Table II. Incidence of micronucleated erythrocytes in the bone marrow of mice

Compound	Concentration (mg/kg)	Micronuclei per 1000 polychromatic erythrocytes in Polychromat. erythroc.	Normochromat. erythroc.	Mode of application
None	—	3.8	3.5	—
ETU	450	3.0	2.5	i.p.
	150	2.0	3.5	
$NaNO_2$	150	2.8	2.0	p.o.
	50	2.5	1.3	
N-nitroso-ETU	125	32.0	4.5	i.p.
ETU + $NaNO_2$	600	50.9	2.6	p.o.
(3:1 w/w)	200	28.4	3.1	
Trenimon	0.12	53.5	1.2	i.p.
	0.06	36.7	2.7	
	0.03	18.0	5.0	

erythrocytes, where they are not expelled together with the nucleus. As an added advantage of this test, erythrocytes which are younger than 24 to 30 h stain blueish (polychromatic erythrocytes) instead of red (normochromatic erythrocytes). This allows one to distinguish between micronuclei formed during (and possibly because of) the treatment of the animal and those formed before. By counting micronuclei in poly- and normochromatic erythrocytes separately, the increase of the number of micronuclei due to the treatment is immediately evident. Furthermore false positive results due to viral infections or other conditions resulting in a higher incidence of micronuclei in single animals can thus easily be recognized and excluded.

Our experiments – summarized in Table 2 – showed clearly that the single compounds were mutagenically inactive. Only the single assay with N-nitroso-ETU was seen to increase the number of micronuclei. It was then presumed that the formation of this derivative would also take place in the acid environment of the stomach upon feeding the animals with a mixture of ETU and sodium nitrite. If the so-formed N-nitroso derivative could reach the target cells within the bone marrow, an increase in the number of micronuclei should – and indeed could – be seen. This dramatic increase in the mutagenic activity of 2 substances, when given together, demonstrates again the possibility of a noxious interaction of the food additive sodium nitrite with aminic or amidic food components: Chemical reactions combine in vivo the 2 more or less harmless substances forming a dangerous product, which is then distributed throughout the organism. Having reached a target site, this compound may cause either a mutagenic alteration in a germ cell or malignant transformation of a somatic cell. In the former case, the damage is transmitted to the offspring and to future generations, whereas in the latter case already the exposed individual might develop cancer. The involuntary and indiscriminate uptake of food additives and food contaminants can thus have more or less immediate as well as far reaching effects. The claim that over 80% of the cancer cases are of environmental origin could then well become substantiated by more research in to the areas of chemical interactions. Also more attention has been paid in the last few years to the genetic effects of environmental chemicals. Certainly the time has come to investigate with all possible means the genetic consequences of such combinations and in vivo interactions. Our findings presented here show clearly that the possibility of mutagenic effects through in vivo occurring chemical interaction of two apparently harmless substances has to be taken into account when testing chemicals for safety purposes.

Zusammenfassung. N-Nitroso-Äthylenthioharnstoff induziert Mutationen in *Salmonella typhimurium*. Diese Verbindung wird im Magen von Mäusen aus Natriumnitrit und Äthylenthioharnstoff gebildet; ihre Mutagenität wird mit Hilfe des Mikrokerntests gezeigt.

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A Negative Cooperative Binding Process Between Chloramphenicol and Sodium Dodecyl Sulphate to Bovine Serum Albumin: A Possible Effect on Drug Absorption

Surfactant influence on drug absorption is a well established fact[1]. The mechanism for this effect has been related to different factors, such as drug solubilization above the critical micelle concentration (CMC)[2], wetting of the membrane surface[3–6], structural modifications of the membrane[7,8] and interactions with the dosage form[9,10]. Any influence of premicellar concentrations on membrane transport is generally ascribed to changes in membrane permeability following the interaction of surfactants monomers with one or more biomembrane constituents.

A previous attempt[11] to find a possible explanation to the effect that surfactant monomers might have on

membrane transport brought us to the conclusion that a competitive binding to the protein constituents of the membrane is at least partially responsible in many cases of enhanced drug absorption. A closer examination of those data and additional experimental evidence, suggested that an allosteric rearrangement of the membrane protein, triggered by the binding of one or more permeating species, rather than a competitive binding process, might be responsible for the increased transport rate of the drug.

It can be visualized that a molecule different from the permeant, a transport effector (or inhibitor) molecule bound to the allosteric sites[12] of the protein constituents of the biomembrane, might induce a number of specific structural changes[13], which in turn would affect the transport rate via the degree of binding of the permeant.

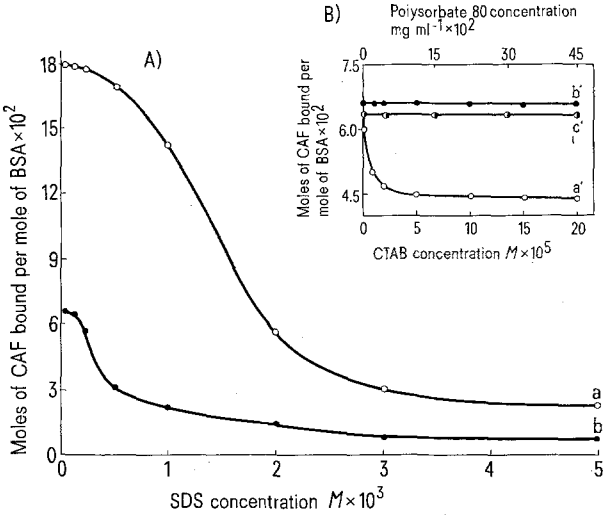

Fig. 1. A) Binding curves for the interaction of CAF with BSA (2% w/v) in phosphate buffer (pH = 6.8) at 25°C in the presence of increasing amounts of SDS (CMC at 25° = 7.9×10^{-3} *M*). Plots a (○—○) and b (●—●) refer to different initial concentrations of the antibiotic, i.e., 6.2×10^{-5} *M* and 3.1×10^{-5} *M* respectively. B) Binding curves of CAF to BSA (2% w/v) at 25°C in the presence of increasing amounts of surfactants. Plots a' (○—○) and b' (●—●) refer to CTAB (CMC at 25° = 9.0×10^{-4} *M*) at pH 6.8 (phosphate buffer) and pH 2.2 (dilute hydrochloric acid) respectively. Plot c' (◑—◑) is related to polysorbate 80 (CMC at 25°C = 1.4×10^{-5} g/ml⁻¹) at pH 6.8 (phosphate buffer). In all cases, the initial concentration of the antibiotic was 3.1×10^{-5} *M*.

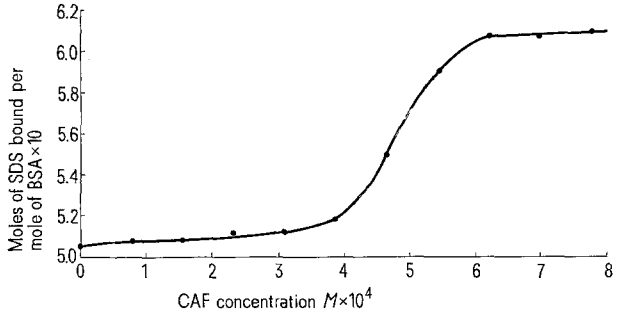

Fig. 2. Equilibrium dialysis curve for the interaction of SDS with BSA (2% w/v) in phosphate buffer (pH 6.8) at 25°C in the presence of increasing amounts of CAF. The initial concentration of surfactant was 1.0×10^{-4} *M*.

Materials and methods. Crystallized bovine serum albumin (BSA) from BDH Chemical Ltd, was dialized against water for 5 days at 4°C and then lyophilized. Sodium dodecyl sulphate (SDS), cetyltrimethyl ammonium bromide (CTAB) from BDH Chemical Ltd, and polysorbate 80 (Fluka, puriss grade) were all used as received. Chloramphenicol (CAF) was a generous gift of the Carlo Erba Co., Milan, Italy. All other reagents were of analytical grade.

Equilibrium dialysis. Solutions at pH 2.2 (ionic strength 0.01) and pH 6.8 (ionic strength 0.2) were obtained with diluted hydrochloric acid and phosphate buffer[14] respectively. It has been demonstrated[15] that the binding of detergent to BSA is independent of ionic strength: the effect appears to be entirely on the concentration of free surfactant monomer. Nevertheless, the equilibrium surfactant concentration in no case exceeded the CMC of detergent at a given temperature and ionic strength[16]. 2 ml of protein solution was placed in a dialysis sac (18/32 type, The Scientific Instrument Centre Ltd) and equilibrated against 5 ml of a solution containing the reagents at 25 ± 0.2°C for 18 h. For each series of dialysis experiments, 2 runs were set up in parallel, each run being the same except for the presence of the second ligand. In this way, an UV-analysis of the solution outside the dialysis sacs allowed us to determine, by direct comparison, the effect of the second ligand on the binding of the first to the protein macromolecule. The UV-analysis of free CAF concentrations at equilibrium was carried out at the wavelength of 278 nm by means of a Beckmann DU-2 spectrophotometer using quartz cells having a path length of 1 cm. Surfactants were found not to interfere with the assay at this wavelength. Free surfactant concentrations were determined by absorption, at 540 nm, of a dye surfactant complex[17]. CAF was found not to interfere with the assay at this wavelength.

Diffusion experiments. The cell and the procedures used were described previously[11]. In all experiments, the concentration of the protein solution (20 mg · ml⁻¹) in the central compartment of the diffusion cell was the same as that used in equilibrium dialysis.

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Permeability constants of CAF across a barrier consisting of a BSA solution (2% w/v)

SDS concentration		CTAB concentration		
$M \cdot 10^5$	$\alpha \cdot 10^6$ cm/sec$^+$	$M \cdot 10^6$	$\alpha \cdot 10^6$ cm/sec$^+$ (pH = 6.8)	$\alpha \cdot 10^6$ cm/sec$^+$ (pH = 2.2)
0	1.13	0	1.13	1.08
10	1.88	10	1.32	1.30
50	2.11	50	1.60	1.33
100	2.16	100	1.70	1.35
500	2.19	200	1.70	1.36
1000	2.22	—	—	—

When water replaced the protein solution as a barrier, $\alpha = 2.41 \cdot 10^{-6}$ cm/sec$^+$.

Results and discussion. To analyze the bonding mechanism involved in the CAF albumin interaction in the presence of premicellar concentrations of surfactants, a number of dialysis experiments were performed at different levels of both surfactant and antibiotic molarity. As shown in Figure 1, by the addition of the anionic surfactant, the antibiotic binding to the protein decreases and anomalies in the shape of the binding curves can be observed.

A sharp discontinuity in the binding isotherm is often referred to as evidence of a 'cooperative' process. In fact, the sigmoid shape of these plots, indicating a non-linear decrease in the binding of CAF following an increase in surfactant concentration, suggests an enhanced negative effect of the surfactant on the degree of association between the antibiotic and serum albumin, i.e., the reversible[18] structural changes induced by SDS would readily decrease the affinity of the binding sites for the antibiotic.

If BSA can exist in a number of structures[18], i.e. folded (F) and unfolded (U), the latter being a function of the anionic surfactant concentration, one might assume a specific binding of the antibiotic and surfactant molecules to the F and U forms respectively. The initial portion of both the interaction curves of Figure 1 represents the binding of CAF to the native protein, while the following portion is related to the binding of the antibiotic to partially unfolded serum albumin. The U form of BSA may be considered as a dynamic collection of structures showing a different binding capacity for the antibiotic, each population of structures being controlled by SDS concentration. Furthermore, the shape of the curves of Figure 1 suggests a non-linear increase of the constant for the reversible structural transition $F \rightleftarrows U$, which seems to be catalyzed by the fraction of protein subunits in the U form.

This effect can be compared to that of an allosteric inhibitor for the enzyme-substrate system as described by MONOD, WYMAN and CHANGEUX[19]. At a lower concentration of te antibiotic (plot b) the effect is less evident, but an increase in CAF molarity reveals the cooperative process (plot a).

An opposite effect was observed when the binding of SDS as a function of CAF concentration was studied, as illustrated in Figure 2, which shows that increasing amounts of surfactant are bound as more antibiotic is added. Since a true competitive inhibitor is a ligand capable of inhibiting the binding of a second ligand by association with the same site of the protein, certainly this is not the case depicted in Figure 2.

At the same time, by the addition of premicellar concentrations of the cationic surfactant CTAB, a decrease in the bound fraction of the antibiotic was observed which can be interpreted in terms of a competitive inhibition (see Figure 1 B, plot a'). This is consistent with the view that this surfactant associates with the protein mainly by electrostatic interaction of the cationic groups with free carboxyl groups of serum albumin[20], with no detecable cooperative effect on the binding of CAF. Further support to this interpretation of the data was obtained in a dialysis experiment performed at pH 2.2, when addition of CTAB did not raise significantly the free antibiotic concentration, since ionic bonding was decreased to a minimum following the net positive charge of the albumin molecule at this pH (see Figure 1B, plot b'). In a similar way, no significant increase in free antibiotic was observed following the addition of premicellar concentrations[21] of polysorbate 80 to the protein solution at pH 6.8 (see Figure 1B, plot c'). The observed difference in bound fraction (plots b' and c') is related to the well known conformational transition of serum albumin due to pH[14, 22]. In fact; it has been demonstrated[11] that more antibiotic is bound to BSA in its threadlike form (pH = 2.2).

In conclusion, both ionic surfactants increase the unbound fraction of the antibiotic in the CAF-BSA system at pH 6.8 but to a different extent, which reflects a different binding pattern.

Differences in the binding mechanism correspond to different flow rates observed in diffusion experiments performed in a model system described elsewhere[11]. Previous results have been completed and data are shown in the Table.

α-values, indicating the rate at which CAF diffuses through the artificial barrier (a 2% w/v serum albumin solution) after an uniform concentration gradient through the diffusion cell has been attained, show that the flux of the antibiotic is related to the extent of its association to the protein, as expected, but it is controlled by the mechanism of binding. A maximum of permeability cor-

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responds to a cooperative binding process, whereas a much less significant effect is determined by a competitive interaction.

It is significant that widely different conformations, such as those observed for BSA at two pH values (2.2 and 6.8 respectively)[22] do not necessarily imply a proportional difference in binding and transport capacities of the protein for the antibiotic[11].

Furthermore, the volume decrease produced by the interaction of SDS with negatively charged BSA[23], which occurs at premicellar detergent concentrations, suggests that, along with binding processes, changes in the geometry of the biomebrane might be significant in determining the transport rate of the permeants.

Résumé. L'étude de l'influence de concentrations prémicellaires des tensioactifs sur l'interaction BSA-CAF permet de conclure qu'il s'agit d'un mécanisme cooperatif suivant le modèle allosterique proposé par MONOD, WYMAN et CHANGEUX[19]. Ce mécanisme représente une possible explication de l'influence des tensioactifs sur l'absorption des médicaments.

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Contractile Response of Halothane-Depressed Isolated Atria to Various Substrates

In our previous report[1] dealing with the mechanism of cardiac depressant action of inhalation anesthetic halothane, it has been demonstrated that: 1. approximately 6 mg/100 ml halothane is required to maintain 50% depression of the force of contraction of isolated rat atria in Krebes-Ringer bicarbonate glucose medium; 2. pyruvate partially restores the contractility of halothane-depressed atria, but has no effect on normal atria. From these findings we concluded that the cardiac depressant action of halothane on rat atria is a manifestation of inhibition of glucose uptake or utilization. The present studies were undertaken to observe the effect of other substrates on halothane-depressed atria in order to substantiate our conclusions. As with the case of pyruvate, lactate and acetate also partially restored the force of contraction of halothane-depressed atria. These data are consistent with the hypothesis that halothane inhibits glucose uptake or utilization in the glycolytic cycle of the myocardium.

Method. Atria from decapitated rats were used as previously described[1,2]. Halothane was administered into the medium by means of anaesthesia[3]. The halothane concentration in the medium was determined at 10 to 30 min intervals with a gas chromatograph throughout the experimental period[3].

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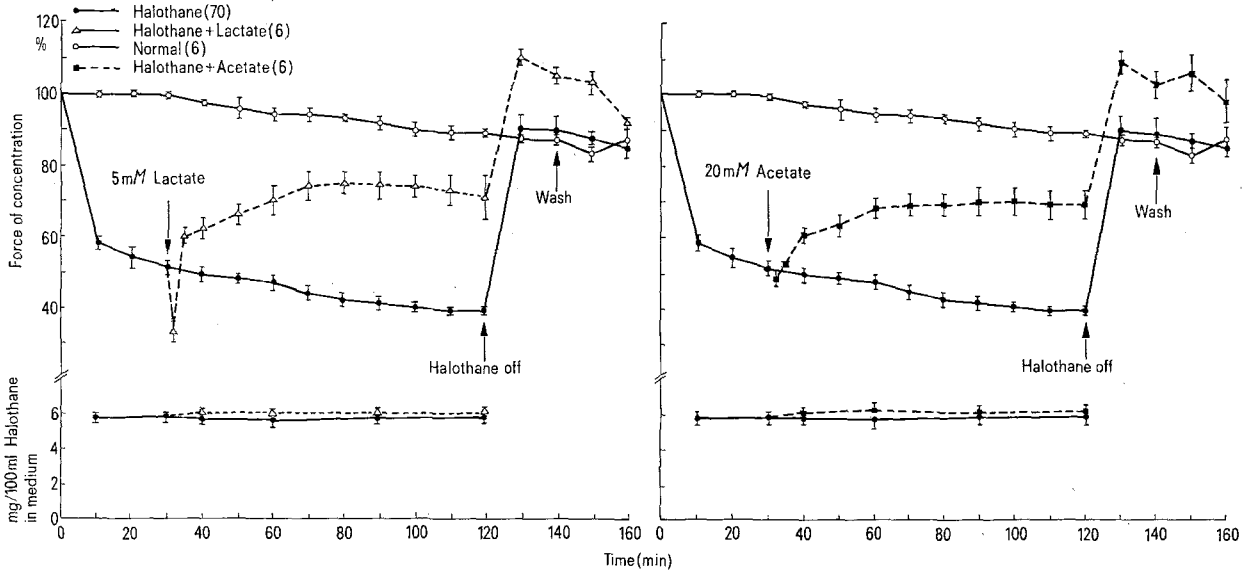

Fig. 1. Effect of lactate (5 m*M*) or acetate (2.5 m*M*) on atria depressed with halothane. In this and subsequent figures, halothane was added at zero time (i.e. following a 60 min equilibration period in the normal Krebs-Ringer bicarbonate glucose medium). Substrates were added 30 min after start of halothane administration. Vertical bars represent 1 SE of the mean.