also suggest a causal relationship between the lower vitamin E content of the SR membranes from fast muscle and the greater susceptibility of these muscles to becoming dystrophic in vitamin E-deficiency.

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2 Author for reprint requests: F.N., Istituto di Patologia Generale, via S. Giacomo, I-40126 Bologna.

3 M. Aloisi, Atti Soc. it. Pat. *11*, 281 (1951).
4 A. Margreth, G. Salviati and S. Salvatori, in: Membranous Elements and Movement of Molecules, p. 25. Ed. E. Reid Ellis Horwood Ltd, Chichester 1977.
5 G. Meissner, G.E. Conner and S. Fleischer, Biochim. biophys. Acta *298*, 246 (1973).
6 G. Rouser and S. Fleischer, Meth. Enzymol. *10*, 385 (1967).
7 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. biol. Chem. *193*, 265 (1951).
8 E. Bonetti and F. Novello, Int. J. Vit. Nutr. Res. *46*, 244 (1976).
9 O.V. Rajaram, P. Fatterpaker and A. Sreenivasan, Br. J. Nutr. *37*, 157 (1977).
10 T.R. Hesketh, G.N. Smith, M.A. Housley, K.A. McGill, N.J.M. Birsdall, J.M. Metcalfe and G.B. Warren, Biochemistry *15*, 4145 (1976).
11 G. Salviati, R. Betto, S. Salvatori and A. Margreth, Biochim. biophys. Acta *579*, 280 (1979).
12 J.A. Lucy, Ann. N.Y. Acad. Sci. *103*, 4 (1972).

Quantification of digoxin by enzyme immunoassay: synthesis of a maleimide derivative of digoxigenin succinate for enzyme coupling

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Summary. We synthesized the m-maleimidobenzoyl derivative of digoxigenin-3-0-succinate (through a p-phenylenediamine bridge) as a hapten derivative directed towards coupling to sulfhydryl groups of β-galactosidase. Prepared enzyme conjugate had about 97% of the enzyme labeled with the hapten derivative while retaining full enzyme activity. The enzyme immunoassay for digoxin we prepared showed a maximum sensitivity of 30 pg per assay (c.v. = 3%) with minimal cross-reaction with digotoxin (3.8%). Our method for hapten conjugation to β-galactosidase is highly efficient and is simple and easily replicated.

Digoxin is used in the treatment of chronic heart diseases. Due to the relatively narrow limits between therapeutic and toxic effects, the accurate determination of plasma or serum digoxin levels is of high importance. The incidence of intoxication is greatly reduced by digoxin blood level monitoring during therapy. The determination of digoxin can be carried out by radioimmunoassay[2-6] and enzyme immunoassay[7]. Employing a highly efficient coupling of a m-maleimidobenzoyl derivative of digoxigenin succinate to the enzyme, we were able to develop an enzyme immunoassay (EIA) for the measurement of digoxin by using β-galactosidase as an enzyme label. We describe here the synthesis of our novel maleimidobendzoyl derivative of digoxigenin succinate and the preparation of an enzyme conjugate for the development of a digoxin EIA.

Materials and methods. Chemicals. Digoxigenin, digoxin and digotoxin were obtained from Sigma Chemical Co., St. Louis, MI 63178. Maleic anhydride, m-aminobenzoic acid and p-phenylenediamine were obtained from Aldrich Chemical Co., St. Louis, MI 63178.

Enzyme. Beta-galactosidase from *E. coli* was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Indiana 46250.

Preparation of digoxigenin-3-0-succinate. Digoxigenin-3-0-succinate was synthesized following the procedure of Oliver et al.[8]. Both 0.7 g of succinic anhydride and 2.0 g of digoxigenin were dissolved in 12 ml of pyridine. The solution was protected from light and allowed to react at room temperature for 3 months. The solution was then poured into 75 ml of cold 2 N H_2SO_4. The solid product was isolated by filtration and washed with cold water. It was then redissolved in 150 ml of chloroform-methanol (2:1). The chloroform-methanol solution was washed once with 25 ml of 1 N H_2SO_4, and 3 times with water. 25 ml of methanol was added after each washing. The organic phase was dried over anhydrous sodium sulfate and taken to dryness on a rotary evaporator. The residue was dissolved in 15 ml of hot ethanol, and hot water was added to turbidity. The solution was allowed to cool to room temperature and then left at 4 °C for 48 h. The resultant crystals were isolated by filtration and washed 3 times with cold ethanol-water (3:2). The final product was a white powder with melting point of 190–197 °C. Esterification at position 3 was confirmed by its immunoreactivity to digoxin antiserum as reported in the results and discussion section.

Production of anti-digoxin antibody. Digoxin-bovine serum albumin (BSA) conjugate was prepared according to the procedure of Smith et al.[11]. 1 mg of the immunogen was dissolved in 0.5 ml of saline and emulsified in an equal volume of complete Freund's adjuvant. Goats were then immunized by i.m. injections 3 times at 2-week intervals and boosted bimonthly thereafter. For booster injection, incomplete adjuvant was used in the preparation of emulsion. The animals were bled 10 days after the 3rd primary injection and after each booster injection.

Preparation of enzyme-hapten conjugate. Conjugation of DSA-MB to β-galactosidase was done by the procedure similar to that described previously[9]. A solution of DSA-MB 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 nmoles). The mixture was incubated for 2 h at room temperature. Following overnight dialysis in the same 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 eluate containing the peak of enzyme activity were used for the digoxin assay. Beta-galactosidase activity was assayed by the method of Dray et al.[12] using O-nitrophenyl β-D-galactopyranoside as substrate.

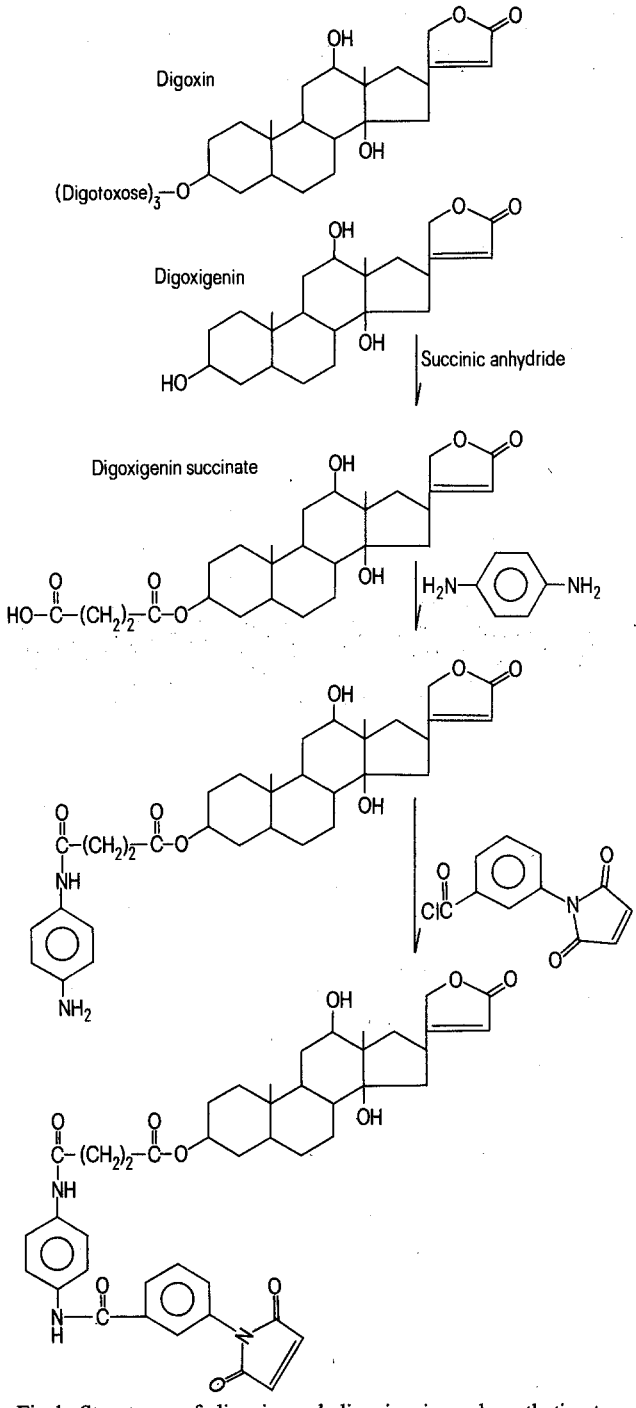

Fig. 1. Structures of digoxin and digoxigenin and synthetic steps leading to the final product, the m-maleimidobenzoyl derivative of digoxigenin succinate conjugated through p-phenylenediamine linkage. Synthesis of p-(digoxigenin-3-0-succinamido)aniline: digoxigenin-3-0-succinate (50 mg) was dissolved in 2 ml of tetrahydrofuran (THF) and 25 ml of tributylamine was added. The solution was then cooled to -10 °C and 25 ml of isobutylchloroformate was added and incubated at -10 °C for 1 h. The precipitate was filtered off at -5 °C and the supernatant was added dropwise to the cooled (-5 °C), stirred solution of THF (2 ml), containing 200 mg of p-phenylenediamine. The solution was incubated further for another 2 h at room temperature. Synthesis of the product ($R_f = 0.3$) was monitored by using ethyl acetate as solvent. A spot of the product gave a positive ninhydrin reaction and fluoresced under UV-light. Product isolation was carried out by column chromatography using ethyl acetate as eluting solvent. Melting points 172–178 °C. Synthesis of m-maleimidobenzoyl derivate of p-(digoxigenin-3-0-succinamido)aniline (DSA-MB): p-(digoxigenin-3-0-succinamido)aniline (50 mg) was dissolved in THF (5 ml) and 50 mg of Na_2CO_3 was added. m-Maleimidobenzoic acid (30 mg) prepared by the procedure described previously[9] was dissolved in 2 ml of THF and added dropwise to the solution of p-(digoxigenin-3-0-succinamido)aniline. The solution was refluxed for 30 min and monitored on TLC ($R_f = 0.34$) using the same solvent system. The product showed a negative ninhydrin reaction and fluoresced under UV-light. The product isolation was carried out by silica gel column chromatography using chloroformethyl acetate (3:7) as solvent system. Meltin points 250–256 °C. The presence of maleimide group in the product was confirmed by its ability to react with cysteine using the method of Grassetti and Murray[10].

Results and discussion. When the maleimide derivative of digoxigenin-3-0-succinate was conjugated to β-galactosidase, about 97% of the enzyme was labeled with the hapten derivative. The number of hapten derivatives per enzyme molecule was not directly examined. However, since no more sulfhydryl group, as examined by the method of Grassetti and Murray, was found n β-galactosidase after the hapten-enzyme conjugation reaction, about 10 hapten derivatives are thought to be conjugated per molecule of β-galactosidase because 1 molecule of β-galactosidase is reported to contain about 10 sulfhydryl groups[13]. No reduction in enzyme activity was observed after the conjugation reaction. The results obtained here were consistent with those obtained previously for other haptens[9,14]. Antiserum was diluted 5000-fold and 0.1 ml of that was used for the routine assay. Displacement with the unlabeled digoxin in EIA showed a maximum assay sensitivity of 0.6 ng/ml with a coefficient of variation of about 3%. The solid phase -

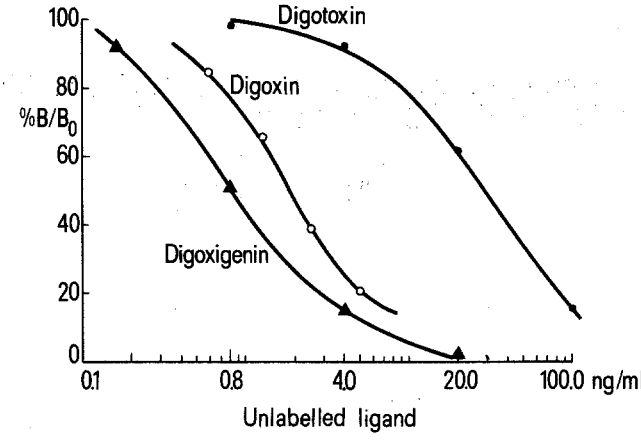

Fig. 2. Inhibition curves of digoxin, digoxigenin and digotoxin with goat anti-digoxin antiserum. Digoxin standard: a stock solution of 1 mg/ml of digoxin dissolved in dimethyl sulfoxide was serially diluted with phosphate buffer-BSA (pH 7.3, 0.05 M, 0.1% BSA) and 10-ml vol. of the standard solutions (0–5 ng/ml) were stored at −20 °C. EIA procedure: a volume of 50 µl of a standard or plasma sample was added to a glass tube (12×75 mm) containing an appropriate dilution of enzyme conjugate in 0.4 ml phosphate buffer-BSA. After mixing, 100 µl of goat anti-digoxin antiserum was added and incubated overnight. Solid phase - rabbit anti-goat immunoglobulin antibody (0.4 ml, Immunobeads from BIO-RAD Laboratories, Richmond, CA 94804) was then added and, after mixing, incubated for 2 h. After incubation, the solid phase was precipitated by centrifugation at 2000×g for 10 min. The precipitated solid phase was washed 3 times with 1 ml phosphate buffer-BSA. The enzyme activity in the precipitate was assayed according to the procedure described previously. Incubation time for enzyme activity was 120 min. Amount of o-nitrophenol produced at the end of incubation was measured by a Gilford Stsar III spectrophotometer at 420 nm wavelength.

second antibody precipitation method we used thus gave reliable and reproducible results. Our results on cross-reactivity studies (figure 2) showed that cross-reactions of 142% and 3.8% were obtained, respectively, for digoxigenin and digotoxin. These also suggested that the EIA is specific for digoxin and the digoxigenin was succinylated at 3 positions during the succinylation reaction.

Maleimidobenzoyl derivatives have been used as cross-linking agents for protein-enzyme conjugation[14-16]. More recently, we reported m-maleimidobenzoyl derivatives of haptens as convenient derivatives for the preparation of enzyme-hapten conjugates[9,17].

In conventional enzyme-hapten conjugations, haptens were conjugated primarily to γ-amino groups of lysine residues of the β-galactosidase molecule, resulting in a substantial reduction of both enzyme activity and water solubility[12,18,19]. In digoxin-enzyme conjugation procedures, a γ-amino group of a lysine residue in an enzyme has been covalently linked to a terminal sugar moiety of digoxin through a diazo linkage. This procedure is rather tedious and difficult to replicate[11,20].

Our method of hapten conjugation to β-galactosidase is highly efficient and simple, and is easily replicated because sulfhydryl groups of the enzyme are involved in the hapten conjugation reaction. The present report also demonstrates that digoxigenin can be employed successfully for enzyme-hapten conjugate preparation for the development of EIA in digoxin quantification. The clinical application of this method is currently under study and will be reported elsewhere.

1 Author for reprints requests.
2 N.P. Kubasik, B.B. Brody and S.S. Barild, Am. J. Card. *36*, 975 (1975).
3 J.J.W. Zeegers, A.H.J. Maas, A.F. Willebrands, H.H. Kruyswijk and G. Jambroes, Clin. chim. Acta *44*, 109 (1973).
4 L.S. Berk, J.L. Lewis and J.C. Nelson, Clin. Chem. *20*, 1159 (1974).
5 W.F. Line, S.J. Siegel, A. Kwang, C. Frank and R. Ernst, Clin. Chem. *19*, 1361 (1973).
6 Z.J. Kuczala and G.S. Ahluwalia, Clin. Chem. *22*, 193 (1976).
7 R.H. Drost, T.A. Plomp, A.J. Teunissen, A.H.J. Maas and R.A.A. Maes, Clin. chim. Acta *79*, 557 (1977).
8 G.C. Oliver, Jr, B.M. Parker, D.L. Brasfield and C.W. Parker, J. clin. Invest. *47*, 1035 (1968).
9 N. Monji, H. Malkus and A. Castro, Biophys. biochem. Res. Commun. *85*, 671 (1978).
10 D.R. Grassetti and J.F. Murray, Jr, Archs Biochem. Biophys. *119*, 41 (1967).
11 T.W. Smith, V.P. Butler, Jr, and E. Haber, Biochemistry *9*, 331 (1970).
12 F. Dray, J.-E. Andrieu and F. Renzud, Biochim. biophys. Acta *403*, 131 (1975).
13 K. Wallenfels and R. Weil, in: The Enzymes, 3rd edn, vol.7, p.617. Boyer, 1972.
14 T. Kitagawa and T. Aikawa, J. Biochem. (Tokyo) *76*, 233 (1976).
15 K. Kato, H. Fukui, Y. Hamaguchi and E. Ishikawa, Eur. J. Biochem. *62*, 285 (1976).
16 K. Kato, H. Fukui, Y. Hamaguchi and E. Ishikawa, J. Immun. *116*, 1554 (1976).
17 N. Monji and A. Castro, Experientia *36*, 347 (1980).
18 S. Comoglio and F. Celada, J. immun. Meth. *10*, 161 (1976).
19 T. Ogihara, K. Miyai, K. Nishi, K. Ishibashi and Y. Kumahara, J. clin. Endocr. Metab. *44*, 91 (1977).
20 R.C. Lauer and B.F. Erlanger, Immunochemistry *11*, 533 (1974).

Characteristic changes of cerebellar proteins associated with cerebellar hypoplasia in jaundiced Gunn rats and the prevention of these by phototherapy

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Summary. In the cerebellar particulate fractions from Gunn rat homozygotes 3 protein bands with apparent mol.wts of 250,000, 50,000 and 33,000 in SDS-polyacrylamide gel disc electrophoresis underwent major changes, and phototherapy of the newborns could effectively prevent the changes.

Hyperbilirubinemic homozygous Gunn rats[2] have been known to show a marked cerebellar hypoplasia[3-6]. In view of the inhibitory effect of bilirubin on the protein synthesizing machinery in Gunn rat cerebellum[7,8], the first attempts were made to characterize cerebellar protein patterns and to investigate the effect of phototherapy in Gunn rat homozygotes (jj), using SDS-polyacrylamide gel disc electrophoresis (SDS-PAGE) and comparing the patterns with those from control non-jaundiced heterozygotes (j+).

Materials and methods. Newborn jj and j+ rats from the same dam were allotted to irradiated and non-irradiated groups. The irradiated group was isolated from the dam and subjected to blue light irradiation (phototherapy) for 5 h/day (09.00-14.00 h) from the 4th-7th day of life at an energy level of about 2.4×10^4 erg/cm^2/sec near the animals.[6]. The non-irradiated group was treated in the same way without irradiation. The rats were sacrificed on the 31st day of life, and the cerebellar homogenates were prepared at 4 °C with 9 vol. of 0.32 M sucrose-10 mM phosphate buffer, pH 7.0. The subcellular fractions obtained by differential centrifugation at 4 °C were termed P_1 (1000 × g for 10 min; twice washed), P_2 (12,000 × g for 20 min; twice washed), P_3 (105,000 × g for 60 min) and S (final supernatant). SDS-PAGE in a linear gradient of 5-15% polyacrylamide was performed according to Ames[9], except that the gels were polymerized at 30 °C with 0.0125% ammonium persulfate in final concentration, and the electrophoresis was carried out at 4 °C at 1 mA/gel tube. Protein was determined by the method of Lowry et al.[10] with bovine serum albumin as a standard. The mol.wt estimation was made using the following protein subunits as internal and external standards: thyroglobulin (330,000) and ferritin (220,000 for half of the native protein and 18,500) from Pharmacia, RNA polymerase (165,000, 155,000 and 39,000)