased hepatic DNA synthesis ($P < 0.01$, Fig. 1A). Treatment with TPA attenuated [$^3$H]TdR incorporation ($P < 0.01$) in liver compared to vehicle-treated controls, an effect consistent with the observations reported by Tsukamoto and Kojo [14]. However, the ability of TPA to decrease [$^3$H]TdR in liver was inhibited by PRL administration. In the thymus, a tissue in which PRL functions as a co-mitogen, the effect of TPA on decreasing [$^3$H]TdR incorporation was similarly antagonized by PRL treatment (Fig. 1B). In both the liver and thymus, PRL and TPA administration significantly ($P < 0.05$) increased [$^3$H]TdR incorporation compared to TPA treatment alone. In contrast, the TPA-produced decrease in [$^3$H]TdR incorporation in kidney and heart was not restored to control levels by co-administration of PRL (Fig. 1, panels C and D). In these tissues, the level of [$^3$H]TdR incorporation produced by the combined regimen did not differ from the effect of TPA administration alone.

Administration of PRL to rats increased hepatic DNA synthesis, similar to the previously reported effect of lactogen treatment in liver [5]. We reported recently that PRL administration is coupled to translocation of PKC from the cytosol to the hepatic membrane first detected by 15 min [13]. In addition, we found that partial hepatectomy increases serum PRL within 1 min with significant PKC translocation detectable at 30 min, the earliest time measured. TPA, which is thought to directly activate PKC by substituting for endogenous diacylglycerol, stimulates the expression of biochemical markers of $G_1$ cell cycle progression [10], but in the liver appears to inhibit entry into S phase. This interpretation is supported by the present study, and also by the reported ability of phorbol ester to inhibit DNA synthesis in regenerating rat liver [14]. In the latter, TPA had no effect on control DNA synthesis. This apparent discrepancy may be due to the treatment protocol employed by these investigators who administered phorbol esters as a single dose 16 hr prior to harvesting the liver for