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Chloramphenicol metabolism in isolated rat hepatocytes*

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In man, 90 per cent of a single dose of chloramphenicol (CAP) appears in the urine within 24 hr, chiefly as CAP-3-glucuronide [1]. The liver is the main site of glucuronidization, and an enzyme which conjugates CAP to glucuronic acid has been found in the microsomal fraction of rat liver homogenates [2]. Inability to glucuronidate CAP, as seen in some newborns [3] and in some patients with severe liver disease [4], results in the accumulation of potentially toxic levels of unmetabolized drug.

Prior studies of CAP metabolism have used whole animals [1], liver slices [5], liver homogenates [2], and liver microsomal preparations [2]. A more direct approach to the hepatic biotransformation of CAP involves the use of isolated viable hepatocytes. Over the past decade, several workers have developed techniques [6–8] for the isolation of intact hepatocytes which have been used to investigate various metabolic and synthetic functions of the liver [8]. Despite obvious advantages [9], such preparations have been used in only a few studies of drug metabolism [9, 10]. In this report, we describe studies of CAP metabolism in isolated rat hepatocytes.

Materials and Methods

Collagenase was obtained from Worthington Biochemical Corp., Freehold, NJ. β-Glucuronidase (bovine type 1B) was obtained from Sigma Chemical Co., St. Louis, MO. Radiolabeled CAP (dichloroacetyl-1,2-[^{14}C]CAP) was purchased from New England Nuclear, Boston, MA. Thin-layer chromatography (t.l.c.) was performed on LQ6D plates from Quantum Industries, Fairfield, NJ.

Isolation of Hepatocytes. A modification of the method of Capuzzi *et al.* [11] was used. Male Sprague–Dawley albino rats, fed *ad lib.* and weighing 175–200 g, were anesthetized with i.p. injections of sodium pentobarbitol, 50 mg/kg. The livers were perfused *in situ* via the portal vein with Ca-free modified Ringer's buffer, pH 7.4, containing Na (146 mM), K (5.5 mM) Mg (1.1 mM), Cl (119 mM), H_2PO_4 (1.1 mM), HCO_3 (25 mM), SO_4 (1.1 mM), glutamate (5 mM), pyruvate (5 mM) and glucose (11 mM). To the buffer were added 10% (v/v) fresh steer erythrocytes which were oxygenated by the "lung" of the Vanderbilt perfusion apparatus. After a 3.5-min initial cleansing perfusion at 14 ml/min, a recirculating perfusion was established by cannulation of the inferior vena cava. Collagenase was added to the perfusate at a concentration of 0.01% (w/v), and the perfusion was continued for 20 min. The livers were then removed, gently dispersed, and shaken for 10 min in the above buffer containing collagenase. The resulting cell suspension was passed through a 160 mesh silk cloth. The cells were washed three times in the above buffer containing Ca (2.4 mM). This procedure produced a suspension composed almost exclusively of hepatic parenchymal cells which appeared intact by light microscopy. Cell viability by trypan blue exclusion was 50–95 per cent after 1 hr of incubation.

Incubation of hepatocytes with CAP. Cells (0.4–2.0 × 10^6/ml) and various concentrations of CAP (sp. act. 1.0 mCi/m-mole) were incubated at 37° in 25-ml Nalgene flasks using the above buffer (plus Ca, 2.4 mM). Incubation volume was 3 ml. The buffer was bubbled with 95% O_2–5% CO_2 and the flasks were flushed with 95% O_2–5% CO_2 and stoppered prior to incubation. Incubations were terminated by centrifugation (1000 *g* × 3 min) and removal of the supernatant fluid.

Detection of metabolites. One-ml aliquots of the supernatant fluid were evaporated to dryness with a stream of N_2, taken up in methanol and chromatographed in a solvent system of n-butanol–pyridine–water (2 : 1 : 1). Detection of aryl amines and nitro compounds was accomplished using published methods [12]. Glucuronides were detected by u.v. fluorescence and by staining with a napthoresorcinol reagent [2]. To determine the distribution of radioactivity, the t.l.c. plates were cut in fractions which were counted by liquid scintillation.

Incubation of CAP metabolite with β-glucuronidase. Metabolites extracted with methanol from unstained t.l.c. plates were incubated with β-glucuronidase (3 mg/ml) in 0.2 M acetate buffer at pH 5.0 for 17 hr. Controls contained heat-inactivated enzyme or no enzyme. After incubation, solutions were boiled briefly and chromatographed as above. The plates were analyzed for radioactivity.

Results and Discussion

The results of a typical incubation of hepatocytes (10^6 cells/ml) with CAP (10 μg/ml) for 1 hr are shown in Fig. 1. The supernatant fluid contained only two radioactive compounds separable by t.l.c. with R_f values of 0.55 and 0.69. Both gave positive staining for the presence of a nitro group. The compound at R_f 0.69 co-chromatographed with authentic CAP. We believe that the compound at R_f 0.55 is CAP-3-glucuronide since it gives a u.v. fluorescence and stains with napthoresorcinol, suggesting the presence of a glucuronide moiety. To determine if it could be cleaved by β-glucuronidase, this metabolite was extracted from the appropriate region of an unstained t.l.c. plate with methanol and then incubated with the enzyme. A single radiolabeled compound which co-chromatographed with authentic CAP was produced. In control experiments with heat-inactivated enzyme or no enzyme, the peak at R_f 0.55 persisted. Urine from patients receiving CAP and, therefore, presumed to contain CAP-3-glucuronide [1] was chromatographed and found to have a nitro compound at R_f 0.5 which also stained with napthoresorcinol. This evidence strongly suggests that the major metabolite produced by isolated hepatocytes is CAP glucuronide.

As seen in Fig. 1, an unlabeled nitro compound was detected at R_f 0.45. Although it stains much less intensely than the glucuronide, this compound is probably a CAP metabolite since control incubations without CAP produce no nitro compounds. The CAP used in these experiments was labeled in the dichloroacetyl group. Since this compound is unlabeled, it may represent the free base of CAP (D(-)threo-2-amino-1-[*p*-nitrophenyl]-1, 3-propandiol) which lacks the dichloroacetyl moiety and

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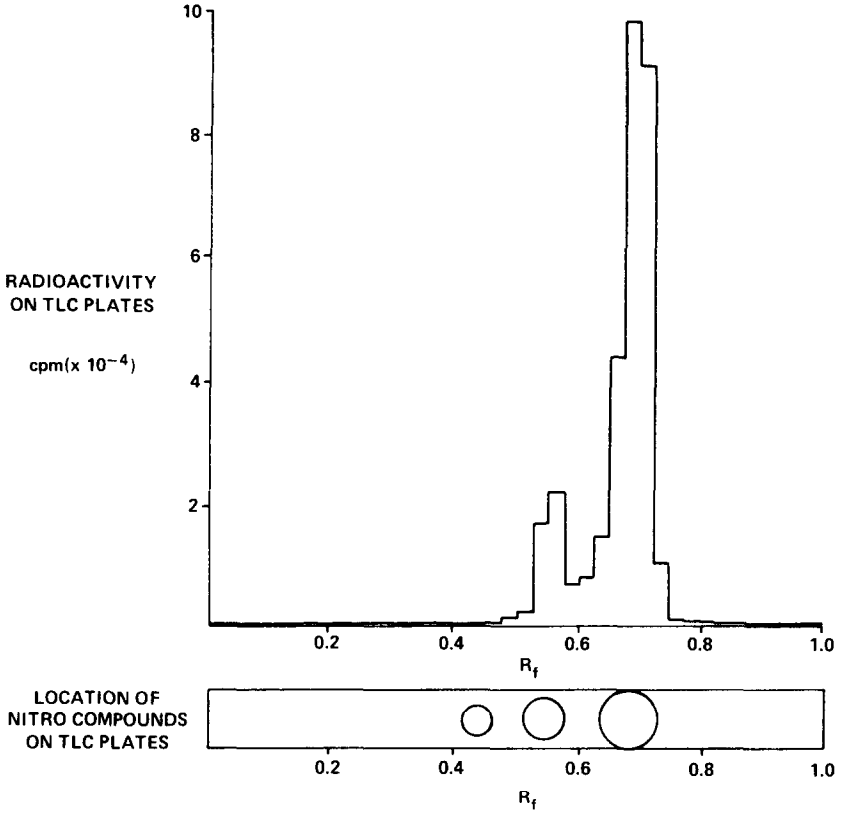

Fig. 1. Location of radioactivity and of nitro compounds on t.l.c. plates. One ml of supernatant fluid from the incubation of liver cells (10^6 cells/ml) with [^{14}C]CAP (10 μg/ml, sp. act. 1.0 mCi/m-mole) for 1 hr was concentrated and chromatographed. The plate was analyzed for radioactivity and for nitro compounds, as described in the text.

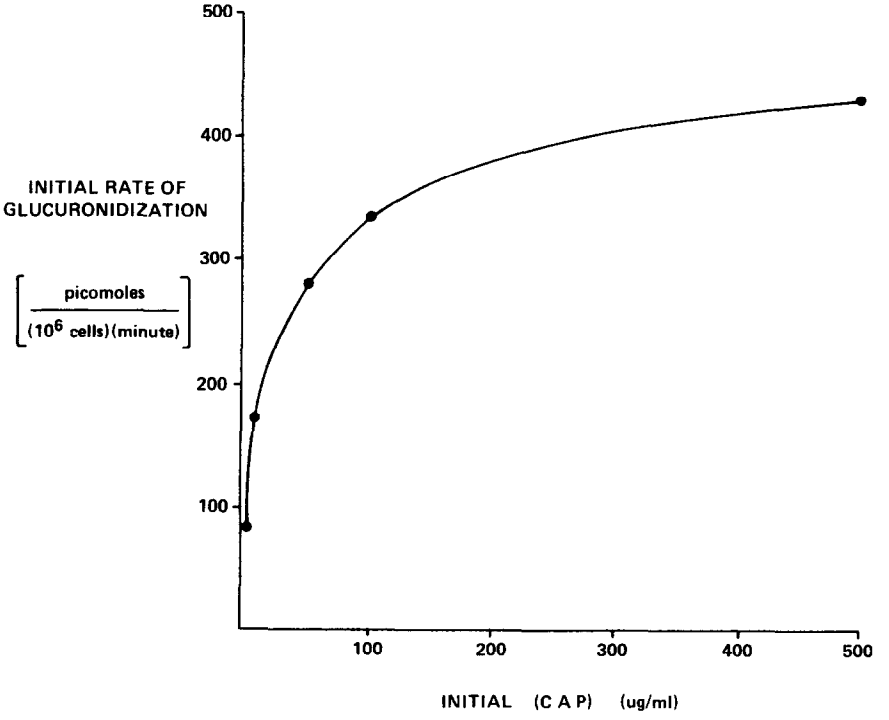

Fig. 2. Effect of [CAP] on rate of glucuronidization. Hepatocytes (0.4 × 10^6 cells/ml) were incubated with various concentrations of CAP (sp. act. 1.0 mCi/m-mole) for 30 min. Supernatant fluids were analyzed for radioactivity in the glucuronide peak. Plotted points are the averages of duplicate determinations. Duplicates agreed within 10 per cent.

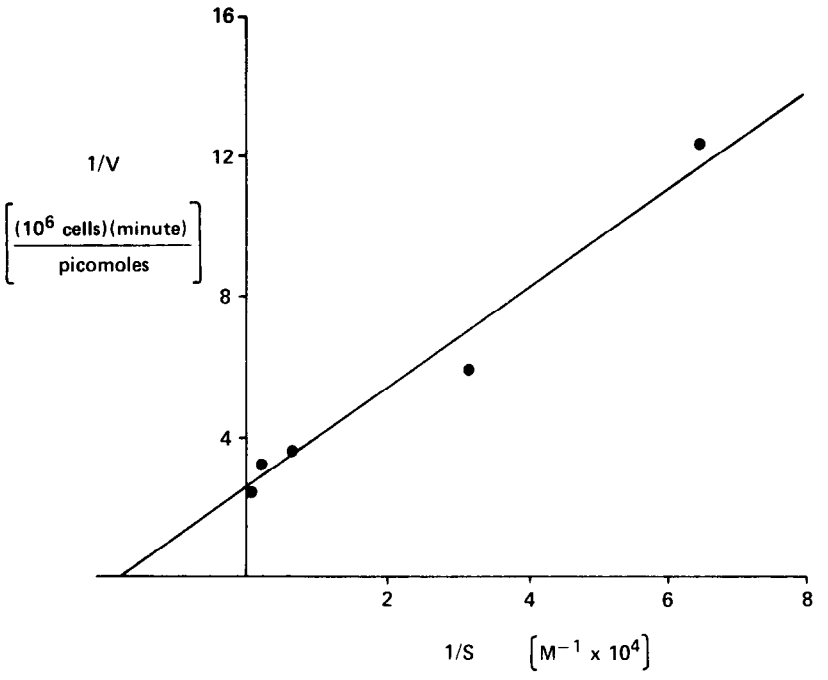

Fig. 3. Lineweaver–Burk plot of the data from Fig. 2.

which has been detected in small amounts in the urine of patients on CAP [13].

Aryl amines formed by the reduction of the nitro group of CAP were not detected in the supernatant fluid. In the rat, CAP is predominantly excreted in the stool, largely in the form of aryl amines [5]. It appears that reduction must occur outside the liver, probably by bacteria in the gut after biliary excretion of the glucuronide [14].

By determining the amount of radioactivity in the glucuronide peak, a study of the kinetics of CAP metabolism by isolated hepatocytes was made. The formation of glucuronide was linear with respect to cell concentration in the range of cell concentrations used and was linear with respect to time for the first hour of incubation. Using 30-min incubations, the effect of CAP concentration on the initial rate of glucuronidation was studied. Results are shown in Fig. 2. The rate approaches a plateau with increasing concentrations. A Lineweaver–Burk plot of the data is shown in Fig. 3. The V_{max} is 420 pmoles/min/10^6 cells. The K_m is 6.4×10^{-5} M (20.7 μg/ml).

If one assumes that 10^6 cells is equivalent to 0.16 mg of microsomal protein [15], then the above values for V_{max} and K_m may be compared with those previously found in our laboratory [2] for rat liver microsomal preparations: V_{max}= 1129 pmoles/min/mg of protein or 179 pmoles/min/10^6 cells and $K_m = 4.0 \times 10^{-4}$ M. The microsomal values were obtained using an arbitrarily fixed concentration of UDPGA, the second substrate of the conjugation reaction. In our study, UDPGA concentration was not manipulated or measured. Presumably, the values obtained in this hepatic cellular system reflect rates at "physiologic" concentrations of UDPGA.

The information generated in the present study may be of practical value in providing data for the estimation of the hepatic extraction ratio of CAP by the method of Rane *et al.* [16]. Using their formula for intrinsic clearance [$Cl_{in} = v/S = V_{max}/K_m + S$) and using actual substrate concentrations (since the assumption of $S << K_m$ is not met in our system), our estimate of the extraction ratio of CAP ranges from 0.003 at 100 μg/ml (3.1×10^{-4} M) to 0.018 at 1 μg/ml (3.1×10^{-6} M). From these ratios, one could predict that liver blood flow is not quantitatively important as a determinant of the duration of CAP action.

Thus, the use of isolated hepatocytes provides a novel approach to the study of CAP metabolism. CAP glucuronide is the major metabolite formed by rat liver cells. The kinetics of glucuronidization in this system suggest a low hepatic extraction ratio for CAP.

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