A number of characteristics of UCH have been assessed: orange red colour with the Pauly reagent, and lilac colour with the Dragendorff reagent; mobility towards the cathode on high voltage electrophoresis, $E_{1\cdot2} = 0{\cdot}95$–$0{\cdot}98$ His, $E_{5\cdot8} = 0{\cdot}84$–$0{\cdot}9$ His.

R_f values in paper chromatography: 0·3–0·35 in *n*-butanol acetic acid water (4:1:5); 0·52–0·57 in *n*-butanol 35% methylamine (8:3); 0·52–0·55 in 1-pentanol pyridine water (40:40:10); 0·48–0·50 in methylethylketone pyridine water 35% methylamine (65:15:10:0·5); and finally 0·28–0·3 in 20% KCl.
—R_f values in thin-layer chromatography on silica gel: 0·15–0·2 in *n*-butanol acetic acid water (4:1:5); 0·63–0·67 in *n*-butanol 35% methylamine (8:3); and 0·29–0·31 in *n*-butanol ethanol 35% methylamine (22:7:1).

The content in *N*-urocanylhistamine of a crude extract of total soft tissues prepared from a large batch of snails was approximately 200 μg/g fresh tissue.

Dye-secreting gastropods belonging to the families *Muricidae*, *Thaididae* and *Drupae* contain in their soft tissues, more precisely in their hypobranchial gland, more or less conspicuous amounts of murexine and/or dihydromurexine, i.e. of the choline esters of urocanic acid and imidazolepropionic acid, respectively.

From present data on imidazolehistamines obtained in *Drupa concatenata* it appears evident that each of the above two imidazole acids may occur in the gastropod body either linked to choline, with a rather labile ester bond, or to histamine, with a much more tenacious peptide bond.

Research is in progress to investigate whether the new histamine derivatives are used by *Drupa concatenata*, a carnivorous gastropod, in the capture of the prey.

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REFERENCE

1. M. Roseghini, A. C. Alcala and T. Vitali, *Experientia* **29**, 490 (1973).

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The effect of sulfisoxazole (Gantrisin) and albumin on bilirubin conjugation in cultures of a clonal cell line with liver-like functions

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The elimination of bilirubin from the organism involves several processes: uptake of bilirubin by the liver cells, conjugation of bilirubin with glucuronic acid, and excretion of bilirubin-glucuronide. Variations in each of these processes may influence the total rate of bilirubin elimination.[1–3] In the blood, unconjugated bilirubin is transported bound to albumin.[4,5] The uptake of bilirubin into the liver cells seems to involve the dissociation of bilirubin from the albumin molecule before bilirubin enters the cells.[6]

Several drugs are bound to serum albumin under *in vivo* conditions, and it is well known that one drug can displace another from its binding to albumin. It has also been shown that various substances, endogenous as well as exogenous, influence the binding of bilirubin to albumin.[7–9] Sulfisoxazole (Gantrisin) is extensively bound to albumin (at a plasma concentration of 100 ng/ml approx. 84 per cent are protein

bound)[10] and has been shown to increase bilirubin toxicity in hyperbilirubinemic newborns.[11] It is difficult, however, to study the effect of various drugs on bilirubin toxicity and metabolism *in vivo*.

Cultures of a clonal strain of rat hepatoma cells (MH_1C_1)[12] have been shown to perform many liver specific functions. They synthesize a number of rat serum proteins[13-15] and they are able to conjugate bilirubin[16] as well as several drugs[17,18] with glucuronic acid. In the absence of the complex metabolic interactions which take place in the whole animal, it is thus possible to study the metabolism of bilirubin, and we found in a previous study[19] that the rate of bilirubin conjugation was highly dependent on the molar bilirubin/albumin ratio. With a constant bilirubin concentration the highest rate of bilirubin conjugation occurred at a molar ratio of 1:1. An excess of bilirubin depressed the formation of bilirubin glucuronide, possibly due to a toxic effect of an increased amount of bilirubin in the cells,[19] in agreement with earlier results.[20]

In the present report we describe the effect of sulfisoxazole (Gantrisin) on bilirubin conjugation and on alanine incorporation at different bilirubin/albumin molar ratios in such a clonal cell line.

The incorporation of alanine into cellular macromolecules was used as a parameter of metabolic, and probably toxic effects of increased levels of bilirubin in the cells.[19]

The cells used in the present experiments were derived from the transplantable Morris rat hepatoma No. 7795. The MH_1C_1 cells were grown as noncomplete monolayers in Falcon plastic tissue culture flasks (250 ml) in Dulbecco's modified Eagle's medium supplemented (unless otherwise stated) with 2·5 per cent fetal calf serum and 15 per cent horse serum. Penicillin 100 U/ml, streptomycin 0·1 g/ml, and nystatin 60 U/ml were added. Subcultures (1:2 to 1:4 split) were made after incubation with 0·25 per cent trypsin in a buffered 0·02 per cent EDTA solution. Full-grown or nearly full-grown cultures were used (protein content 2–4 mg per flask), usually 6–8 days after subculture. Ten to sixteen replicate subcultures were made for each set of experiments.

Human albumin, lyophilized (Kabi), was dissolved in the cell culture medium without serum. Unconjugated bilirubin (Koch Light Laboratories) was dissolved (4 mM in 0·05 N NaOH) immediately before each experiment. Sulfisoxazole (Gantrisin) was kindly supplied from Hoffmann–La Roche & Co., Basel. Solutions (10–25 mM in 0·05 N NaOH) were freshly made immediately before each experiment. (U-^{14}C) l-alanine (Amersham, sp. act. 1·0 m Ci/m-mole) was purchased through Norsk Atominstitutt.

At the beginning of each experiment the growth medium was removed, and the cells washed once with fresh prewarmed medium without added serum. Previously prepared solutions of bilirubin and albumin adjusted to pH 7·8 were then added to give a final bilirubin concentration of 100 μM. The albumin concentration was varied from 50 to 200 μM, and the molar bilirubin/albumin ratio thus varied between groups of flasks. For each set of experiments replicate flasks from the same subculture were used, and duplicates were made for each bilirubin/albumin ratio. Sulfisoxazole was added to some of the flasks (in duplicate) at final concentrations from 0·1 to 2·5 mM. The flasks were incubated at 37°, and samples were removed at intervals for measurement of total bilirubin[21] and conjugated bilirubin.[22] Before incubation, the media were gassed with 5% CO_2 in air. After this procedure the pH of all the flasks was between 7·6 and 7·7. No alteration in pH could be detected in the medium during the time of incubation.

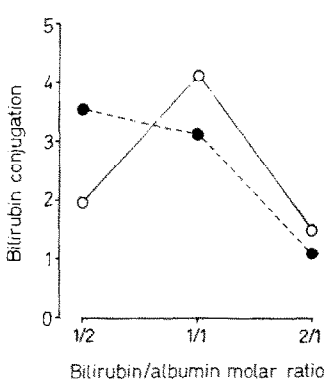

FIG. 1. Effect of varying the molar bilirubin/albumin ratio and addition of sulfisoxazole on bilirubin conjugation by MH_1C_1 cells. The total bilirubin concentration was kept constant at 100 μM. The albumin concentration varied from 50 to 200 μM. Ordinate: values for conjugated bilirubin given in nmoles/mg cell protein and hour, and represent mean values of six experiments. (●) 1·0 mM of sulfisoxazole added, (○) no sulfisoxazole added.

Cell protein was determined using Oyama and Eagle's modification of Lowry's method with bovine serum albumin (Sigma) as standard.[23] Sulfisoxazole and metabolites (N_4-acetyl and glucuronide derivatives) were measured according to method of Rieder.[24] Macro-molecules of cells cultured for 8 hr in the various media were labelled for 40 min with 0·5 μCi (0·005 mM) of U-^{14}C alanine per flask. The incorporation of the labelled precursor was estimated as previously described.[19]

Preliminary experiments showed that conjugated bilirubin appeared in the culture medium at a constant rate during the first 9 hr of incubation when unconjugated bilirubin (100 μM with a bilirubin/albumin ratio of 1:1) was added to the medium. The absolute values for conjugated bilirubin production varied considerably from one set of experiments to another, but replicate flasks from the same subculture varied very little (at most 10 per cent). No bilirubin conjugates were found when unconjugated bilirubin was incubated with medium in the absence of of cells.

Cells were incubated with unconjugated bilirubin for 8 hr, and samples were withdrawn for assay after 4 and 8 hr. An average of about 10 per cent of the bilirubin added was conjugated during 8 hr of incubation (when the molar bilirubin/albumin ratio was 1:1).

The effect of varying the molar bilirubin/albumin ratio from 2:1 to 1:2 on the appearance of conjugated bilirubin, with and without 1 mM of sulfisoxazole added, is shown in Fig. 1. Conjugation of bilirubin in the absence of sulfisoxazole is optimal at a bilirubin albumin ratio of 1:1. In the presence of sulfisoxazole (final concentration 1·0 mM), the optimal conjugation occurs at a bilirubin/albumin ratio (1:2) with an even better efficiency than the conjugation obtained without sulfonamide (Fig. 1).

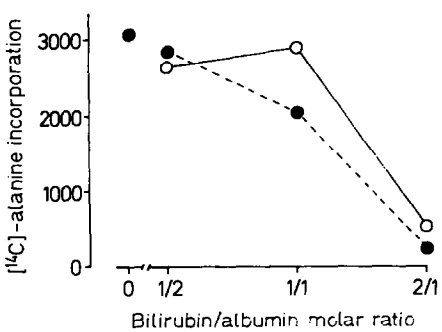

FIG. 2. Effect of varying the molar bilirubin/albumin ratio and addition of sulfisoxazole on the incorporation of ^{14}C-labelled alanine by MH_1C_1 cells. The bilirubin concentration was kept constant at 100 μM. The albumin concentration varied from 50 to 200 μM. The results are given as counts per minute and mg cell protein and represents mean values of four experiments. (●) 1·0 mM of sulfisoxazole added. (○) no sulfisoxazole added. Control experiments without bilirubin with and without sulfisoxazole gave identical results.

In the absence of sulfisoxazole, incorporation of alanine, an indicator for the toxic action of unconjugated bilirubin on liver cells, is affected only at a bilirubin/albumin ratio of 2:1 (Fig. 2) whereas in the presence of sulfisoxazole, such a depression takes place already at a ratio of 1:1.

Plotted on a logarithmic scale, the amount of bilirubin conjugated (at a bilirubin/albumin ratio of 1:2) increases linearly with the concentration of sulfisoxazole added in the culture medium and reaches about three-fold the control value in the presence of 2·5 mM sulfisoxazole (Fig. 3).

Experiments where sulfisoxazole (1 mM) was added alone to the culture medium were carried out to find out if any of the sulfonamide had been metabolized, as it has been shown[25] that substrates may compete for glucuronic acid conjugation in these cells. After 24 hr of incubation less than 1 per cent of the sulfonamide was glucuronidated and no acetylation had taken place.

The rate of bilirubin conjugation in intact animal as well as in isolated cells has been shown to be influenced by drugs in several ways. Barbiturates and related compounds are known to increase bilirubin-UDP-glucuronyltransferase activity and thus lower the serum bilirubin concentration in patients with reduced activity of this enzyme.[26] On the other hand, flavaspidic acid has been shown to reduce the capacity of hepatoma cells to conjugate bilirubin.[16]

As suggested by Lathe,[6] uptake of bilirubin by the liver cells involves dissociation of bilirubin from the albumin molecule and uptake of the unbound pigment into the cells. As previously reported[19] and confirmed by the present experiments, alterations in the molar bilirubin-albumin ratio of the culture

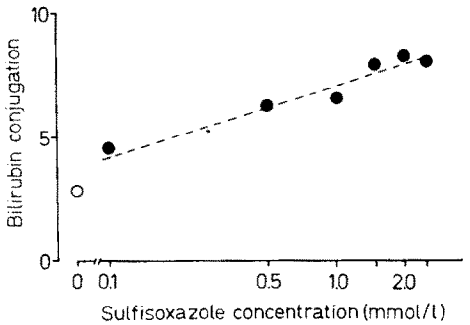

FIG. 3. Log dose-response relationship between the concentration of added sulfisoxazole and bilirubin conjugated by MH_1C_1 cells. The molar bilirubin/albumin ratio was kept constant at 1/2, and the bilirubin concentration was 100 μM. (●) Sulfisoxazole added. (○) no sulfisoxazole added.

medium has a marked influence on the rate of bilirubin conjugation by rat hepatoma cells. This indicates that the rate of bilirubin conjugation by intact cells is determined by the extent of binding of bilirubin to albumin.

Sulfisoxazole has been shown to displace bilirubin from its binding to albumin[8,9] and to increase the incidence and mortality of kernicterus in newborns.[11] In the present report sulfonamide was also found to influence markedly the bilirubin conjugation by MH_1C_1 cells. It is suggested that sulfisoxazole decreases the binding of bilirubin to albumin in the culture medium and increases the binding to cells or its uptake into cells. At a molar bilirubin albumin ratio of 1:2, the reserve capacity of the albumin molecule to bind bilirubin at the primary binding site would be still sufficient to keep the concentration of bilirubin in the cells below toxic levels: addition of sulfisoxazole increases cellular uptake and thereby conjugation of bilirubin. At a molar bilirubin/albumin ratio equal to or greater than 1:1 the reserve binding capacity at the primary binding site of the albumin molecule is exhausted. The affinity for bilirubin at the secondary binding sites on the albumin molecule is low[2,7] and bilirubin is almost insoluble in water. Addition of sulfisoxazole at bilirubin/albumin ratios equal to or greater than 1:1 may therefore lead to a displacement of bilirubin from albumin to cellular components, so that toxic levels of bilirubin in the cells are obtained causing a reduction in both bilirubin conjugation and ^{14}C-labelled alanine incorporation.

On the basis of the present experiments it seems possible that at certain bilirubin albumin ratios drugs which cause displacement of bilirubin from serum albumin may enhance the bilirubin conjugation also *in vivo*.

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REFERENCES

1. C. CATZ and S. J. YAFFE, *Pediat. Res.* **2**, 361 (1968).
2. L. M. GARTNER and I. M. ARIAS, *Pediat. Res.* **3**, 171 (1969).
3. A. J. LEVI, Z. GATMAITAN and I. M. ARIAS, *Lancet* **ii**, 139 (1969).
4. E. P. DI LEO, C. A. BUTTARO and B. BIANCONI, *Panminerva Medica* **2**, 10 (1960).
5. N. H. MARTIN, *J. Am. chem. Soc.* **71**, 1230 (1949).
6. G. H. LATHE, *Clin. Chem.* **11**, 309 (1965).
7. D. BRATLID, *Scand. J. clin. Lab. Invest.* **30**, 107 (1972).
8. D. BRATLID, *Scand. J. clin. Lab. Invest.* **30**, 331 (1972).
9. G. B. ODELL, *J. Pediat.* **55**, 268 (1959).

10. J. Rieder, *Arzneimittel-Forsch.* **13,** 81 (1963).
11. W. A. Silverman, D. H. Andersen, W. A. Blanc and D. N. Crozier, *Pediatrics* **18,** 614 (1956).
12. U. I. Richardson, A. H. Tashijian and L. Levine, *J. cell. Biol.* **40,** 236 (1969).
13. A. H. Tashijian, F. C. Bancroft, U. I. Richardson, M. B. Goldlust, F. A. Rommel and P. Oener, *In Vitro* **6,** 32 (1970).
14. H. E. Rugstad, H. Prydz and B. Johansson, *Exptl. Cell Res.* **70,** 41 (1970).
15. G. Gaudernack, H. E. Rugstad, I. Hegna and H. Prydz, *Exptl. Cell Res.* **77,** 25 (1973).
16. H. E. Rugstad, S. H. Robinson, C. Yannoni and A. H. Tashijian, *J. Cell. Biol.* **47,** 703 (1970).
17. E. Dybing and H. E. Rugstad, *Acta Pharmac. Toxic.* **31,** 153 (1972).
18. A. Winsnes and H. E. Rugstad, *Acta Pharmac. Toxic.* **33,** 161 (1973).
19. D. Bratlid and H. E. Rugstad, *Scand. J. clin. Lab. Invest.* **29,** 461 (1972).
20. S. O. Lie and D. Bratlid, *Scand. J. clin. Lab. Invest.* **26,** 37 (1970).
21. J. Fog, *Scand. J. clin. Lab. Invest.* **10,** 241 (1958).
22. F. P. Van Roy and K. P. M. Heirwegh, *Biochem. J.* **107,** 507 (1968).
23. V. I. Oyama and H. Eagle, *Proc. Soc. exp. Biol. Med.* **91,** 305 (1956).
24. J. Rieder, *Chemotherapy* **17,** 1 (1972).
25. H. E. Rugstad and E. Dybing, *Acta Pharmac. Toxic.* **34,** 65 (1974).
26. J. F. Crigler and N. J. Gold, *J. clin. Invest.* **45,** 998 (1966).
27. J. Jacobsen, *FEBS Lett.* **5,** 112 (1969).