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Inhibition of chemical carcinogenesis: early increase in tissue uptake and macromolecular binding of [^{3}H]-labelled metabolites of the hepatocarcinogen 3′-methyl-4-dimethylaminoazobenzene in rat liver *in vivo* in the presence of the tumour inhibitor chloramphenicol

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The antibiotic chloramphenicol (CAP)* inhibits tumour induction in several tissues by a variety of carcinogens [1–4], including 3′-methyl-4-dimethylaminoazobenzene (3′MeDAB) [5], but the mechanism of protection is unknown. Many other inhibitors of chemical carcinogenesis apparently protect against tumour induction by inhibiting the metabolic activation and/or subsequent covalent binding of carcinogens to molecules in the target tissues [6]. However, CAP had no effect on levels of protein-bound 3′MeDAB metabolites when both compounds were pair-fed to rats for up to 20 days [7], although both protein [8, 9] and nucleic acid binding [9] of acetylaminofluorene derivatives in rat liver were apparently depressed by CAP. Although CAP modifies several early effects of 3′MeDAB feeding on the liver [5, 7, 10–14], it had no effect on the time of maximum liver protein binding of 3′MeDAB metabolites with an azo linkage [7] at 10 days.

CAP may inhibit tumour induction by competing for critical intracellular binding sites of the carcinogen [8, 9]. To test this hypothesis, the effects of [^{14}C]CAP on the uptake, subcellular distribution and covalent binding of [^{3}H]3′MeDAB metabolites to DNA, RNA and protein from liver subcellular fractions were investigated very soon after the commencement of feeding both compounds and at the time of maximal liver protein binding.

Male Sprague–Dawley rats (200–250 g) were random-bred from local departmental stock. Weight-matched pairs were housed in individual cages and pair-fed [7] diets containing [^{3}H]3′MeDAB alone or in combination with 2% [^{14}C]CAP for 4 or 10 days. Unlabelled 3′MeDAB and CAP, and [^{3}H]3′MeDAB (sp. act. 119 mCi/mmole, labelled on toluidine ring) were obtained as described previously [10]. (Dichloroacetyl-l-^{14}C)-CAP (sp. act. 7.18 mCi/mmole) was purchased from the Radiochemical Centre, Amersham, U.K. The specific activities of [^{3}H]3′MeDAB and [^{14}C]CAP in the diets used for the 4-day feeding were 119 mCi/mmole and 32.3 μCi/mmole, respectively, and in the 10-day feeding 47.6 mCi/mmole and 9.66 μCi/mmole, respectively. Despite the isotope dilution in the 10-day diets, approximately half of the rats died during the feeding period. A plan to feed further groups of rats for 20 days was therefore abandoned.

Rats were killed by exsanguination under light ether anaesthesia, and livers were excised and homogenized in 2 vol. of a buffer containing 0.25 M sucrose, 0.05 M Tris–HCl, 0.025 M KCl and 0.005 M $MgCl_2$ (pH 7.5). Differential centrifugation of the homogenates yielded crude nuclear (755 *g*, 15 min precipitate), crude mitochondrial (10,000 *g*, 15 min precipitate), microsomal (100,000 *g*, 60 min precipitate) and cytosol fractions (100,000 *g*, 60 min supernatant fraction). Purified nuclei were isolated from the crude nuclear pellets by the method of Blobel and Potter [15]. RNA was isolated from the mitochondrial, cytosol, microsomal and purified nuclear fractions by the phenol/sodium dodecyl sulphate (SDS) method described by Muramatsu [16]. Protein was precipitated from the first two portions of the phenol phase obtained from the deproteinization of each of the RNA samples by adding 1.5 vol. of acetone at 4° overnight. The RNA and protein preparations were washed repeatedly with organic solvents (ethanol or ethanol and acetone, respectively) at 20° and 60° until negligible radioactivity was detected in the supernatant fractions. Nuclear DNA was extracted from the crude nuclear fraction by the method of Phillips and Blunck [17]. Protein was redissolved in 0.01 M phosphate (pH 7.1)/1% SDS/1% mercaptoethanol and RNA and DNA were hydrolysed in 0.3 M KOH at 60° for 60 min and 5% (w/v) trichloroacetic acid (TCA) at 100° for 30 min, respectively. Protein, RNA and DNA contents of the subcellular fractions and purified macromolecules were determined by the method of Lowry *et al.* [18], u.v absorption [19] and the indole method [20], respectively, except for the RNA contamination of DNA which was determined by the orcinol method [21]. Protein of the purified nuclear fraction and the proteins extracted from the subcellular fractions were freed of substances interfering with the Lowry reaction by quantitative precipitation of protein with deoxycholate/TCA [22] prior to the Lowry assay. The mean (± S.E.M.) percentage yields of extracted protein, RNA and DNA were 89.8 ± 5.2 per cent, 49.8 ± 2.6 per cent and 38.1 ± 3.9 per cent, respectively, of the total tissue fraction contents. The levels of protein contamination of the extracted RNAs and DNA were 4.7 ± 0.8 per cent and 3.8 ± 0.7 per cent, respectively; the level of DNA contamination of nuclear RNA was 10.9 ± 4.3 per cent. RNA contamination of DNA and DNA or RNA contamination of protein could not be detected. The yields of extracted macromolecules and the levels of cross contamination did not differ in preparations from rats fed either diet.

For determination of isotope uptake into the liver, aliquots (0.1 ml) of tissue were heated in 0.3 M KOH (0.9 ml) at 60° for 60 min, acidified with conc. HCl (0.1 ml) and added to a scintillation mixture (8.0 ml) which comprised toluene/Triton X-100 (2/1), 0.4% 2, 5-diphenyloxazole and 0.05% 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene. Covalent binding of isotope to the acid-insoluble material (generally >90 per cent protein) of the various fractions was estimated by precipitating acid-insoluble material with TCA/acetone/ethanol [23], washing the precipitate extensively with TCA, ethanol and acetone at 20° and 60° and redissolving it in 0.3 M KOH at 60° for 60 min. Aliquots of the KOH solution were added to scintillation mixture as described for isotope uptake into the liver. Bovine serum albumin (2 mg/0.5 ml) was added to the purified nuclear fraction as a carrier prior to acid precipitation. Covalent binding of isotope to purified protein and DNA preparations was determined by adding aliquots directly to scintillation mixture, whereas RNA preparations were first acidified with conc. HCl (0.01 vol.). The [^{3}H] and [^{14}C] channels were set according to the procedure described by Neame and Homewood [24].

When both [^{14}C]CAP and [^{3}H]3′MeDAB were fed to

* Abbreviations used: CAP, chloramphenicol; 3′MeDAB, 3′-methyl-4-dimethylaminoazobenzene; SDS, sodium dodecylsulphate; TCA, trichloroacetic acid

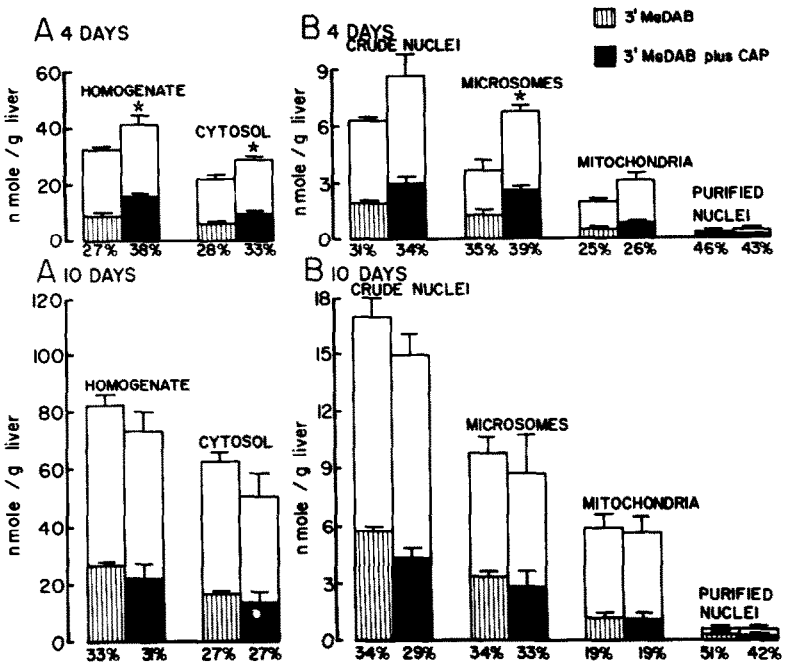

Fig. 1. The levels of [^{3}H]3′MeDAB present in subcellular fractions of rat liver and of [^{3}H]3′MeDAB metabolites covalently bound to acid-insoluble material of these fractions following the pair-feeding of 0.06% [^{3}H]3′MeDAB alone or in combination with 2% [^{14}C]CAP for either 4 or 10 days. Liver subfractionation and measurement of total and covalently bound [^{3}H]3′MeDAB metabolites were carried out as described in the text. The levels of [^{3}H]3′MeDAB present in homogenate and subcellular fractions of liver (entire columns) and of covalently bound [^{3}H]3′MeDAB metabolites (shaded lower sections) are expressed as nmoles [^{3}H]3′MeDAB/g liver, and represent the mean ± S.E.M. values obtained from three rats for each group. The value given below each column represents the ratio of covalently bound [^{3}H]3′MeDAB metabolites to the total level of [^{3}H]3′MeDAB present in each fraction, expressed as a percentage. The asterisk (*) represents values significantly greater than the [^{3}H]3′MeDAB group; $P<0.05$ (Student's one-tailed t-test).

rats for four days at levels that inhibit 3′MeDAB-induced hepatocarcinogenesis, there was an increase rather than a decrease in total liver content and content in all liver subcellular fractions of [^{3}H]3′MeDAB derivatives (Fig. 1). Covalent binding of [^{3}H]3′MeDAB metabolites to acid-insoluble material from each subcellular fraction and to DNA, RNA and protein from these subcellular fractions was likewise increased (Fig. 2). The increases were statistically significant for liver content of [^{3}H]3′MeDAB ($P< 0.05$), and for content in and for covalent binding of [^{3}H]3′MeDAB metabolites to acid-insoluble material from total homogenate, cytosol and microsomal fractions (Fig. 1). The pattern of results was similar regardless of the manner in which the data were expressed (content/g liver or content/total liver, data not shown). Covalent binding of [^{3}H]3′MeDAB metabolites to mitochondrial and microsomal RNA (binding/mg RNA, Fig. 2) or to purified nuclear RNA and to RNA and protein from both cytosol and microsomal fractions (binding/g liver, data not shown) was also significantly increased after four days on the diet containing both [^{14}C]CAP and [^{3}H]3′MeDAB.

The percentage of the total [^{3}H]3′MeDAB present in each of the subcellular fractions that was covalently bound to acid-insoluble material (figures under columns, Fig. 1) was not increased to the same extent as the absolute levels of binding (per g liver or per total liver) when both [^{14}C]CAP and [^{3}H]3′MeDAB were fed. The increased [^{3}H]3′MeDAB metabolite binding in these rats is therefore probably secondary to the increased [^{3}H]3′MeDAB content of the liver rather than to any specific increase in 3′MeDAB activation to binding metabolites or in the availability of binding sites. The increased liver content of [^{3}H]3′MeDAB may be a consequence of the relative decrease in the activity of mixed function oxidases after four days of 3′MeDAB feeding [10], which would be expected to depress the conversion of 3′MeDAB to inactive nonbinding derivatives. Liver uptake of [^{3}H]3′MeDAB could also be increased at this time, as liver uptake of leucine is increased [25] and liver plasma membrane-associated enzyme activities are altered [26] when 3′MeDAB and CAP are fed to rats.

At least some of the factors that contributed to the increased liver [^{3}H]3′MeDAB content in rats fed both [^{14}C]CAP and [^{3}H]3′MeDAB for four days were obviously altered by ten days, when the increase was no longer apparent. Likewise, at ten days the content of [^{3}H]3′MeDAB in liver subcellular fractions, the covalent binding of [^{3}H]3′MeDAB metabolites to acid-insoluble material and the DNA, RNA and protein from the subcellular fractions were similar in rats fed both [^{14}C]CAP and [^{3}H]3′MeDAB to the levels in rats pair-fed [^{3}H]3′MeDAB (Figs. 1 and 2). This finding was likewise independent of the manner in which the data were expressed. Thus at the time of maximum protein binding of 3′MeDAB metabolites in rat liver [7], CAP has no apparent effect either on the absolute levels of 3′MeDAB binding to macromolecules or on the subcellular distribution of this binding.

The failure of CAP to inhibit binding of [^{3}H]3′MeDAB metabolites to macromolecules isolated from liver subcellular fractions when both compounds were administered

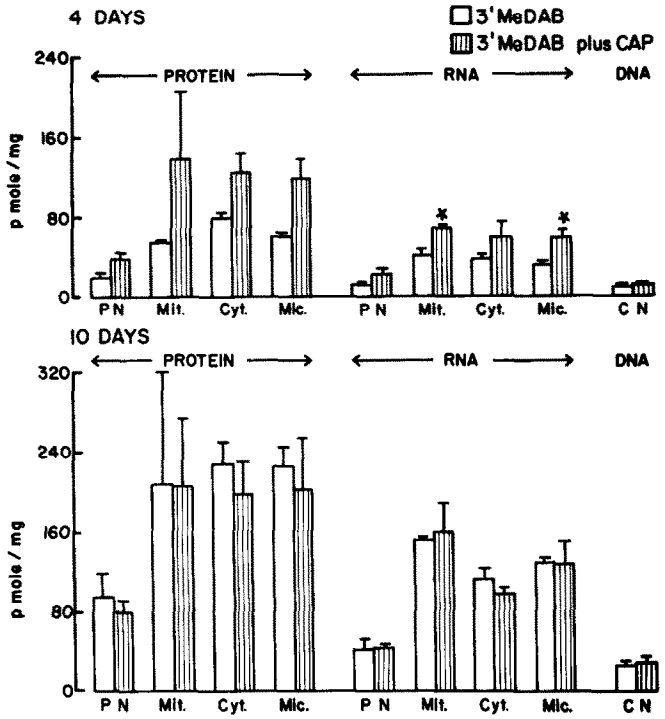

Fig. 2. The levels of [^{3}H]3′MeDAB metabolites covalently bound to protein, RNA and DNA extracted from liver subcellular fractions prepared from rats pair-fed 0.06% [^{3}H]3′MeDAB alone or in combination with 2% [^{14}C]CAP for 4 or 10 days. Liver subfractionation, protein and nucleic acid extraction, and measurement of [^{3}H]3′MeDAB metabolite covalent binding to these molecules were carried out as described in the text. The levels of covalent binding are expressed as pmoles [^{3}H]3′MeDAB bound/mg protein, RNA or DNA and represent the mean ± S.E.M. values obtained from three rats of each group. CN, crude nuclei; PN, purified nuclei; Mit., mitochondria; Cyt., cytosol; Mic., microsomes. The asterisk (*) represents values significantly greater than the [^{3}H]3′MeDAB-fed group, $P<0.05$ (Student's one-tailed t-test).

concurrently *in vivo* for ten days confirms earlier reports of the general lack of effect of CAP on protein-bound dye levels in the livers of rats fed 3′MeDAB [7] and on metabolic activation of [^{3}H]3′MeDAB to RNA-binding metabolites by a cell-free system from rat liver [10]. However, in the earlier study using a colorimetric assay [7], no effect of CAP on 3′MeDAB metabolite binding after four days on the diets was observed. Possible explanations of the discrepancy between that report and the present one are that the [^{3}H]3′MeDAB metabolite binding measured here includes that of some derivatives lacking an azo linkage [27, 28] that would not be estimated by the colorimetric determination of protein-bound dye [29]. Furthermore, the binding assay using [^{3}H]3′MeDAB has been claimed to be more sensitive than the colorimetric procedure [27].

[^{14}C]CAP was present in all subcellular fractions of the liver of the rats fed [^{14}C]CAP and [^{3}H]3′MeDAB and was apparently covalently bound to the acid-insoluble material and to the extracted DNA, RNA and protein of each of these fractions (data not shown). The tissue content and levels of binding of [^{14}C]CAP were between 20 and 200 times greater than the corresponding levels for [^{3}H]3′MeDAB metabolites in the same subcellular fractions, although the pattern of subcellular distribution and covalent binding was similar for both [^{3}H]3′MeDAB and [^{14}C]CAP. This finding confirms that of an earlier report that CAP derivatives bind to proteins of liver and other tissues [30]. Some binding is due to the production of an unstable electrophilic oxamyl chloride derivative by oxidative dechlorination of CAP [31, 32], although recent evidence indicates that labelled CAP breakdown products are reutilized for synthesis of endogenous macromolecules with consequent overestimation of [^{14}C]CAP binding [33]. Nevertheless, the sites involved in authentic [^{14}C]CAP binding are clearly not those to which [^{3}H]3′MeDAB metabolites bind, for the levels of [^{3}H]3′MeDAB metabolite binding *in vivo* were not depressed by concurrent [^{14}C]CAP feeding to the rats.

There are other reports in the literature describing inhibitors of azo dye carcinogenesis that do not decrease carcinogen binding [34, 35]. These compounds might affect noncovalent cellular interactions of 3′MeDAB or its metabolites that could be relevant to carcinogenesis, but in at least some instances the mechanism of inhibition probably operates at some subsequent stage in carcinogenesis. CAP may be able to prevent the expression of critical effects of carcinogen-induced damage, for it can phenotypically suppress the expression of some prokaryotic mutants [36]. In rat liver, CAP reverses the decreased liver RNA/DNA ratio [7, 12], normalizes the altered phase distribution of mitosis [5, 12] and increases both nuclear RNA synthesis [13] and RNA polymerase activity [14] when it is fed concurrently with 3′MeDAB for 4–20 days.

In conclusion, the antibiotic CAP, when fed concurrently with 3′MeDAB, fails to decrease the level of binding of [^{3}H]3′MeDAB metabolites to liver macromolecules in the early stage of a dietary regimen that inhibits 3′MeDAB-induced hepatocarcinogenesis. After only four days of feeding, the binding of 3′MeDAB metabolites was actually increased. Our findings suggest that CAP is not inhibiting

metabolic activation of 3'McDAB or competing with 3'MeDAB metabolites for intracellular binding sites. Possibly, CAP could inhibit carcinogenesis by phenotypically suppressing the expression of carcinogen-induced damage to the cell. Inhibition of 3'MeDAB-induced hepatocarcinogenesis in the rat by CAP may therefore prove to be a suitable experimental model for the steps in tumour induction subsequent to carcinogen activation and binding to cellular macromolecules.

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