PMN [29]. SOD itself has been found to be anti-inflammatory [10]. This property has been related to a scavenging effect on O_2^- and a protective effect against the autoxidation of stimulated PMN [11]. Our findings regarding the effect of SOD on the functional activity of PMN show that PMN behave quite differently in the presence of SOD as compared to PMN treated with Cu(II)–Sal_2. This could mean that SOD, due to its relatively high molecular weight, remains outside the cells and does not interfere with the oxidative system of the cells, whereas the complex's small size favours its permeability across biological membranes. Consequently it reaches the active site of the oxidative system of PMN. Observations concerning the lag time necessary for the complex to inhibit the respiratory burst in PMN support this assumption. An exact determination of the enzymatic system, sensitive to the inhibitory effect of the complex as well as its intracellular localization, will help to understand oxidative metabolism in PMN.

In summary, phagocytosis-induced metabolic activation in human polymorphonuclear leucocytes has been found to be strongly inhibited by salicylate–copper complex (Cu(II)–Sal_2) whereas under similar experimental conditions salicylate exhibits no effect. These results are in agreement with the hypothesis suggesting that copper chelate is an active form of the drug.

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An impurity, N^{10}-methylfolate, associated with methotrexate inhibits folate binding in milk and serum

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High-affinity folate binding proteins are known to be present in milk, serum, leukocytes and various tissues [1]. The biological function of these binders is not fully understood, but they may serve as transport or storage proteins for folates [1]. Of particular interest is the theoretical possibility that at least some of the pharmacological actions elicited by the antileukemic drug methotrexate may be ascribed to interference with the specific protein binding of naturally occurring folate derivatives. However, previous studies concerned with the effect of methotrexate on high-affinity folate binding gave somewhat conflicting results, probably due to differences in the choice of method and concentration of methotrexate. Thus, inhibition of folate binding by methotrexate was reported in milk [2–4], serum [2, 5], leukocyte lysates [6], intestinal and renal brush border membranes [7, 8], whereas other studies showed no inhibition either in hog kidney [9] or in leukocyte lysates [10, 11]. In the latter study [11] methotrexate was purified as described by Gallelli and Yokoyama [12].

The present study deals with the effect of methotrexate,

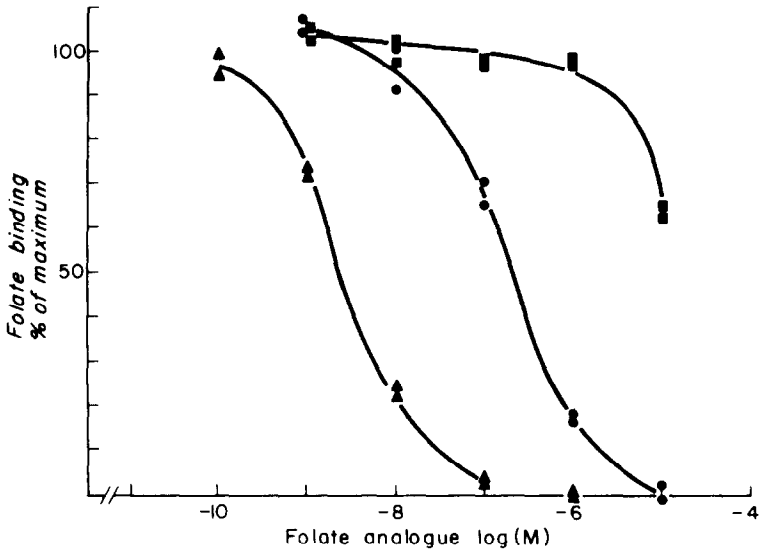

Fig. 1. Effect of folate analogues on high-affinity folate binding in cow's milk. Unpurified methotrexate (●), purified methotrexate (■), peak IV (▲). The [^{3}H]folate concentration used was that (10^{-9} M) required for saturation of binding. Each point represents the results of a single dialysis experiment in 0.17 M Tris buffer (pH 7.4, 37°) with high-affinity folate binding protein. For isolation of binder, cf. Refs. [15 and 16].

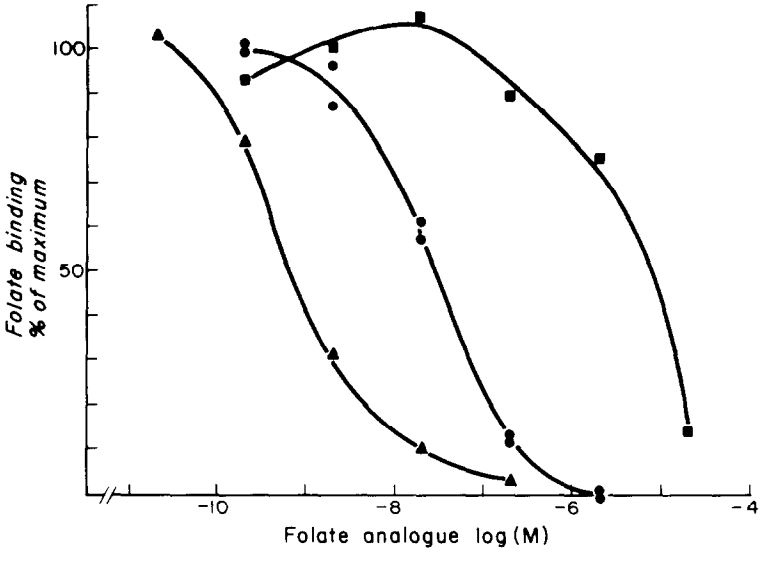

Fig. 2. Effect of folate analogues on high-affinity folate binding in serum (for details and symbols, cf. Fig. 1). The [^{3}H]folate concentration used was that (2×10^{-10} M) required for saturation of binding. For isolation of high-affinity folate binding protein, cf. Ref. [14].

purified and unpurified, on high-affinity folate binding in milk and serum. Binding experiments were performed over a wide range of potential inhibitor concentrations under standardised equilibrium conditions [13, 14].

[^{3}H]Folic acid with a specific activity of 5.0 or 20–41 Ci/mmole was supplied by the Radiochemical Centre, Amersham, U.K. Methotrexate and N^{10}-methylfolate, both supplied by Lederle, were purified on a DEAE-Sepharose CL-6B (Pharmacia, Uppsala, Sweden) column (2 cm^2 × 40 cm) eluted with a linear gradient (0.1–0.4 M) of NH_4HCO_3 (pH 8.3). In accordance with Gallelli and Yokoyama [12], who originally described the chromatographic procedure for purification of methotrexate, we observed four peaks in the elution pattern. A major peak, III (93%)* represented methotrexate, while impurities (decomposition or hydrolysis products) appeared as the peaks I, II and IV [12]. Peak IV (2–3%) was previously identified as N^{10}-methylfolate by Gallelli and Yokoyama [12]. In accordance with their findings most (93%) of

* Concentrations of methotrexate (peak III) and impurities (peaks I, II and IV) were estimated from the absorptivity of each peak at 257 nm.

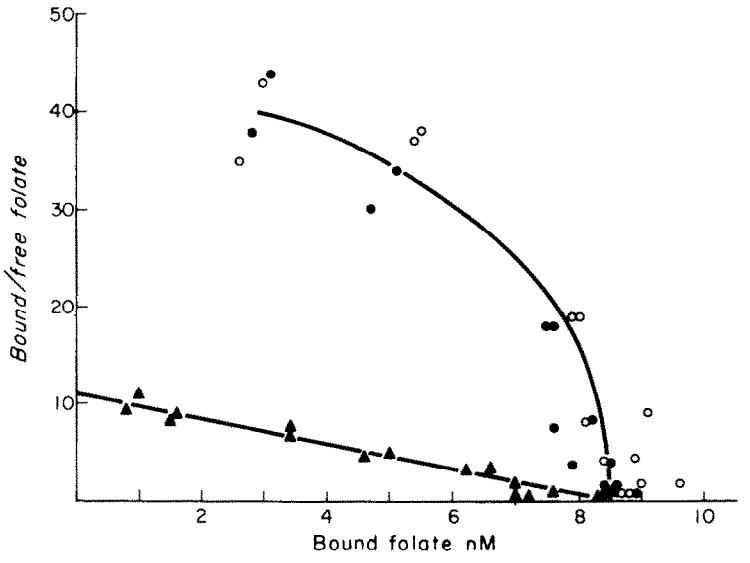

Fig. 3. Scatchard plot showing the effect of folate analogues on high-affinity folate binding in cow's milk. For details, cf. Fig. 1. Control experiments (○). Experiments in the presence of purified methotrexate, 100 nM (●) or peak IV, 2 nM (▲).

the N^{10}-methylfolate was found to elute at the position of peak IV.

Methods for the isolation of high-affinity folate binding proteins in milk and serum developed in our laboratory are described in detail elsewhere [14–16]. [^{3}H]Folate binding was studied in equilibrium dialysis experiments at 37° in 0.17 M Tris buffer of pH 7.4, as reported elsewhere [13–16].

Folate binding in milk was studied in the presence of methotrexate (purified and unpurified), the impurities associated with methotrexate (the peaks I, II and IV) and N^{10}-methylfolate (identical to peak IV). As shown in Figure 1, unpurified methotrexate was a weak inhibitor of folate binding, the molar methotrexate : folate ratio being 100 : 1 at 50 per cent inhibition. Furthermore, inhibition was not due to methotrexate itself since this drug in its purified form had virtually no inhibitory potency (methotrexate : folate ratio exceeded 10^4 at 50 per cent inhibition), but to the impurity peak IV (3%), the molar ratio peak IV : folate being 1 : 1 at 50 per cent inhibition. The effect of N^{10}-methylfolate was indistinguishable from that of peak IV. The other impurities (the peaks I and II) did not inhibit folate binding. Parallel experiments on high-affinity folate binding in serum gave similar results (Fig. 2).

We have previously shown that positive cooperativity, a typical characteristic of folate binding in milk, disappears in the presence of unpurified methotrexate (100 nM) which seemed to act as a competitive inhibitor of folate binding [3, 4]. These experiments were now repeated with purified methotrexate, the impurity, peak IV, and N^{10}-methylfolate. A Scatchard analysis of some of the binding data is shown in Fig. 3. Obviously, purified methotrexate (100 nM) had no inhibitory effect on folate binding which displayed positive cooperativity as indicated by a downward concavity of the Scatchard plot also in the presence of this drug. However, at an estimated concentration of 2 nM the impurity, peak IV, inhibited folate binding (Fig. 3). Curves from control experiments and experiments in presence of the impurity (peak IV) seemed to converge towards a common intersection point on the abscissa (maximum folate binding). The unaffected binding maximum in the presence of the impurity (peak IV) indicates that folate binding has changed to a simple non-cooperative type. The effect of N^{10}-methylfolate could not be distinguished from that of the impurity.

In conclusion, purified methotrexate had no inhibitory effect on folate binding in milk serum, whereas the impurity N^{10}-methylfolate associated with methotrexate acted as a potent competitive inhibitor. Obviously, this finding invalidates conclusions drawn from previous work with unpurified methotrexate. Furthermore, it has some interesting structure–activity implications which may be of great value in future studies on the molecular mechanisms of high-affinity folate binding.

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Restriction of hexobarbital metabolism by *t*-butyl hydroperoxide in perfused rat liver

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Hepatic monooxygenation reactions, catalysed by cytochrome P-450, can be supported by organic hydroperoxides as was found with isolated microsomal fractions [1–4]. Further, redox effects of organic hydroperoxides on NADH, NADPH and cytochrome b_5 have been described with such fractions [5–9]. The physiological or toxicological relevance of these effects in more complex experimental systems has so far not been studied extensively.

Experiments with isolated hepatocytes [8] and with isolated perfused liver [10] revealed no effect of added *t*-butyl hydroperoxide on the steady state level of the cytochrome P-450–CO or cytochrome P-450–substrate complexes. In view of the substantial oxidation of NADPH to $NADP^+$ during the reduction of organic hydroperoxides by intact liver [11] the question arises whether added organic hydroperoxide might be capable of supporting cytochrome P-450–dependent drug oxidations in intact cells or organs.

In the present work, the effect of *t*-butyl hydroperoxide on the metabolism of hexobarbital was studied in perfused livers from phenobarbital-pretreated rats.

Materials and methods

Hemoglobin-free liver perfusion. Livers from male Wistar rats, 130–190 g body weight, fed on stock diet (Altromin) and pretreated for at least 7 days with sodium phenobarbital (1 mg per ml of drinking water) were perfused as described previously [12]. The perfusion medium consisted of 115 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM Na_2SO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 1.2 mM L-lactate and 0.3 mM pyruvate (as sodium salts), equilibrated with O_2/CO_2(19/1, v/v). The temperature was maintained at 33°. The perfusion was non-recirculating (open system). Additions were made from stock solutions by means of precision micropumps into the perfusate entering the portal vein.

Assays. The concentration in influent and in effluent perfusate was determined for *t*-butyl hydroperoxide [13], oxygen [12, 13] and hexobarbital ([14]; slightly modified as described in [15]).

Chemicals. t-Butyl hydroperoxide was a gift from Peroxid Chemie, Höllriegelskreuth, München, and sodium hexobarbital was a gift from Bayer, Leverkusen. All other chemicals were from Merck, Darmstadt, or Boehringer, Mannheim.

Results and discussion

Previous experiments had shown that the half-maximal effect for extra O_2 uptake upon infusion of hexobarbital was 59 μM [16]. Therefore, in the present work hexobarbital concentrations of approx. 0.2 mM were employed. As shown in Fig. 1, the uptake of hexobarbital under such condition is about 0.2 μmoles/min per gram of liver; it was 0.21 ± 0.02 μmoles/min per gram (mean ± S.E.M.) in 10 different perfusions. The extra O_2 uptake was 2.01 ± 0.18 μmoles O_2 per μmole of hexobarbital taken up, as determined in 7 different perfusions, similar to our previous results [16]. Since the extra O_2 uptake occurs also in the presence of antimycin A and is suppressed upon addition of 0.12 mM metyrapone [16], it is attributable to the enhanced flux through the monooxygenase system. Thus, it is calculated that 4 μmoles extra NADPH is utilized by the monooxygenase system per μmole of hexobarbital metabolized.

The infusion of *t*-butyl hydroperoxide leads to a substantial decrease of the rate of hexobarbital metabolized (Fig. 1), concomitant with a decrease of O_2 uptake by the liver (not shown). The effect is reversible. The extent of the restriction of hexobarbital metabolism increases with the rate of infusion of the hydroperoxide (Fig. 2). Since the addition of *t*-butyl hydroperoxide in the absence of hexobarbital also leads to a slight decrease in O_2 uptake [11], the inhibitory effect on O_2 uptake in the present experiments may be of composite nature and, therefore, was not analysed further for the present purposes. The addition of *t*-butanol, the product of *t*-butyl hydroperoxide reduction, had no effect on O_2 uptake or on the NAD(P)H-dependent fluorescence even at a high concentration of 2 mM [11].

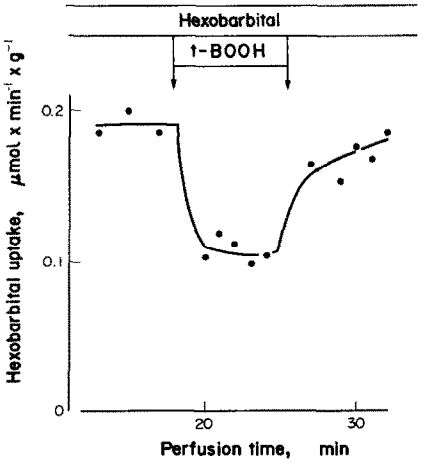

Fig. 1. Reversible inhibition of hexobarbital metabolism by *t*-butyl hydroperoxide in perfused liver. Influent concentrations were: sodium hexobarbital, 0.19 mM; *t*-butyl hydroperoxide (*t*-BOOH), 0.56 mM.