more. However, the 2nd ^{110m}Ag peak which spreads from fractions 27 to 36 contains high ^{110m}Ag activity/mg protein. Interestingly no protein peak is discernible in this region, though the fractions do contain protein as estimated by the Lowry method. The protein in the fractions moved as a single band during polyacrylamide gel-electrophoresis.

The gel-filtration estimate of the mol.wt of Ag BP using globular proteins as markers is in the vicinity of 9500 daltons (figure 2). Mol.wt estimates of Cd-Zn containing metallothioneins carried out by a number of workers by a variety of methods have ranged from 6600 to 12,000[13,14]. It was hypothesized that higher estimates were due to the occurrence of dimerization. However, recent work suggests that metallothionein behaves as a linear rather than a globular molecule during gel-filtration; such behaviour leads to a higher estimate of mol.wt when globular proteins are used as markers[14]. Further work is in progress in our laboratory to check the truth of this hypothesis employing Ag BP as a test molecule.

One of the most important properties of metallothioneins studied so far is their heat stability[15]. We have observed that heating liver supernatant at 64 °C for 15 min causes precipitation of only 20% of the total ^{110m}Ag present in the fraction. Heated supernatant, upon fractionation with Sephadex G-75, showed a single ^{110m}Ag peak corresponding to that of the Ag BP of the 2nd peak in unheated supernatant (data not shown).

When Ag BP was allowed to pass through a Sephadex G-25 column preequilibrated at pH 2.2 all of the ^{110m}Ag activity was recovered in the void volume of the column as shown in figure 3. The stability of the Ag BP complex at this extremely acidic pH is suggestive of the presence of much stronger bonds between Ag^+ and protein. In this respect Ag BP differs from Cd-thionein in which Cd^+ dissociates from the protein at pH 2.2 but is similar to Hg-thionein which is stable at this pH[16].

It is known that the binding of metal to protein often causes a shift in absorption maxima as compared to those for the free apoprotein[17]. The UV-scan obtained by us for Ag BP (figure 4) shows 2 absorption maxima, 1 major peak at 260 nm and another at 225 nm. Cadmium-containing and zinc-containing metallothioneins have their absorption maxima at 254 nm and 215 nm respectively. It has been suggested that the absorption maxima observed in cadmium- and zinc-containing metallothioneins are due to the metal mercaptides[17]. However, our observations on the absorption of UV-light by Ag BP need to be investigated further before it can be said with certainity that the observed λmax of Ag BP is due to silver mercaptides. However, the low absorption at 280 nm does suggest a lack of aromatic amino acids in Ag BP.

The properties of avian hepatic Ag BP studied in the present report indicate its similarity to other metallothioneins. Further studies are required to ascertain whether avian hepatic AG BP performs functions similar to those of mammalian metallothioneins.

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Early changes of cyclic nucleotide levels in a mitogenic reaction in the rat mesentery[1]

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Summary. By measuring simultaneously cAMP and cGMP we found a biphasic time course with regard to cGMP and the cGMP/cAMP ratio very early in a mitogenic reaction in vivo. This is a new finding.

Accumulating evidence indicates that the endogenous cyclic nucleotides cAMP and cGMP play a part in the regulation of proliferation[2-4]. These cyclic nucleotides often, but not always[5], show alterations in their intracellular concentrations very early in the process of mitogenesis in a variety of mammalian cells in cell culture[6]. These alterations often are concomitant, reciprocal, and mono-phasic.

The aim of the present investigation was to study simultaneously the concentrations of cAMP and cGMP as soon as possible and at intervals up to 32 min after induction of mitogenesis in intact tissue in vivo. The tissue chosen was the rat mesenteric membrane. Proliferation was induced by i.p. injection of compound 48/80. This caused rapid secretion of mast cells followed by prompt mitogenesis in 2 cell types, namely fibroblast-like and mesothelial-cell-like cells[7,8].

Materials and methods. Animals: male Sprague-Dawley rats (Anticimex AB, Stockholm, Sweden) weighing 170–200 g (mean ± SE was 183.6 ± 2.4 g) fed standard pellets (AB Astra Ewos, Södertälje, Sweden) and water ad libitum were used. The animals were kept under carefully standardized laboratory conditions[7] for at least 5 days before being used in experiments.

Induction of proliferation: compound 48/80 (Sigma Chemical, Co., St. Louis, USA) dissolved in saline was injected i.p. in a single dose of 1 µg/g b.wt (1 ml/100 g) at 09.00 h in unanaesthetized rats. For technical details see[7,8].

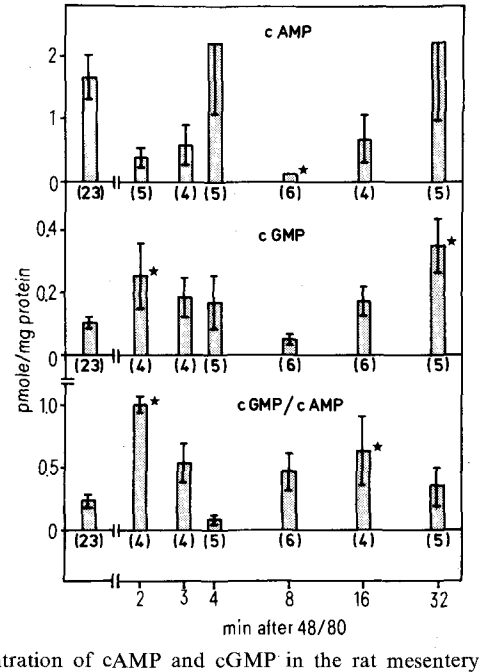
At intervals after injection of 48/80 the unanaesthetized

animals were decapitated and the abdomen immediately opened. A medium-sized square of mesentery free from large vessels was excised without delay, submerged in Frigen ($CFCl_3$) at −80 °C, and placed in a small ampoule with a stopper at −80 °C. The time between decapitation and freezing of the specimens was about 2 min in each animal, in test animals as well as controls. Specimens were collected at 2, 3, 4, 8, 16, and 32 min after injection of 48/80. Untreated animals were used as controls. 2 specimens from the same interval were pooled to give 1 sample. Altogether 53 samples including 23 control samples were analyzed for cAMP and cGMP contents.

cAMP and cGMP: the frozen mesentery preparations were homogenized in 5% perchloric acid and centrifuged. The supernatant was applied to columns of AG-1-X8 (200–400 mesh, formate form). After eluation of the cyclic nucleotides with formic acid, the eluates were lyophilized and dissolved in sodium acetate. The cyclic nucleotides were then measured by radioimmunoassay[9]. The sensitivity of the assay was enhanced by acetylating the samples as described by Harper and Brooker[10]. Commercial antibodies from Becton-Dickinson Immunodiagnostics (Orangeburg, N.Y., USA) were used for the measurements of cAMP and cGMP.

Statistics: the significance of the difference between 2 or more means was assessed by Student's t-test, by Scheffé-tests[11], and by conventional F-tests.

Results. Following the i.p. injection of 48/80, cAMP showed a marked decrease at 2 and 3 min, a slight increase above control levels at 4 min, a marked decrease at 8 min ($p \leq 0.04$), a slightly higher value at 16 min, and an increase to above the control value at 32 min (figure). The difference between the values at (2+3) min and 4 min was statistically significant ($p \leq 0.015$), as was the difference between 4 and 8 min ($p \leq 0.015$). The time course therefore seemed to be biphasic. However, judging by an F-test, the difference between treatment means were not significant and consequently the t-tests reported above may be viewed with caution. Furthermore, the more conservative Scheffé-test showed no significant changes in cAMP at all.



Concentration of cAMP and cGMP in the rat mesentery at different times after initiation of a mitogenic reaction achieved by i.p. injection of compound 48/80. Figures in brackets indicate number of samples. Altogether 53 samples were analyzed, 23 of which were controls. * indicates statistically significant difference according to Student's t-test compared to controls. A biphasic time course was suggested for each of cAMP, cGMP, and the cGMP/cAMP ratio (for details see Results). Abscissa: min after 48/80, modified log scale.

As shown in the figure, the time course of the changes in cGMP values following injection of 48/80 differed from that for cAMP. A marked increase for cGMP was found at 2 min ($p \leq 0.02$). This increase was slightly reduced at 3 and 4 min. At 8 min the level was somewhat lower than that in the controls. A moderate increase appeared at 16 min. The difference between 8 and 16 min was significant ($p \leq 0.04$). At 32 min there was a significant increase compared to control values ($p \leq 0.001$). Significant differences between treatment means were demonstrated in an F-test ($p \leq 0.05$), and the Scheffé-test showed statistically significant differences between controls and 2 min, and between 2 and 8 min ($p \leq 0.05$).

The ratio cGMP/cAMP calculated for each individual sample showed a statistically significant increase at 2 min ($p \leq 0.001$) and at 16 min ($p \leq 0.03$) compared to controls (see figure). A trough appeared at 4 min. The difference between 3 and 4 min was significant ($p \leq 0.025$), as was that between 4 and 8 min ($p \leq 0.05$). A biphasic time course was thus apparently also established for this variable. The difference between treatment means were significant ($p \leq 0.01$) according to an F-test. The conservative Scheffé-test also demonstrated significant differences between controls and 2 min, and between 2 and 4 min ($p \leq 0.01$).

Discussion. We investigated the earliest possible changes in the cyclic nucleotides cAMP and cGMP after induction of a mitogenic reaction in the mesentery of normal rats. Mitogenesis was initiated by an i.p. injection of compound 48/80, which causes mast-cell secretion that appears within seconds and probably is complete within 20 sec. The time of starting of the reaction was thus well defined. Mitogenesis then promptly occurs in 2 types of cells, namely fibroblast-like and mesothelial-cell-like cells. Both show increased DNA synthesis within 16 h and increased mitotic activity within 24 h[8]. Untreated rather than saline-treated animals were used as controls since i.p. injection of saline causes slight secretion of histamine of mast cells and low but significant proliferation in both cell types[12,13]. The briefest interval between the injection of 48/80 and freezing of the tissue for later analysis was about 2 min. This membranous tissue is very thin[14] and can be rapidly frozen entire. Made up of connective tissue cells only[8,12], it has a simple anatomical structure which facilitates interpretation of the results.

Compound 48/80 is a polymeric mast-cell-activating agent causing release of heparin, histamine, and serotonin, and possibly also of proteolytic enzymes from rat mast cells. At high concentrations the agent suppresses cAMP-phosphodiesterase, and at very high concentrations, also cGMP-phosphodiesterase[15]. With the concentration used in the present study these effects of 48/80 seem negligible.

In experiments in vitro using slices of guinea-pig lung tissue Sohn et al.[16] reported increased levels of both cAMP and cGMP within 20 sec during 'anaphylaxis'. This illustrates that the earliest changes in the cyclic nucleotides could have appeared before the 1st observation point in the present study in vivo. They also found a roughly parallel time course for both cyclic nucleotides, and therefore no marked change in the cGMP/cAMP ratio. An intricate problem is that of interpreting the physiological meaning of cyclic nucleotide changes in lung slices, as the cell population of this tissue is so heterogeneous.

The earliest changes recorded in the present study were a transient increase in cGMP and a roughly concomitant fall in cAMP, resulting in an increase in the cGMP/cAMP

ratio. A similar pattern regarding initial changes in cyclic nucleotides has been reported in fibroblast-like connective-tissue cells in cell cultures when proliferation was initiated by different stimuli such as serum and a Ca^{2+}-ionophore[17,18]. The subsequent changes in the cyclic nucleotides showed a more complex pattern in the present in vivo study, however, than has been reported from experiments in vitro. Using a conventional statistical method such as Student's t-test we found a biphasic time course for each of cAMP, cGMP, and the cGMP/cAMP ratio within the observation time of 32 min. The more conservative Scheffé-test, however, did not substantiate this conclusion. It should be observed that fibroblast-like and mesothelial-cell-like cells are present in roughly equal numbers (52% and 48%), but that more fibroblast-like cells than mesothelial-cell-like cells are stimulated to proliferate with the treatment given[8]. This difference in mitogenic responsiveness between the 2 cell types is noteworthy in relation to the apparently biphasic time course for changes in cyclic nucleotides found here. The results appear to be compatible with a monophasic course for either cell type. The pattern of changes in cyclic nucleotides in the present study is quite different from what has been reported by Whitfield et al.[19] on proliferation of hepatocytes in rats in vivo. They found no alteration in the concentration of cyclic nucleotides during more than 30 min following partial hepatectomy or initiation of liver cell proliferation by i.v. injection of a mixture of triiodothyronine (T3), amino acids, glucagon, and heparin. After about 35 min they demonstrated a significant increase in the concentration of cAMP but no change in cGMP was found at all.

The time course of cyclic nucleotide changes in the present investigation is of interest from various points of view, even though it remains uncertain whether or not it is these changes that determine the subsequent DNA-synthesis and mitotic activity. One important point is that biphasic courses for both cAMP and cGMP (and the cGMP/cAMP ratio) were suggested. Without early and closely spaced observation times the results could have been very difficult to interpret. These findings may explain some of the apparently conflicting reports on changes in cAMP and cGMP in mitogenesis in previous papers[4].

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A species comparison of 2,4-dinitrotoluene metabolism in vitro[1]

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Summary. Postmitochondrial supernatants prepared from livers of mice, rats, rabbits, dogs, and monkeys metabolized 2,4-dinitrotoluene. The pattern of metabolites was characterized in both sexes of the species examined. In addition, the pattern of metabolites was altered by varying incubation conditions and pretreating male rats with phenobarbital or SKF 525-A.

This study was undertaken to compare the ability of livers from various species to metabolize 2,4-dinitrotoluene (2,4-DNT) and to characterize the pattern of metabolites produced. Dinitrotoluene is used as a dye intermediate. In the production of toluene diisocyanate for polyurethane foams, it is reduced to the diamine. Dinitrotoluene has also been used as a gelatinizing and waterproofing agent in the production of explosives.

In mice, rats, and dogs, the subacute toxicity of 2,4-DNT includes methemoglobinemia and atrophy of the testes with aspermatogenesis[2]. During a 24-month feeding study, rats receiving 2,4-DNT developed hepatocellular carcinomas and an increased incidence of tumors of mammary and s.c. tissues[3,4]. In contrast, mice that received 2,4-DNT mainly developed cystic renal tumors[4]. In another study, the reduced derivative of 2,4-DNT, 2,4-diaminotoluene, produced hepatocellular carcinomas in rats[5].

The results of the present study will be valuable in understanding how different species metabolize 2,4-DNT and, accordingly, will contribute to a better understanding of its toxicity.

Material and methods. In the present study, CD rats, CD-1 mice, New Zealand albino rabbits, beagle dogs, and rhesus monkeys were used. The animals were killed, and their livers were removed, weighed, and homogenized in 6 vol. of 1.15% KCl containing 50 mM tris HCl buffer (pH 7.4). The homogenate was centrifuged at 10,000 × g for 30 min, and the resulting postmitochondrial supernatant was used for all incubations, which were conducted in 25-ml Erlenmeyer flasks. The reaction mixture contained 5 mM glucose-6-phosphate; 5 mM magnesium chloride; 0.8 mM nicotinamide adenine dinucleotide phosphate; 1 mM 2,4-dinitrotoluene [ring-^{14}C (U)] (^{14}C-2,4-DNT) which was purchased from New England Nuclear (Boston, Massa-