to which human beings are exposed. A brief examination of the table presented by Veljković and Lalović, and of additional appropriate examples, strongly suggests that the authors may have deceived themselves, and that they have certainly misled the scientific community.

Thus, they listed dimethyl sulphate as a noncarcinogen, when it has a well-known reputation for carcinogenicity[2]; it is certainly as potent as urethane[3], which appeared in the authord list of carcinogens. Calculation of Z* values, shown in parentheses, would place the following organics below $Z^* = 3.20$ and thus in the class of carcinogens: Ethane (1.75), butane (1.86), acetic acid (3.00), and ethanol (2.22). On the other hand, the known carcinogen N-nitroso-N-methylurea[4] (3.33), would be classed as a noncarcinogen. If one considers covalent inorganic compounds, in view of the authors' inclusion of hydrazine (2.33), then water (2.67) and ammonia (2.00) must appear among the list of carcinogens.

Finally, it appears that Veljković and Lalović[1] arbitrarily assigned $Z = 1$ to halogen elements; no rationale was presented for this. It would appear that the a priori knowledge that many organohalogen compounds are carcinogens prompted this modification to the basic concept, rather than any insight arising from accepted physical theory.

Our examination of the 'quasi-valence number' criterion for defining chemical carcinogenicity strongly indicates that it is untenable and should therefore be discarded. Nothing in succeeding papers of Veljković, in particular that dealing with the cytostatic activity of organic compounds[5], provides a reason to alter this assessment.

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PRO EXPERIMENTIS

A novel method in enzyme immunoassay: Maleimide derivative of hapten for enzyme coupling

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Summary. Meta-maleimidobenzoyl derivative of L-thyroxine methyl ester (MBTM) was synthesized and coupled to β-galactosidase at molar ratio of over 5 to 1. More than 97% of the enzyme was found to be labeled with MBTM. A thyroxine enzyme immunoassay was carried out with sensitivity in the 0–10 µg/100 ml range.

Maleimide derivatives have been used for coupling enzymes to proteins, such as immunoglobulins with N,N-o-phenylenedimaleimide[1,2] and insulin with m-maleimidobenzoyl-N-hydroxysuccinamide ester[3].

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 enzyme, resulting in either low efficiency of coupling[4] or reduction of enzyme activity[5]. In order to avoid such disadvantages, we report here the synthesis of m-maleimidobenzoyl derivative of hapten for coupling to sulfhydryl groups of the enzyme. A high efficiency of binding to the enzyme, high yields, and minimum loss of both enzyme activity and immunoreactivity were found.

Preparation of m-maleimidobenzoyl derivative of thyroxine methyl ester for development of thyroxine enzyme immunoassay is described (figure 1). β-galactosidase was used for several reasons: 1. it can be obtained in highly purified form, 2. it has a high catalytic number, 3. conjugation to sulfhydryl groups of the enzyme does not reduce enzyme activity, 4. the enzyme and its conjugates are stable up to 1 year when stored at 4 °C, and 5. it is not present in human or animal biological fluids.

Chemicals. L-thyroxine and o-nitrophenyl-β-D-galactoside were obtained from Sigma Chemical Co., β-galactosidase from *E. coli* from Boehringer Mannheim Biochemicals, and meta-aminobenzoic acid, benzaldehyde, and maleic anhydride from Aldrich Chemical Co.

Antiserum. Antiserum to thyroxine was produced in rabbits by injection of thyroxine conjugated to bovine serum albumin prepared by the method of Gharib et al.[6]. Cross reactivity with triiodothyroxine was minimal. Goat anti-rabbit immunoglobulin antibody was obtained from Calbiochem.

Synthesis of m-maleimidobenzoic acid (MBA). Meta-carboxymaleanilic acid was first prepared from meta-aminobenzoic acid and maleic anhydride following the method of Parola[7]. It was then cyclized with acetic anhydride to give MBA following the procedure of Searle[8].

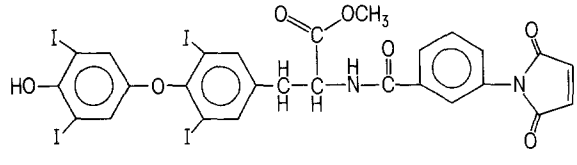

Fig. 1. Synthesis of m-maleimidobenzoyl derivative of L-thyroxine methyl ester. MBA (200 mg) was dissolved in 3 ml of thionyl chloride ($SOCl_2$) and refluxed for 30 min. Excess $SOCl_2$ was then evaporated under diminished pressure. The m-maleimidobenzoyl chloride (MBC) was kept overnight in a vacuum desiccator. The dried MBC was dissolved in 10 ml of tetrahydrofuran (THF) and added dropwise to a stirred THF solution containing L-thyroxine methyl ester (400 mg) and a slurry of sodium carbonate (400 mg). The reaction mixture was refluxed for 30 min. At this point linkage was complete and m-maleimidobenzoyl L-thyroxine methyl ester produced was examined by TLC using Eastman chromatogram 13179 as an eluting plate and ethyl acetate as eluting solvent. The reaction mixture was then filtered and the solvent removed under diminished pressure to yield crude pale-yellow product. The MBTM was purified by silica gel column chromatography (1.5 × 30 cm) using chloroform as eluting solvent. The isolated white powder of MBTM gave a single spot on TLC, $R_f = 0.56$, using ethyl acetate as solvent. The presence of maleimide group in the isolated product was confirmed by IR and by its ability to react with cysteine using the method of Grassetti and Murray[10]. Melting points 137–141 °C.

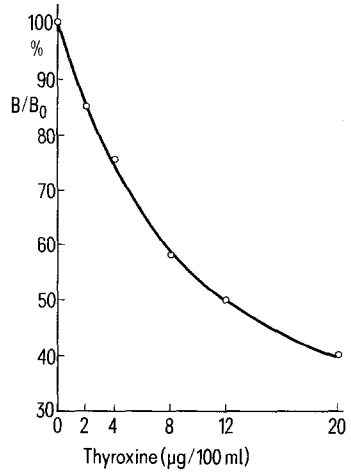

Fig. 2. Thyroxine assay. Immunoassay for thyroxine was developed using MBTM-enzyme conjugate as label and second antibody for separation of bound from unbound. To a glass tube (16×100 mm) 100 µl of a 100-fold dilution of the peak fraction of MBTM-enzyme conjugate eluted from Sephadex G-25 column was added, followed by addition of 500 µl of phosphate buffer (pH 7.0, 0.05 M) containing 0.5 mM 8-anilino-1-naphthalene-sulfonic acid-sodium salt. 50 µl of each of a series of standards containing 0, 2, 4, 8, 12 and 20 µg/100 ml of L-thyroxine in serum (Syva Corp.) were added, followed by 100 µl of a 1:400 dilution of rabbit anti-thyroxine antiserum. After incubation for 45 min at room temperature, 100 µl of 1:20 dilution of normal rabbit serum was added, followed by addition of 100 µl of goat anti-rabbit IgG. After vortex mixing, the tube was incubated for 15 min in ice water and centrifuged for 10 min at 3000 rpm. The pellet was washed twice with phosphate buffer and resuspended in 0.5 ml phosphate buffer-BSA (pH 7.5, 0.05 M, 0.1% BSA). The enzyme activity was assayed using o-nitrophenyl-β-D-galactoside as substrate. O-nitrophenol produced at the end of incubation time was measured by Gilford Stasar III spectrophotometer at 420 nm wavelength.

Synthesis of L-thyroxine methyl ester. L-thyroxine methyl ester was prepared by the method of Ashley and Harington[9].

Conjugation of β-galactosidase to maleimidobenzoyl L-thyroxine methyl ester (MBTM). 50 µl of a solution of MBTM in THF (0.2 mg/ml, 10 nmoles) was added to 1.5 ml of 0.05 M phosphate buffer (pH 7.0) containing β-galactosidase (0.5 mg, 0.93 nmole). The mixture was incubated for 2 h at room temperature. Following overnight dialysis in phosphate buffer, the mixture was chromatographed on a Sephadex G-25 column (1.5×40 cm). The fractions of eluate containing the peak of enzyme activity were used for the thyroxine assay. β-galactosidase activity was assayed by the method of Dray et al.[4] using o-nitrophenyl β-D-galactoside as substrate.

When MBTM and β-galactosidase conjugation was carried out at molar ratios of over 5 to 1, more than 97% of the enzyme was found to be conjugated with MBTM as examined by double antibody precipitation method in excess of anti-thyroxine antibody. The number of moles of MBTM conjugated per enzyme was not determined. Since it is known that β-galactosidase possesses about 10 sulfhydryl groups per molecule[11], the maximum number of MBTM attached per enzyme is 10. The practical limit of solubility of MBTM in the conjugation solvent is 10 µg/ml, restricting the molar ratio of MBTM to enzyme. The conjugation reaction was carried out immediately after dissolution of MBTM in the buffer.

Enzyme activities examined before and after the conjugation step did not show any difference, suggesting full retention of the enzyme functional groups. With the final antiserum dilution of 2400-fold, a reproducible thyroxine enzyme immunoassay was successfully demonstrated. Incubation times of the assay were 45 and 15 min respectively for competitive binding of thyroxine and MBTM-enzyme conjugate and double antibody precipitation steps (figure 2). The highest sensitivity in the assay was observed at 0–10 µg/100 ml range.

We believe that our novel approach in the use of the maleimide derivative of hapten is a convenient and efficient way for conjugating hapten to an enzyme without reduction of enzyme activity. Our model of enzyme-hapten conjugation procedure could be extended to many haptens and can improve assay sensitivity and precision.

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A rapid micro radial electrophoretic method of protein separation on cellulose acetate membranes[1]

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Summary. A new rapid micromethod for protein separation under a radial electric field is described. As many as 12 rabbit serum samples could be separated in 4–6 min.

Cellulose acetate membranes are used in various methods, like double diffusion, electrophoresis and immunoelectrophoresis[2–5]. These techniques have been adopted to micro methods also[6].

In the course of investigations on micro analytical techniques applicable to biological materials of pathodiagnostic importance, we developed a simple, quick and reproducible procedure of protein separation on cellulose acetate membranes. The interesting feature of the procedure is that, probably for the first time, a circular electrophoretic separation is successfully employed for protein separation. An inexpensive apparatus was designed and fabricated in our laboratory for this purpose. It consists of a perspex sheet with mini tanks and copper strips as electrodes (figure 1).