However, the DHA reducing ability was not regained. This might be due to irreversible denaturation of Hb by 2-ME[15]. Hb denaturants like urea also caused complete inhibition of DHA reduction by Hbs from guinea-pig, rat and cat. The inhibition was, however, released when urea was removed by gel filtration. This would suggest that not only the presence of reactive SH groups but also the native conformation of the Hb molecule was needed for DHA reduction. Presumably, in the native conformation 2 reactive SH groups come into suitable proximity for the reduction of a DHA molecule.

In order to find out whether disulfide linkage formation took place within the same peptide chain or between 2 chains, SDS polyacrylamide gel electrophoresis (in presence and absence of 2-ME) of both DHA-treated and untreated guinea-pig Hb was done. This resulted in the appearance of a single band in the same position on both the gels, corresponding to the mol. wt of 16,000, indicating the formation of intrachain disulfide bonding. Similar results were obtained with rat and cat Hbs. The α chain of the major cat Hb has 3 reactive SH groups whereas the β chain has only one[14]. This suggests that the DHA reducing SH groups of cat Hb are present in the α chain. This was confirmed by hybridization experiments between cat Hb and human HbA, which is incapable of reducing DHA. Among the hybrids $\alpha_2^{cat}\beta_2^{human}$ and $\alpha_2^{human}\beta_2^{cat}$, prepared according to the method of Taketa et al.[14], only $\alpha_2^{cat}\beta_2^{human}$ was able to reduce DHA to AA.

Sequence studies of the α and β chains of major rat Hb[12,13] indicate α cys at positions 13, 104 and 111 and β cys at 93 and 125. From looking at the Hb model one observes that β 93 and 125 are very distant from each other. Therefore, as in cat Hb, in rat Hb also the DHA reducing SH groups are tentatively suggested to be present in the α chain. Considerable reactivity of the cys at α 13 has been reported[12]. Dog Hb also posses 2 cys at α 104 and 111, of which α 111 is reactive to mercurals, but α 104 is not[16]. Considering these facts it would appear that in rat Hb α 111 and α 13 may be involved in DHA reduction. Indirectly, in rat Hb α 111 and α 13 are presumably in close proximity.

The results presented in this communication indicate that DHA may be used as a preliminary probe to estimate vicinal intrachain reactive SH groups in mammalian Hbs.

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Effect of glucocorticoids on the appearance of gamma-glutamyl transpeptidase activity in primary cultures of adult rat hepatocytes[1]

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Summary. Primary cultures of adult rat liver parenchymal cells showed a progressive rise of gamma-glutamyl transpeptidase (GGT) activity (E.C. 2.3.2.2.) after the first 5 days of culture. The presence of dexamethasone and other synthetic glucocorticoids in the culture medium partially prevented this increase.

On adaptation to culture conditions, adult rat liver cells lose many specific functions, and show phenotypic characteristics of fetal liver[3,4]. Thus, it has been shown that GGT activity increases in primary cultures of adult rat hepatocytes[5]. This enzymatic activity, widely distributed in mammalian tissues, is low in adult liver but is present at higher levels in the fetal liver and in hepatomas[6,7].

Attempts to protect cultured hepatocytes from this dedifferentiation have led to the use of different support systems[5,8,9] and culture media supplemented with serum and/or hormones[10-13]. Glucocorticoids, which are known to inhibit fibroblastic cell growth (14, 15), seem to have a beneficial effect on cultured hepatocytes, increasing their survival and maintaining their polygonal epithelial morphology[16-18]. In this work we have studied the effect of several glucocorticoids on the expression of GGT activity in primary cultures of adult rat hepatocytes. The results show that these hormones maintain some adult liver characteristics of cultured hepatocytes for a longer period of time.

Materials and methods. Hepatocytes were isolated from livers of normally fed male adult Sprague-Dawley rats (200–300 g), by perfusion with collagenase (type I, Sigma Chemical Co., St. Louis, Mo. USA) according to Hue et al.[19]. Rats were anesthetized with ketamine clorhydrate (25 mg per 100 g b.wt). Ham's F-12 medium (Flow Laboratories, Irvine, U.K.), supplemented with 20% heat-inactivated fetal calf serum and antibiotics, was used[4]. Monolayers were maintained in plastic tissue culture dishes (Falcon Plastics, Los Angeles, Cal., USA) at 37 °C. Culture medium was changed daily. Glucocorticoids were added to the culture medium at the moment of seeding. Hepatocytes were harvested at the indicated times, and were homogenized in a medium containing 5 mM phosphate buffer pH 7.4, 10% glycerol and 1% Triton X-100. GGT activity was assayed at 25 °C according to the procedure described by

Sirica et al.[5]. 1 unit of specific activity is the amount of enzyme required to produce 1 µmole of p-nitroanilide per min and per mg of protein. Protein was determined by the method of Lowry et al.[20], using bovine serum albumin as standard. DNA was measured by the method of Burton[21], with calf thymus DNA as standard. The amount of DNA of cultured hepatocytes expressed in µg per mg of cellular protein was of 11 ± 0.9. This value was obtained from 7 different preparations of adult rat liver cells cultured under the above-mentioned conditions, and it is similar to those of 10.8 and 10.56 obtained by Seglen[22] and by Shibko et al.[23], for isolated rat liver cells and whole rat liver, respectively.

Results and discussion. Figure 1 shows that GGT activity of rat hepatocytes cultured under standard conditions was very low during the first 3 days (0.25 mU/mg protein), after which the enzyme activity progressively rose during the following days of culture, reaching a value of 81 mU/mg of protein on the 10th day. The presence of dexamethasone (10^{-7}M) in the culture medium practically blocked the rise of GGT activity. This blockage disappeared when the glucocorticoid was withdrawn from the culture medium on the 5th day. At this point, GGT activity began to rise, though at a slower rate than in control hepatocytes, reaching a value of 15 mU/mg of protein on the 10th day of culture.

Hepatocytes were also cultured for 7 days in the presence of different glucocorticoids and GGT activity was measured. The table shows that both dexamethasone and triamcinolone, even at the lowest concentration used (10^{-8}M), were effective blockers of the increase of GGT activity. Although they were also effective, 16-methyl-prednisolone, betamethasone and hydrocortisone showed a dose-dependent response in the concentrations assayed; the last 2 glucocorticoids were markedly less effective in blocking the rise in GGT activity.

Light microscopic morphology showed that hepatocytes cultured for 9 days in the presence of dexamethasone maintained their characteristic polygonal shape, a granular cytoplasm and a close apposition with neighbouring cells

Gamma-glutamyl transpeptidase activity of adult rat hepatocytes cultured in the presence of different glucocorticoids

Additions	GGT activity (mU/mg of protein)		
	10^{-6} M	10^{-7} M	10^{-8} M
Dexamethasone	3.77	3.26	3.83
Triamcinolone	3.53	2.82	3.00
16-methyl-prednisolone	3.67	4.71	9.16
Betamethasone	7.31	13.65	-
Hydrocortisone	9.36	16.76	24.24
Control	18.39		

GGT activity was measured on the 7th day of culture. Each value is the mean of duplicate assays of 2 culture dishes.

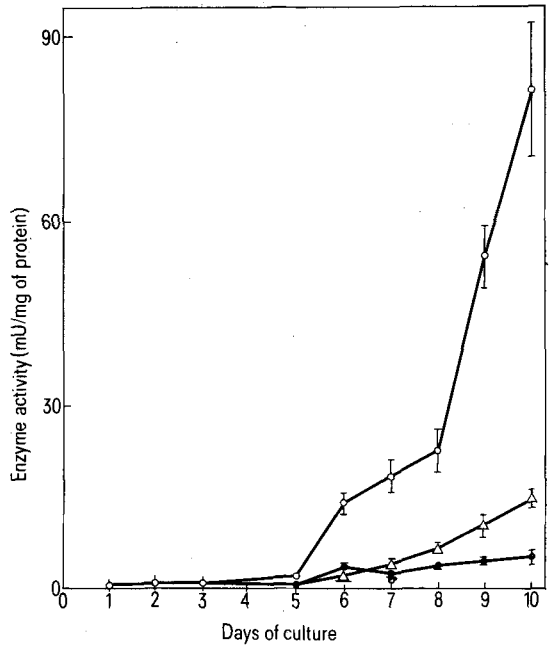

Figure 1. Gamma-glutamyl transpeptidase activity of adult rat hepatocytes cultured in the presence (●) and in the absence (○) of dexamethasone. (△) Dexamethasone was removed on the 5th days of culture. Glucocorticoid concentration was 10^{-7} M. Values are means ± SEM of triplicate dishes, from 1 representative experiment.

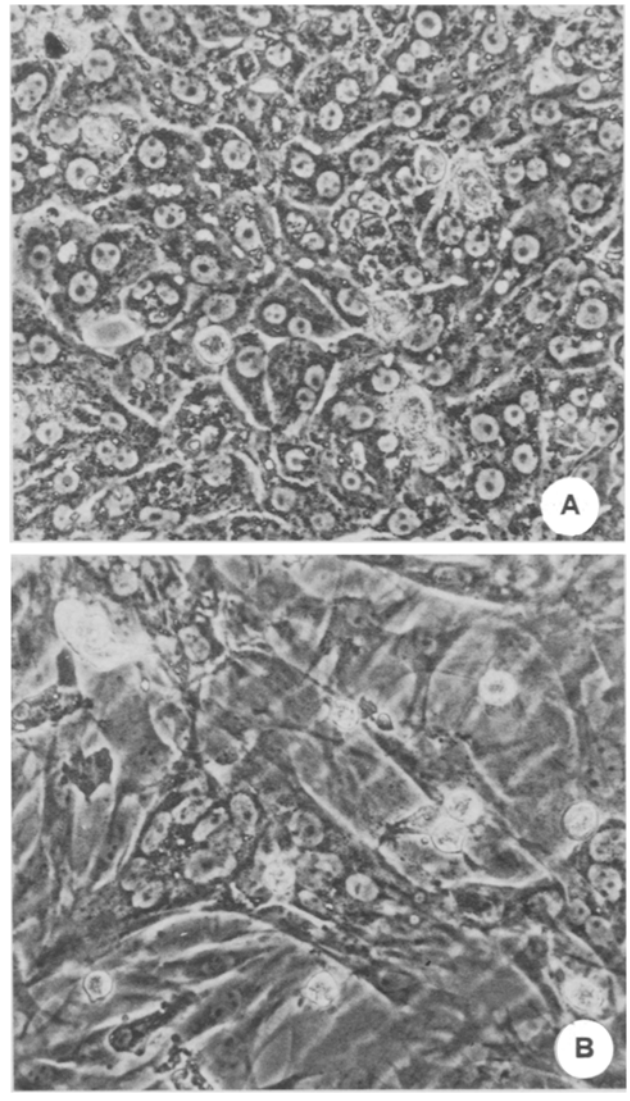

Figure 2. Adult rat hepatocytes maintained in primary cultures for 9 days. *A* Hepatocytes cultures in the presence of 10^{-7} M dexamethasone. *B* Hepatocytes cultured in the absence of added glucocorticoids. Cells were observed under phase contrast. × 400.

(fig. 2A). In contrast, hepatocytes cultured in the absence of glucocorticoids for the same period of time adopted a flattened shape and were very poorly granulated (fig. 2B). Our data confirm the previous report of Sirica et al.[5] on the increase of GGT activity which occurs in adult rat hepatocytes maintained in primary cultures. Although these authors found no decrease in the number of cells exhibiting GGT activity when hepatocytes were cultured in the presence of 10^{-5}M dexamethasone, we found that this glucocorticoid practically blocked the rise in GGT activity. There is no clear explanation for this apparent discrepancy, but it is possible that the histochemical method used by Sirica et al. for the detection of GGT activity, was not adequate or sufficiently sensitive to detect the partial block in the rise of this enzyme activity produced by the glucocorticoids.

The biochemical basis of the known beneficial effect of glucocorticoids on the survival of cultured hepatocytes[13-18] has been poorly studied. It appears that these hormones delay the substitution of aldolase B by aldolase A in primary cultures of adult rats hepatocytes[24,25]. These findings, in connection with the blocking effect of the glucocorticoids on the rise of GGT activity, could suggest a role for these hormones in the control of the spontaneous dedifferentiation of liver cells in culture.

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Interaction of organic dyes with hepatic microsomal drug-metabolizing monoöxygenases in vitro

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Summary. Organic dyes such as malachite green, methylene blue, fuchsin, safranine T, neutral red, phenazine methosulphate, riboflavin, dichlorophenolindophenol, phenolphthalein, and fluorescein, inhibit hepatic microsomal mixed-function oxidases and, partly, enhance, partly, inhibit hepatic microsomal NADPH-dependent cytochrome c and neotetrazolium reductases, in contrast to other inhibitors of drug metabolism which do not affect cytochrome c reductase but only interact with cytochrome P-450.

Most inhibitors of the biotransformation of drugs and other xenobiotics by liver microsomal monoöxygenases interact with cytochrome P-450, the terminal oxidase of the microsomal NADPH-dependent electron transport system[3] and thus inhibit drug monoöxygenation, but they do not interact with microsomal NADPH-cytochrome c reductase, the other constituent of this electron transport system[4-9]. This paper shows that, on the other hand, some organic dyes behave in a distinct way insofar as they not only inhibit the overall mixed-function oxidation reactions but have effects on the reductase, too. Parts of these findings have been previously reported, in a preliminary way[6,10].

Experimental. The following dyes were used: dichlorophenolindophenol (sodium salt), fluorescein (sodium salt), fuchsin (NB), malachite green, methylene blue (B), phenolphthalein, and riboflavin, from Merck AG, (Darmstadt, FRG), neutral red and safranine T, from Riedel-De Haën (Seelze, FRG), phenazine methosulphate and neotetrazolium chloride from Schuchardt (München, FRG). Aminopyrine, anisic acid N,N-diethylaminoethyl ester hydrochloride ('anisic ester'), methylayapanine, and 4-nitroanisole were from Hoechst AG (Frankfurt, FRG), 4-methylumbelliferone from EGA-Chemie (Steinheim, FRG). All biochemicals came from Boehringer (Mannheim, FRG), and buffer substances, reagents, and solvents were purchased from Merck (Darmstadt, FRG) and Riedel-De Haën (Seelze, FRG).

For the enzyme studies, rat liver 13,000 × g supernatants were prepared from crude potassium chloride homogenates by centrifugation, and rat and guinea-pig liver microsomes