

**PREPARATION AND CHARACTERIZATION OF LIGAND-MODIFIED
LABELLED LIPOSOMES FOR SOLID PHASE IMMUNOASSAYS**

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ABSTRACT

Small unilamellar vesicles conjugated with an enzyme label and with specific ligands for biological molecules may prove to be useful as signal enhancement vehicles in the development of enzyme-linked immunoadsorbent assays and other detection applications. Bifunctional vesicles have been prepared by covalently attaching horseradish peroxidase (HRP) and monoclonal antibodies to the outside of the lipid bilayer. The reaction conditions were optimized to obtain 7-12 antibody molecules and 100-200 HRP molecules per vesicle. The enzyme retained 70-80% of its specific activity after immobilization with no apparent change in vesicle stability. These bifunctional vesicles were used in a non-competitive immunoassay for D-Dimer, a fibrin dimer formed at the early stages of thrombogenesis. The assay results using vesicles led to a detection limit for D-Dimer in human plasma which was five times lower than what was achieved using a conventional enzyme-antibody conjugate assay. HRP labelled (bifunctional) liposomes can also be used in competitive assays for the detection of small ligands in bulk solution. HRP and biotin-conjugated vesicles were prepared and used in competitive assays for biotin in free solution. The lowest detection limit for biotin using vesicles as the signal generation mechanism was found to be a factor of 10 lower than what could be observed with a traditional biotin-HRP conjugate. A model has been developed for the competition between a small ligand in solution and a large ligand-conjugated vesicle for binding sites on a solid surface.

INTRODUCTION

Most previous applications of liposomes to immunodiagnostics have been based on homogeneous assays which are generally turbidimetric or lytic (1-3). There are only a few examples where liposomes have been used in solid phase or heterogeneous immunoassays (4-8). All of the latter have utilized encapsulated fluorescent groups which are released by the addition of surfactants after adsorption of the vesicles to specific antibody sites on the solid surface. Because of the large number of encapsulated fluorors, signal enhancements of an order of ten have been reported when liposome-based competitive assays for small ligands (digoxin, theophylline) are compared to assays done with single fluorescent-labelled ligand. It is also desirable to develop increased sensitivity and detection limits for enzyme-linked immunoadsorbent assays (ELISAs¹). In this paper, we report on the use of liposomes whose surfaces have been modified by the attachment of horseradish peroxidase (HRP) and either small ligands (biotin) or large ligands (D-Dimer monoclonal antibodies) in both competitive and non-competitive (sandwich) ELISAs. The competitive assay configuration is illustrated schematically in Figure 1, where a liposome with a large number of enzyme labels and a small ligand covalently attached to the outer membrane is competing for antibody sites on the surface with a small ligand (analyte) in free solution. An earlier paper (9) described the preparation and characterization of biotinilated vesicles and how they adsorb and interact with antibody-coated surfaces as a model of competitive assays for small ligands. In this manuscript, it is demonstrated that the large number of enzyme molecules on the surface of the liposomes can result in higher signals and lower analyte detection limits in both competitive and non-competitive assays.

MATERIALS AND METHODS

Competitive Assay for Biotin

Horseradish peroxidase (HRP) type VI-A, biotin-conjugated horseradish peroxidase (B-HRP), and goat affinity-purified polyclonal anti-biotin antibody (ABA) were obtained from Sigma. Biotinilated dimyristoyl phosphatidyl ethanolamine (DMPE-LC-Biotin) was synthesized from biotinyl-imidohexanoyl-N-hydroxysuccinimide ester, or long-chain biotin, obtained from Pierce, using the method of Bayer and Wilcheck (10). Biotinilated small unilamellar vesicles

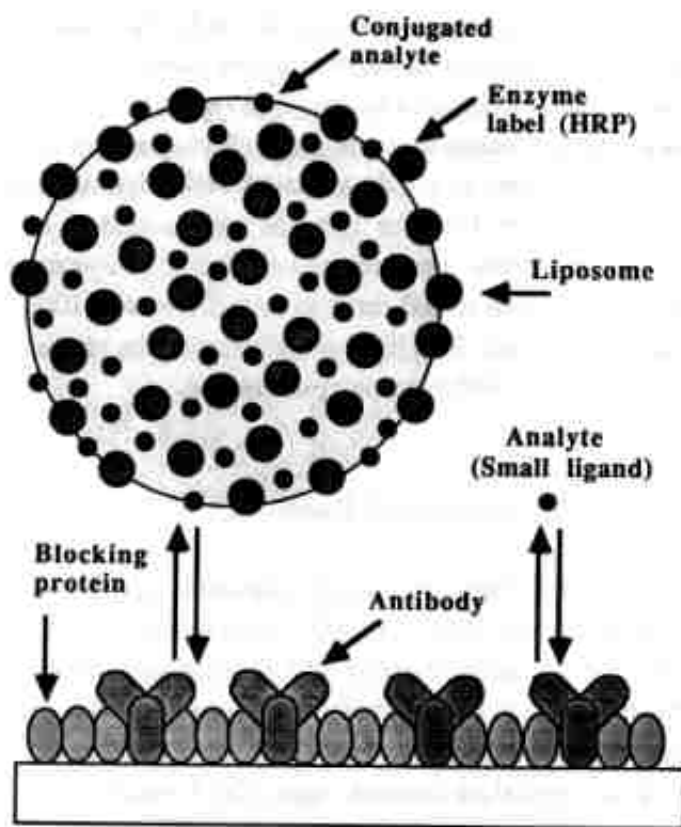


FIG 1: Representation of the use of liposomes conjugated with a large number of small ligands and enzyme molecules in a liposome-mediated solid phase competitive immunoassay.

were prepared with molar ratios of dimyristoyl phosphatidyl choline (DSPC), cholesterol, DMPE and DMPE-LC-Biotin of (42.4:37.5:20:0.1 mole%) using standard sonication procedures (11). HRP was covalently linked to the liposomes using the periodate oxidation method (12) with some modifications (9). The HRP-conjugated biotinylated vesicles are designated as HBVs. Enzyme activity measurements were carried out using 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and H_2O_2 as substrates as described by Gallati (13). Phospholipid concentrations were determined by a phosphomolybdate assay (14), and vesicle diameters by quasi-elastic light

scattering (15). Competitive assays were carried out by first adsorbing ABA to polystyrene 96-well flat bottom microtiter plates from Costar, using ABA concentrations in phosphate buffered saline (PBS) from 0.5 to 10 $\mu\text{g/ml}$. The plates were blocked using technical grade 0.5 wt% casein (Sigma) in PBS for three hours at room temperature. Aliquots of HBVs containing biotin at concentrations ranging from 1 pM to 100 μM were added to the wells and incubated at 37 C for two hours. For comparison, similar assays were done with B-HRP. The wells were then rinsed three to four times, and 100 μl of ABTS substrate solution were added. The light absorbance of the product at 410 nm was measured in a Biotek EL 340 microtiter plate reader.

Non-competitive (Sandwich) Assay for D-Dimer

D-Dimer and anti-D-Dimer monoclonal antibodies 5-4-C and 8-8-G were provided by Organon Teknika, Treyburn, NC. Small unilamellar liposomes were prepared by sonication as described above with DSPC:Cholesterol:DMPE ratios of (40:40:20 mole%). HRP and antibody-conjugated vesicles (HAVs) were prepared by the simultaneous coupling of anti-D-Dimer monoclonal antibody 5-4-C and HRP using the periodate method, with 10-13 mg/ml of HRP and 1-3 mg/ml of antibody in a liposome solution containing approximately 1 mg/ml of phospholipid in a 50 mM citrate buffer at pH 6.0. Vesicles were separated from the unreacted protein using gel permeation chromatography on a Sepharose CL-6B column. Enzyme activities, liposome diameters, and phospholipid concentrations were measured as described above. The amount of antibody bound to the vesicles was determined by radiolabelling the anti-D-Dimer 5-4-C antibody with ^{14}C -formaldehyde prior to attachment to the liposome surface, and measuring the resulting counts of the HAVs using a scintillation counter. Microtiter plates were coated with anti-D-Dimer antibody 8-8-G using approximately 40 $\mu\text{g/ml}$ of antibody in 50 mM carbonate-bicarbonate buffer, pH 9.6 for 21 hours at 4 C with shaking. The wells were blocked with 1 wt% BSA for one hour at room temperature. Antigen samples were diluted in 10 mM carbonate/bicarbonate buffer (pH 9.6), applied to the wells, and incubated at 37 C for one hour. After washing three times with PBS, HAVs were introduced into the wells and incubated for one hour at 37 C. The wells were washed six times with PBS to remove unbound and non-specifically bound liposomes. 100 $\mu\text{l/well}$ of substrate (3,3',5,5' tetramethyl-benzidine-dihydrochloride (TMB), urea

hydrogen peroxide) solution was added to the plate and incubated at room temperature for 30 minutes. The reaction was stopped with 2N sulfuric acid and the light absorbance at 450 nm was read in a Biorek EL 340 microtiter plate reader. The results of the liposome-mediated assay were compared to the results obtained with an HRP-anti-D-Dimer antibody (5-4-C) conjugate prepared in our laboratory.

RESULTS AND DISCUSSION

Competitive Assay for Biotin

The biotinylated HRP-conjugated vesicles (HBVs) were found to have an average diameter of approximately 1480 Å, and approximately 550 HRP molecules/vesicle. Based on the amount of DMPE-LC-Biotin inserted into the liposomes, it is estimated that approximately 130 biotin molecules/liposome were exposed on the outer surface. The HRP activity was approximately 65% to 84% of the activity of the enzyme in bulk solution prior to immobilization over the pH range of 3.5 to 5.0. The blocking of the polystyrene plates with 1 wt% casein was an important step in reducing non-specific binding of liposomes to the surface. The contribution of non-specific binding to the overall signal during the assays was only 1.9%.

Typical competitive assay results are found in Figure 2 for the case of plates adsorbed with low and high concentration of ABA. Figure 2a shows the ratio of specific signal to the specific signal at zero biotin concentration as a function of biotin concentration for plates where the surface concentration of ABA was 58 ng/cm². As can be seen, under these conditions, the free biotin can compete effectively with the liposomes for surface antibody binding sites. Figure 2b shows the results of a similar assay carried out with plates with high antibody loading (250 ng/cm²). In this case, the vesicles are not easily displaced from the surface, even at extremely high free biotin concentrations. The reason for this is the high binding probabilities that result when a liposome with a large number of ligands (biotin) on the membrane is in close proximity to a surface where there are a large number of binding sites (ABA). When the antibodies are at low density and the binding sites are far apart on the surface, only one or two biotin molecules on the liposome are able to interact with the antibody. When the antibodies are at

high concentration on the surface, the liposomes are able to interact through many biotin molecules with the surface, making the competition with biotin much more difficult.

The solid lines passing through the data in Figure 2 are the results of a model derived by Jones (16) for the competition between small ligands (biotin) and large ligands (liposomes) for surface binding sites. In this model, the fraction of the surface covered by the large ligand is given by the equation

$$\theta = \frac{K^* P(\theta) [L^*]}{(1 + K(1 - \theta) [L])^m} \quad (1)$$

where K^* is the association constant for the large ligand (liposome), K is the association constant for the small ligand (biotin), and $P(\theta)$ is the probability that a large ligand will bind to the surface when the fraction of the surface coverage is θ . Measured values for the association constants for the small and large ligands have been reported previously (9). The quantities $[L]$ and $[L^*]$ represent the small and large ligand concentrations respectively. As the fraction of the surface coverage increases, the binding probability decreases significantly, a phenomenon which has been termed the 'large ligand effect'. An approximate formula that can be used to estimate the effect of geometrical effects on the binding probability has been derived by Schaaf and Talbot (17),

$$P(\theta) = \exp\left(2 - \frac{2}{(1 - \theta)^2} + \frac{7\theta}{8(1 - \theta)^2} + \frac{7}{8} \ln(1 - \theta)\right) \quad (2)$$

The power m in the denominator of equation (1) is a number that is bounded between 0 and 1, and it controls the increased probability of binding when the area of the liposome is sufficiently large to cover several ligand binding sites on the surface. When the antibodies on the surface are so far apart that only one antibody binds to one vesicle, then $m \sim 1$. When several antibodies can bind to a single liposome, then $m < 1$. In Figure 2, it is evident that m takes on smaller values with increasing antibody loading on the plate.

One of the more important measures that can be used to quantify the sensitivity of an assay is the least detectable dose (LDD) of the analyte in solution.

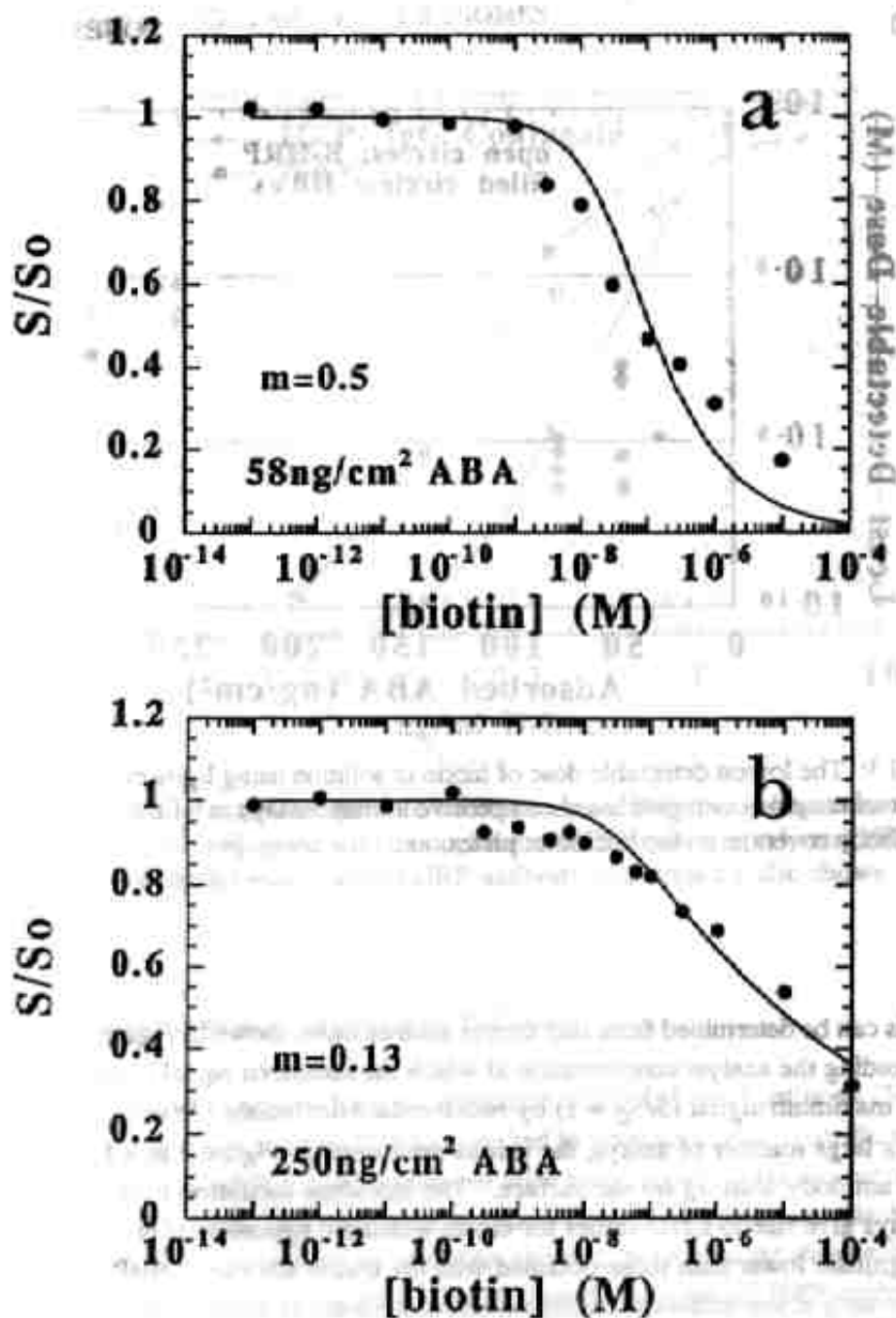


FIG 2: Results of a competitive immunoassay for biotin using HRP and biotin-conjugated liposomes. (a) Results obtained at low surface coverage of antibody on the plate (58 ng/cm^2) and a liposome concentration $[L^*] = 5.65 \times 10^{-11} \text{ M}$ (b) Results obtained at high surface coverage of antibody (250 ng/cm^2) and a liposome concentration $[L^*] = 2.05 \times 10^{-11} \text{ M}$. The solid lines are the results predicted by equation (1) with $K = 3.71 \times 10^7 \text{ M}^{-1}$ and $K^* = 1 \times 10^9 \text{ M}^{-1}$.

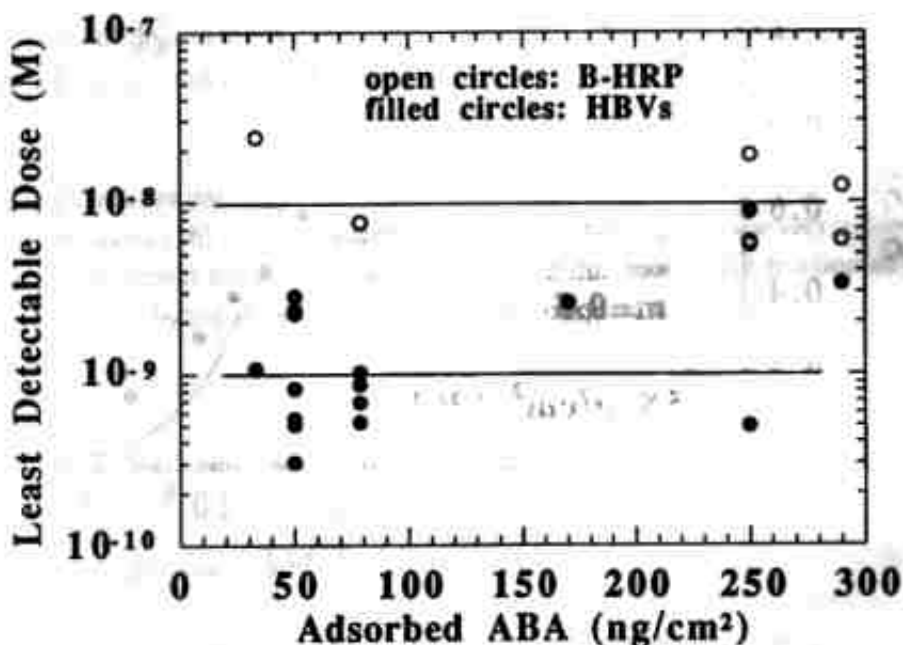


FIG 3: The lowest detectable dose of biotin in solution using liposome-based and normal enzyme-conjugate based competitive immunoassays as a function of antibody coverage on the microtiter plates.

This can be determined from titer curves such as those shown in Figure 2, by recording the analyte concentration at which the measured signal is smaller than the maximum signal ($S/S_0 = 1$) by two standard deviations. When this is done for a large number of assays, the results are shown in Figure 3 as a function of the antibody loading on the surface. The liposome-mediated immunosorbent assays give rise to LDD values for biotin which are approximately an order of magnitude lower than those obtained with the traditional biotin-HRP conjugate, especially at low antibody loadings where there is easier competition between the small ligand in solution and the liposomes. These results clearly demonstrate that enzyme-labelled targeted liposomes can be used in the design of efficient competitive immunoassays.

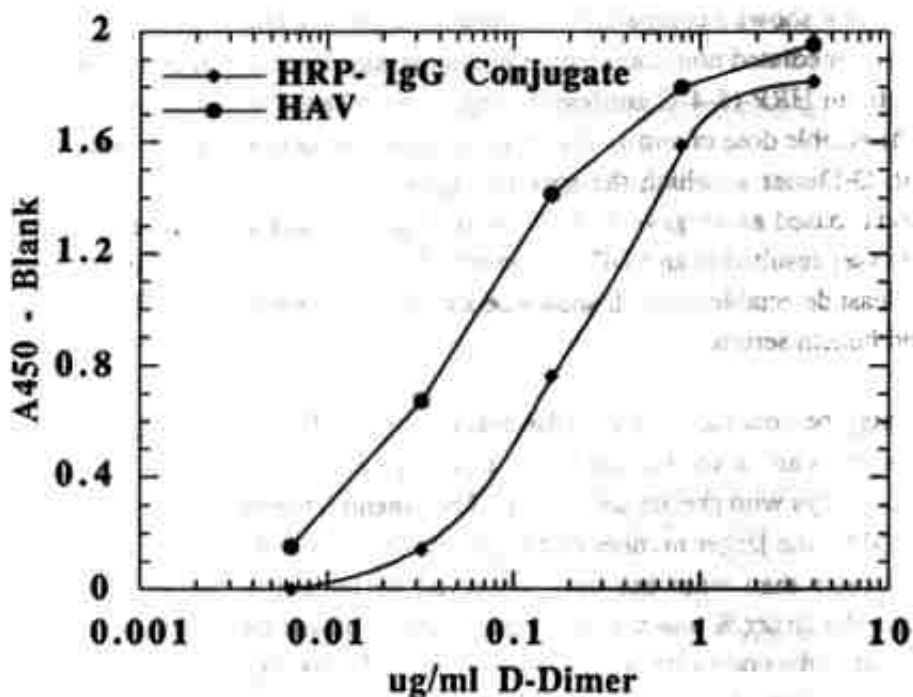


FIG 4: Results of a non-competitive immunosay for D-Dimer in human serum using liposomes conjugated with monoclonal anti-D-Dimer antibody and HRP. The results obtained with a normal HRP-antibody conjugate are also shown.

Non-competitive (Sandwich) Assay for D-Dimer

The protocol described for the preparation of the D-Dimer antibody (5-4-C) and HRP-conjugated vesicles (HAVs) resulted in liposomes with mean diameters of approximately 1000 Å. The number of HRP molecules/vesicles was estimated to be about 103 when 12-13 monoclonal anti-D-Dimer antibodies were attached to the surface (5-4-C). Control experiments showed that the larger the number of antibodies on the vesicle surface, the smaller the number of HRP molecules which could be attached. Blocking of the plates was carried out using a 1 wt% BSA solution, which was found superior to casein or milk protein. It was also found that the larger the antibody coating on the plates (anti-D-Dimer 8-8-G antibody), the larger the resulting signal. As a result, all of the assays were performed on plates exposed to a 40 µg/ml solution of anti-D-Dimer during the preparation.

Figure 4 shows a comparison between the signals obtained with the liposome-mediated non-competitive immunoassay, and an immunoassay carried out with an HRP-(5-4-C antibody) conjugate prepared in our laboratory. The least detectable dose of anti-D-Dimer in serum can be defined as the concentration of anti-D-Dimer at which the specific signal is 0.1. Using this criterion, the liposome-based assay gave an LDD of 0.6 ng/ml, while the normal conjugate-based assay resulted in an LDD of 3 ng/ml. This represents a 5-fold improvement in the least detectable dose. It should be stressed that this assay was carried out in diluted human serum.

It may be concluded from these results that HRP and antibody-conjugated liposomes can also be used to develop non-competitive (sandwich) immunoassays with greater sensitivity. The potential improvement in sensitivity provided by the larger number of enzyme molecules on the liposome surface is even greater than what has been observed so far experimentally, primarily because of a larger % non-specific signal in the liposome-based assays (10-25%), compared to the conjugate-based assays (1-5%). By modifying the surface of the vesicles and the plates to reduce non-specific binding, it may be possible to improve considerably the liposome-based solid-phase immunoassay performance.

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FOOTNOTES

- 1 ABA: Anti-biotin antibody
ABTS: 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid)
B-HRP: Biotin-HRP conjugate
Chol: Cholesterol
DMPE: Dimyristoyl phosphatidyl ethanolamine
DMPE-LC-Biotin: Biotinilated dimyristoyl phosphatidyl ethanolamine

- DSPC: Distearoyl phosphatidyl choline
ELISA: Enzyme-linked immunoadsorbent assay
HAV: HRP and anti-D-Dimer antibody (5-4-C)-conjugated vesicles
HRP: Horseradish peroxidase
HRP-IgG Conjugate: HRP and anti-D-Dimer antibody (5-4-C) conjugate.
LDD: Least detectable dose
PBS: Phosphate-buffered saline
TMB: 3,3',5,5' tetramethyl-benzidine-dihydrochloride

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