with carbol-fuchsin and attached with Permount onto slides.

Preparations were coded and analyzed blind by 1 investigator. A total number of 100 anaphase-telophase cells per tube was scored for chromatin bridges. Mitotic index was determined by counting at least 5000 cells for each experiment. This value was expressed as the number of mitotic plates per 100 cells. Comparison between treated and untreated cells was made by the Student t-test.

Results. The table summarizes the results obtained from the different treatments and controls. In 4 and 8 h caffeine-treated cells, the frequency of chromatin bridges in anaphase-telophase was not statistically different from those of controls. On the other hand, treatments with TEM alone induced a significant increase in the frequency of chromatin bridges ($p < 0.01$). In TEM plus caffeine treatments the percentage of anaphase-telophase cells with chromatin bridges increased again, showing significant differences between controls ($p < 0.001$) and TEM-treated cells ($p < 0.05$).

The analysis of the mitotic index also revealed the effect of both chemicals. The 4 h caffeine treatments induced a little increase in the frequency of dividing cells, whereas in 8 h treatments the mitotic index was slightly lower. However, there were no statistical differences between caffeine-treated and control cells. TEM treatment induced a significant decrease of the mitotic index ($p < 0.001$). Combined treatments with TEM and caffeine exhibited a slight increment of dividing cells, but the mitotic index was statistically lower than that of controls ($p < 0.01$ and $p < 0.001$ respectively).

Discussion. Chromatin bridges at anaphase-telophase arise from: a) Chromosome stickiness; b) dicentric chromosomes showing criss-cross separation of chromatids; c) dicentric chromatids with centromeres oriented toward opposite poles[9,10]. However, the origin of chromatin bridges cannot be determined accurately in anaphase-telophase plates.

The increase in chromatin bridges in treated cultures could be a result of chromosome exchanges induced by TEM and enhanced by caffeine. This assumption is in godd agreement with the well known effects of caffeine in chromosome aberration potentiation[5,6] but it would imply that most of the TEM-induced lesions lead to chromosome exchanges which will be detected at the 1st mitosis after treatment. On the other hand, chromatin bridges at anaphase-telophase could arise from chromosome stickiness. In such a case, the potentiating effect of caffeine might be an indication that, like other subchromatid or chromatid aberrations, entangled chromatin fibrils are repaired by post-replication repair or another mechanism which can be inhibited by caffeine.

Our results do not decisively support any of these hypothesis. The influence of caffeine post-treatment on the frequency of chromatin bridges suggests, however, a relation between metaphase and anaphase-telophase chromosome aberrations and is a further evidence of the relevance of anaphase-telophase analysis for chemical mutagenesis.

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Synthesis of maleimide derivative of cortisol for enzyme coupling in cortisol enzyme immunoassay[1]

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Summary. A new cortisol derivative, cortisol-21-m-maleimidobenzoate (CMB), was synthesized and conjugated with sulfhydryl groups of β-galactosidase (BG). Both CMB-BG and CHS-BG conjugates have a high immunoreactivity to cortisol antibody, and although CHS-BG does not displace well with the added cortisol, CMB-BG does.

We have previously described the use of a novel m-maleimido-benzoyl derivative of thyroxine (T_4) methyl ester for the development of enzyme-labeled immunoassay (EIA)[3]. These studies showed a high efficiency of hapten coupling to sulfhydryl group of β-galactosidase without reduction in both enzyme activity and immunoreactivity, resulting in highly sensitive and reproducible EIA, and indicated the possibility that the same approach for enzyme-hapten conjugation could be applied to other haptens. This report describes the preparation of that m-maleimidobenzoyl derivative of cortisol and use of the compound for the development of EIA.

Materials and methods. Cortisol, cortisol-21-hemisuccinate, cortisone, corticosterone, cortisol-21-acetate and o-nitrophenyl-β-D-galacto-pyranoside were obtained from Sigma Chemical Co. Antiserum to cortisol-21-hemisuccinate (CHS, figure 1)-bovine serum albumin (BSA) was raised in rabbits following the procedure of Ruder et al.[4]. m-Maleimido-benzoic acid was prepared according to the method of Kitagawa and Aikawa[5]. Conversion to its carbonyl chloride (MBC) was done by the procedure described previously[3]. Synthesis of cortisol-21-m-maleimidobenzoate (CMB) was carried out as follows: 100 mg of MBC dissolved in 2 ml of tetrahydrofuran (THF) was added dropwise to 148 mg of cortisol dissolved in 2 ml of THF. The mixture was refluxed for 1 h and the synthesized product was monitored by TLC using Eastman chromatogram 13179 (Eastman Kodak Co.) as an eluting plate and ethyl acetate as an eluting solvent ($R_f = 0.67$). The product isolation was carried out by silica gel column chromatography (1.5 × 30 cm) using chloroform-ethyl acetate (3:1) as an eluting solvent. The isolated product gave a single fluorescent spot on TLC by sulfuric acid spray (10% concentration sulfuric acid in ethanol). Esterification at position 21 was

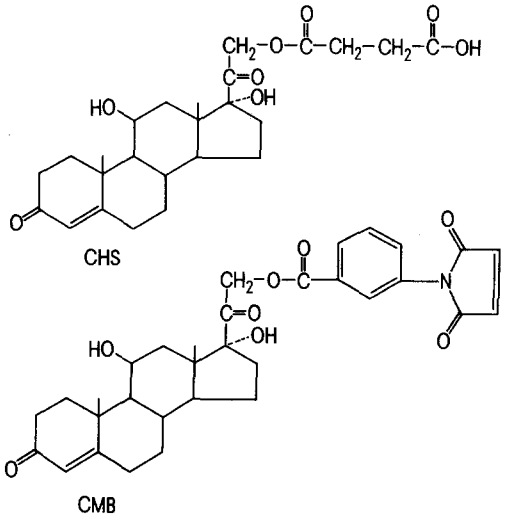

Fig. 1. Structure of cortisol-21-hemisuccinate (CHS) and cortisol-21-m-maleimidobenzoate (CMB).

confirmed by its immunoreactivity to cortisol antiserum as reported in the result section. The presence of a maleimide group on the compound was confirmed by its ability to react with cysteine using the method of Grassetti and Murray[6]. Structure of CMB is shown in figure 1. Melting point was 134–136 °C. For preparation of CHS-β-galactosidase conjugate, CHS was conjugated to β-galactosidase (from *E. coli,* Boehringer Mannheim, Inc.) by mixed acid anhydride method using the procedure of Comoglio and Celada[7].

Conjugation of CMB with β-galactosidase in phosphate buffer (pH 7.0, 0.05 M, buffer A) as well as isolation of the enzyme-hapten conjugate by Sephadex column chromatography was carried out by the same procedure as was reported for T_4 EIA[3]. For preparation of cortisol standards, a stock solution of 100 µg/ml of cortisol in ethanol was serially diluted with phosphate buffer-BSA (pH 7.3, 0.05 M, 0.1% BSA, buffer B) and 10-ml volumes of the standard solutions (0–50 µg/dl) were stored in a deep freezer. These standard solutions were stable for at least 6 months. EIA was carried out by the following procedure: Volumes of 20 µl of standards or plasma samples were mixed with 300 µl of the buffer A in glass tubes (16×100 mm). After thorough mixing, the solutions were heated in a boiling water bath for 10 min. The mixtures were then cooled and 0.4 ml of the buffer B was added, followed by 0.1 ml of the enzyme conjugate solution. After mixing, 0.1 ml of 1:500 dilution of cortisol antiserum (final dilution 1:3000) was added and incubated for 60 min at room temperature. Then, 100 µl of 1:20 dilution of normal rabbit serum was added, followed by 100 µl of goat anti-rabbit IgG (Calbiochem). After mixing on a vortex mixer, the tubes were incubated for 2 h at 4 °C and then centrifuged for 10 min at 3000 rpm. The pellet was washed 2 times with the buffer B. The enzyme activity in the pellet was assayed according to the procedure of Dray et al.[8] using o-nitrophenyl-β-D-galactopyranoside as substrate. Incubation time for enzyme activity was 60 min. Amount of o-nitrophenol produced at the end of incubation was measured by Gilford Stasar III spectrophotometer at 420 nm wavelength.

Results and discussion. CMB was synthesized and conjugated to sulfhydryl groups of β-galactosidase. We compared our method with the conventional conjugation method, i.e., CHS being conjugated to amino groups of β-galactosidase[7]. The latter method resulted in only 40% of the enzyme being

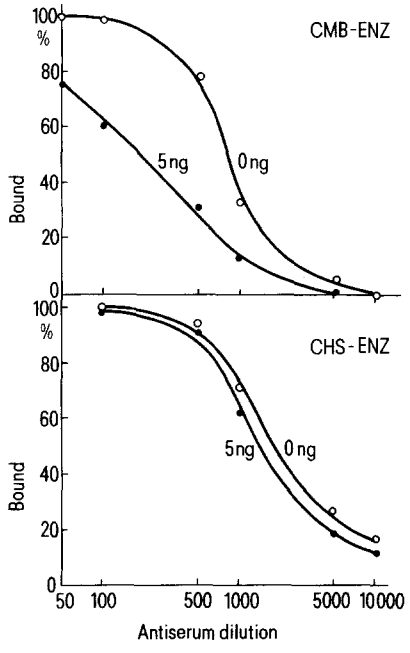

Fig. 2. Cortisol antiserum dilution curves using CMB-β-galactosidase (CMB-ENZ) and CHS-β-galactosidase (CHS-ENZ) as labels and with (5 ng) or without (0 ng) addition of cortisol.

labeled as examined by double antibody precipitation method in excess of cortisol antibody and 20% reduction of enzyme activity, whereas our conjugation method showed full retention of enzyme activity and over 95% of the enzyme being labeled with hapten derivatives. The number of moles of CMB conugated per enzyme was not determined. Since β-galactosidase possesses about 10–12 sulfhydryl groups per molecule, the maximum number of CMB attached per enzyme is 12.

When the immunoreactivities of these conjugates to the produced cortisol antiserum were examined (figure 2), CHS-β-galactosidase conjugate showed excellent immunoreactivity but poor displacement with 5 ng of the added cortisol. CMB-β-galactosidase conjugate, on the other hand, showed not only a high immunoreactivity to the antibody, but also displaced well with the added cortisol, showing maximum sensitivity of 1 µg/dl. Modification around the bridge of cortisol derivative may have resulted in increased sensitivity to cortisol. Cross reactivity to other steroids in the assay were 15% for cortisol-21-acetate, 10% for cortisone and 7% for corticosterone. These immunochemical results well indicated that CMB was esterified at position 21 by m-maleimidobenzoyl group. The measurable circulating cortisol range was 1–50 µg/dl, using 20-µl samples. The assay precisions of CV = 4.7% for intra-assay and CV = 6.5% for inter-assay were obtained. Correlation of serial dilutions of plasma samples containing various concentrations of cortisol with the standard curve indicated a negligible interference on EIA by other plasma constituents.

Maleimide derivatives have been used for coupling enzymes to proteins[5,9–11]. Use of these coupling agents showed no appreciable reduction of enzyme activity due to the involvement of sulfhydryl groups of the enzyme in the coupling process. Coupling of haptens to the enzyme, on the other hand, is often identical to the preparation of hapten-protein conjugates for immunization. Such coupling involves either amino or carboxyl groups of the enzymes, resulting in either low efficiency of coupling[7] or reduction of enzyme activity[8]. In order to obviate such disadvantages,

we synthesized m-maleimidobenzoyl derivative of hapten for coupling to sulfhydryl group of the enzyme, resulting in high efficiency of binding to the enzyme without appreciable reduction in enzyme activity. When we compared this enzyme labeled hapten with the conjugate prepared identical to that of hapten-protein conjugation for immunization, our conjugate preparation resulted in increased assay sensitivity (figure 2). Since similar results have been often observed in radioimmunoassay for the preparation of tyrosine or tyramine derivative of haptens as radioiodinated ligants[12], our studies showed for the first time the importance of modification around the bridge for increased EIA assay sensitivity, although it is also important to ensure that the antigenic groups exposed upon the hapten-protein conjugate employed for immunization are available in the enzyme-coupled hapten complex.

A high sensitivitiy observed in our T_4 EIA reported previously may also be due to the similar modification around the bridge in hapten-enzyme complex formation[3].

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Suppression of cytophilic antibody ('arming' factor) in the sera of patients with prostatic cancer by human seminal plasma

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Summary. The 'arming' of normal peripheral blood leukocytes (PBL) by cytophilic antibody in the sera of prostatic cancer patients is suppressed by pretreatment of PBL with normal human seminal plasma (HuSPl). Suppression of cytophilic antibody by HuSPl extends the spectrum of immunologic reactions on which SPl has an immunosuppressive effect and may provide further insight into the possible role of SPl in the natural history of prostatic cancer.

The immunosuppressive properties of the hormonal and/or secretory milieu or tumour-elaborated factors (in the case of carcinoma) of the prostate have been suggested[2] as 1 explanation for the hypothesized immunologic privilegedness of the prostate[3]. In an initial study of the role of one of these factors as contributory to the privileged status of the prostate, normal human seminal plasma (HuSPl) has been observed to suppress tumour-associated immunity in patients with prostatic cancer[4].

To possibly further elucidate the immunosuppressive effects of HuSPl and its role in tumour-host responsiveness, the effect of HuSPl on a stage-related and disease-specific activating or 'arming' factor (cytophilic antibody) in the sera of prostatic cancer patients[5,6] has been evaluated.

Materials and methods. Peripheral blood leukocytes (PBL) were obtained from 8 normal adult individuals, ranging in age from 26–67 years, by Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) centrifugation using a modification[7] of the method of Boyum[8]. PBL at a concentration of 1×10^7 cells/ml in RPMI 1640 medium (Grand Island Biological Company, Grand Island, New York), containing 100 IU penicillin g/ml and 100 µg streptomycin/ml, untreated and treated with 280 µg/ml of pooled normal HuSPl[4] were 'armed' by incubation at 37 °C for 50 min with 1:2 dilutions of serum from each of 7 patients with localized (stage A) and metastatic (stage D) prostatic cancer[6]. After incubation, cells were washed twice in RPMI 1640 medium and viability assessed by trypan-blue dye exclusion.

Employing a modification[7] of the tube leukocyte adherence inhibition method[9], untreated and 'armed' PBL and PBL treated with HuSPl and 'armed' were reacted with 3M KCl-$(NH_4)_2SO_4$ extracts of allogeneic malignant prostate[7]; and the number of nonadherent cells counted in quadruplicate using a Standard Neubauer haemocytometer.

Delineation of the significance of the effect of HuSPl on the 'arming' of normal PBL and their reactivity with malignant prostate compared with the reactivity obtained with untreated and 'armed' normal PBL was determined by the paired t-test.

Effect of human seminal plasma (HuSPl) on 'arming' of normal peripheral blood leukocytes with serum from patients with localized and metastatic prostatic cancer

Serum from stage[a]	Mean ± SE % nonadherent cells obtained with malignant prostate and normal leukocytes[b]		Significance[c]
	Untreated and 'armed'	Treated with HuSPl and 'armed'	
A (Localized)	24.1 ± 2.7	12.8 ± 1.7	$p < 0.05$
D (Metastatic)	13.3 ± 2.0	7.5 ± 2.0	$p < 0.05$
Significance[c]	$p < 0.05$	$p > 0.05$	

[a] Serum from 7 patients with localized prostatic cancer (stage A) and 7 patients with metastatic prostatic cancer (stage D); [b] from 8 normal adult volunteers; [c] paired 't'-test.