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[54] **OPIATE IMIDATES AND PROTEIN CONJUGATES THEREOF**

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Related U.S. Application Data

[63] Continuation-in-part of Ser. Nos. 304,157, Nov. 6, 1972, , and Ser. No. 143,609, May 14, 1971, abandoned.

[52] **U.S. Cl.** ..... **260/121,** 23/230 B, 260/112 B, 260/112 R, 260/285, 424/1, 424/12, 424/85, 424/88, 195/63, 195/68

[51] **Int. Cl.** **C07g 7/00,** A61k 23/00

[58] **Field of Search** ..... 260/285, 112 R, 121, 112 B

[56] **References Cited**

UNITED STATES PATENTS

3,690,834 9/1972 Goldstein et al. ..... 260/285 X

Primary Examiner—Howard E. Schain

[57] **ABSTRACT**

Novel water soluble opiate poly(amino acid) conjugates are provided, wherein the opiate is bound to the poly(amino acid) by an imidoyl linkage. The conjugates find use in the formation of antibodies or as reagents in immunoassays.

5 Claims, No Drawings

OPIATE IMIDATES AND PROTEIN CONJUGATES THEREOF

CROSS REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of Application Ser. No. 304,157, filed Nov. 6, 1972, and application Ser. No. 143,609, filed May 14, 1971 now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

Because of the wide use and abuse of heroin and other opiate alkaloids, there have been continuing efforts to develop methods which can actively detect heroin and its metabolites in various physiological fluids, e.g. urine, in extremely minute quantities. In order to have accurate detection, it is necessary that compounds having similar structure be distinguished in an accurate manner.

Radioimmunoassay is one of a number of immunoassays which has been reported to be useful in the detection of opiate alkaloids. See U.S. Pat. No. 3,709,868. Other immunoassays, which have also been reported as being effective in detecting heroin and its metabolites, are an assay sold under the trademark FRAT by Syva Corporation, based on a stable free radical detector, and as assay sold under the trademark EMIT by Syva Corporation, based on an enzyme detector.

In order to have a satisfactory immunoassay, it is desirable to be able to produce relatively high concentrations of antibodies in the serum and, the antibodies so produced, should have relatively high affinity for the opiate alkaloids and low affinity for compounds other than the opiate alkaloids. In order to obtain high concentrations of antibodies, it is advantageous to have relatively large amounts of the opiate alkaloid conjugated to the antigenic protein. Furthermore, the link between the opiate alkaloid and the antigenic protein should not adversely affect the recognition by the antibody of the opiate alkaloids, in comparison to other compounds.

2. Description of the Prior Art

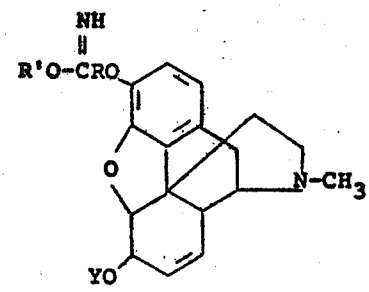
Odell, Competitive Protein Binding, Blackwell, Scientific Publications, Oxford, 1971, discloses a number of haptens conjugated to proteins, see particularly Chapter II beginning at page 25. A wide variety of functionalities are disclosed as useful for conjugation. U.S. Pat. No. 3,709,868 discloses o^3-carboxyalkyl morphine and its conjugate to antigenic proteins. U.S. Pat. No. 3,690,834 discloses an immunoassay technique employing stable free radicals as detector molecules.

SUMMARY OF THE INVENTION

Opiate alkaloid conjugates to poly(amino)—polypeptides and proteins—are provided. An opiate alkaloid is modified at O^3 of the alkaloid with an imidoylalkyl ester group. The imidoyl ester is conjugated to a protein under mild conditions at mild pH's to provide a product which is highly water soluble. The produce finds use either in the formation of antibodies or where the poly(amino acid) is an enzyme, as a detector molecule in an immunoassay.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The imidate ester compounds of this invention have the following formula:



wherein:

Y is hydrogen or acetyl, usually hydrogen;

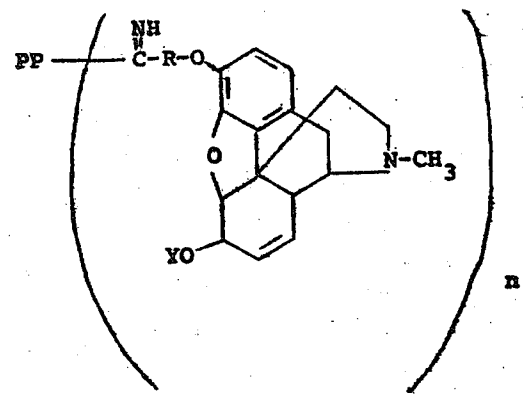
R is alkylene of from 1 to 6 carbon atoms, more usually of from 1 to 4 carbon atoms, and may be branched chain or straight chain, and preferably methylene; and,

R' can be any convenient hydrocarbon group, usually of from 1 to 6 carbon atoms and more usually of from 1 to 3 carbon atoms, the smaller alkyl groups being preferred.

The imidate compounds can be readily prepared by substitution of a halo- or pseudo-haloalkyl cyanide with the O^3-oxygen of morphine or morphine derivative, so as to form the morphinyloxysubstituted alkyl cyanide. The cyanide may then be combined with an alkanol and its alkali metal alkoxide to form the imidate from the cyanide group.

Conjugation of the imidate ester to a poly(amino acid) is carried out in an aqueous medium at mildly basic pH at moderate temperatures. The product may then be isolated by conventional means. Where the poly(amino acid) is an antigen, the product is lyophylized to isolate the desired conjugate. Where the poly(amino acid) is an enzyme, the product will normally be dialyzed.

The conjugate of the opiate alkaloid to the poly(amino acid) will have the following formula:



wherein:

R and Y have previously been defined;

PP is a poly(amino acid), which includes polypeptides and proteins; and,

n is the number of opiate alkaloid groups bonded on the average to the poly(amino acid), PP. *n* will normally be at least 1 and generally range from 10 to 200 for the usual antigenic poly(amino acid). When the poly(amino acid) is an enzyme, *n* will usually be 1 to 25, more usually 2 to 20 on the average.

Various poly(amino acids) find use. The poly(amino acids) which include polypeptides and proteins, are

conjugated with one or more opiate groups. One group of poly(amino acids) is antigenic, so that by bonding the opiate alkaloid to the poly(amino acid), antibodies can be formed to opiate alkaloids and their metabolites. A narrower class of poly(amino acids) which also can be used an antigens but will not normally be used as such, are enzymes which are employed as the detector in an immunoassay system.

Polypeptides usually encompass from about 2 to 100 amino acid units (usually less than about 12,000 molecular weight). Larger polypeptides are arbitrarily called proteins. Proteins are usually composed of from 1 to 20 polypeptide chains, called subunits, which are associated by covalent or non-covalent bonds. Subunits are normally of from 100 to 300 amino acid groups (approximately 10,000 to 35,000 molecular weight). For the purposes of this invention, poly(amino acids) is intended to include individual polypeptide units, or polypeptides which are subunits of proteins. The proteins may be composed solely of polypeptide units or polypeptide units in combination with other functional groups, such as porphyrins, as in hemoglobin or cytochrome oxidase.

The first group of poly(amino acids) which will be considered are the antigenic polypeptides or proteins. These may be joined to the imidate through an amino group. The amidine product can be used for the formation of antibodies to opiate alkaloids or their metabolites. The poly(amino acid) materials which may be used will vary widely in molecular weight, normally being of from about 1,000 to 10 million molecular weight, more usually from 12,000 to 500,000 molecular weight.

With most conventional poly(amino acids), there will not be more than about one opiate alkaloid or derivative group per 1,500 molecular weight of poly(amino acid), usually not more than one group per 2,000 molecular weight. There will be at least one group per 500,000 molecular weight, usually at least one per 50,000 molecular weight. With intermediate molecular weight antigens (50,000 to 1 million) the number of opiate alkaloids or derivative groups will generally be from about 2 to 250, usually from 10 to 100.

With low molecular weight antigens 1,000 to 5,000 molecular weight, the number of opiate alkaloid or derivative groups will be in the range of 1 to 10, usually in the range of 2 to 5, so that there may be as many as one opiate alkaloid or derivative per 500 molecular weight of antigen.

Usually, the number of groups bonded to the poly(amino acid) will be related to the available amino groups, e.g., the number of lysines present. While the opiate alkaloid or derivative may be bonded through the imidoyl group to hydroxyl or mercaptan groups, which are present in the polypeptide, for the most part the bonding will be to amino and, therefore, the compounds are described as amidines.

Amino acids present in poly(amino acids) which have free amino groups for bonding to the imidoyl group include lysine, arginine, histidine, etc.

Various protein types may be employed as the antigenic material. These types include albumin, serum proteins, e.g. globulins, ocular lens proteins, lipoproteins, etc. Illustrative proteins include bovine serum albumin, key-hold limpet hemocyanin, ovalbumin, bovine γ-globulin, etc. Small natural polypeptides which are immunogenic, such as gramicidin may also be employed. Various synthetic poly(amino acids) may also be employed, such as polymers of lysine, glutamic acid, phenylalanine, tyrosine, etc., either by themselves or in combination. Of particular interest is polylysine or a combination of lysine and glutamic acid. Any synthetic poly(amino acid) must contain a sufficient number of active groups, as for example, amino groups provided by lysine.

The second group of poly(amino acids) are the enzymes to which the imidoyl opiate alkaloid may be conjugated. As indicated, the optiate alkaloid derivative modified enzyme is useful for immunoassays. The immunoassay techinque will follow in more detail

Various enzymes may be used such as oxidoreductases, hydrolases, lyases, and the like. These enzymes include esterases, amidases, phosphorylases, carbohydrases, oxidases, reductases and the like. Of particular interest are such enzymes as lysozyme, amylase, dehydrogenases, particularly malate dehydrogenase, lactate dehydrogenase, mannitol-1-phosphate dehydrogenase, and glucose 6-phosphate dehydrogenase, β-glucuronidase, cellulase and phospholipases, particularly phospholipase C. The enzymes will usually have molecular weights in the range of about 1×10^4 to 6×10^5, more usually in the range of about 1.2×10^4 to 3×10^5.

There will usually be at least one opiate alkaloid or derivative group per enzyme molecule, and usually not more than one group per 1,500 molecular weight, usually not more than one group per 2,000 molecular weight. Usually, there will be at least one opiate alkaloid or derivative group per 50,000 molecular weight, and more usually at least one group per 30,000 molecular weight. The modified enzyme will retain on the average at least 10%, more usually at least 30% of the activity of the unmodified enzyme.

Where the opiate alkaloid or derivative is bonded to a poly(amino acid), there need be only one opiate alkaloid or derivative group, but usually there will be at least two groups bonded to the poly(amino acid). With the enzymes, the number of opiate alkaloid or derivative groups will generally be of from 1 to 25, more usually 2 to 20. Usually, there will be at least 2, more usually at least 3, groups per enzyme, when the enzyme is randomly substituted with the opiate alkaloid or derivative groups and preferably not more than 16.

Antibodies

The preparation of antibodies specific for haptenic materials is a well established practice. A thorough description of the procedure may be found in Williams et al., Methods in Immunology and Immunochemistry, Academic Press, New York and London, 1967, page 197 to 385, particularly that portion beginning at 197 and ending at 254.

For preparation of antibodies to haptens, a hapten is conjugated to antigenic material such as a polypeptide or protein, although polysaccharides, particularly containing amino sugars, can also be used.

The particular manner in which the hapten is bonded to the antigenic material, will depend on the functionalities which are available on the haptenic material and the antigenic material, the number of haptenic groups to be conjugated to the antigenic material, and the like. Numerous procedures for the conjugation of a wide variety of haptens have been developed and published.

The antigenic conjugate may be injected in the fluid state: adsorbed to insoluble particles, such as alumina;

or incorporated in matrix materials such as agar, calcium alginate, or Freund's adjuvants ("complete" or "incomplete", depending on whether mycobacteria are incorporated). The adsorbtion to various insoluble colloidal carriers is described in the aforementioned text, the carriers being illustrated by alumina, aluminum phosphate, blood charcoal and the like. Other materials include polyacrylamide gel, bentonite, and protein. As adjuvants, methylated bovine serum albumin and Freund's adjuvant find use. Complete Freund's adjuvant is a water-in-oil emulsion, using emulsion stabilizers such as lanolin, lanolin derivatives e.g. Aquaphor, mannide monooleate and Arlacel A, available from Duke Laboratories, South Newark, Conn. The complete adjuvant is distinguished from the incomplete adjuvant, by having mycobacteria e.g. M.butyricum or M.tuberculosis. the adjuvants are commercially available from Difco Laboratories, Detroit, Mich.

Immunization can be carried out in a variety of ways with a number of different animals. For the most part, for commercial production of antibodies, relatively large animals are employed, such as equine, bovine, porcine, canine, ovine, caprine, rodentia, rabbits and hares. Of particular interest are horses, goats, sheep and cows, that is, the larger domestic animal.

The antigenic material may be injected interperitoneally, intramuscularly, subcutaneously and the like. When employing Freund's adjuvants, usually in combination with saline, the amount of antigen employed will vary depending on the particular antigenic material and the number and period of prior injections. Usually, about 0.1 to 5 mg of antigenic material will be employed per one ml of solution. The total amount of antigenic material and emulsion will depend on the size, nature and weight of the animal employed. The initial injection will normally be at a number of sites, aliquots of the composition being employed.

The first injections of antigen serve to load the animal, and a period of time is allowed to pass before booster injections are introduced, normally from 2 to 5 weeks. Bleeding may occur after each injection, so as to follow the formation of the desired antibody. Depending on the animal, bleedings can be carried out via heart puncture, the carotid artery or external jugular vein. The bleeding will usually be carried out about one week after an injection. The blood may then be combined with a small amount of sodium citrate, the mixture agitated and the erythrocytes settled by standing or centrifugation. The plasma is drawn off and combined with calcium chloride, with clotting resulting. If necessary, thrombin may be added to enhance clotting. After breaking up the clot, the clot is compressed and serum is withdrawn and filtered. Various other procedures are known and can be employed.

The serum can be treated in various ways depending on its subsequent use. The serum may be fractioned, by employing ethanol, neutral salts such as ammonium sulfate or sodium sulfate, or the like. Alternatively, the serum may be chromatographed on various modified cellulose columns, e.g. diethylaminoethylcellulose or carboxymethylcellulose. Or, various physical means may be employed to concentrate the desired antibodies. Usually the product will be dialyzed after dissolution in a buffer, filtered and then isolated.

Numerous preservatives can be employed to stabilize the antibodies and the antibodies will normally be stored at reduced temperatures.

The antibodies are primarily γ-globulin which are found to have a molecular weight of about 150,000. The antibodies will be specific for a particular spatial structure and polar-non-polar distribution. Various structures deviating from an ideal structure will give different binding constants.

EXPERIMENTAL

The following examaples are offered by way of illustration and not by way of limitation.

(All temperatures not so otherwise indicated are in Centigrade)

EXAMPLE A

Preparation of Morphine

Employing an antigen prepared in accordance with Example II, infra, a sheep was injected with 2 cc of a solution with 0.5 cc aliquots at 4 subcutaneous sites and 1 cc intramuscularly in each hind leg. The solution was comprised of 30 mg of the antigen in 1 ml saline, and 3 ml incomplete Freund's adjuvant. Repeated injections were carried out on an approximately monthly basis of a solution containing 30 mg of the antigen, 1 ml saline, and 3 ml incomplete Freund's adjuvant.

The animals were bled about one week after each injection, either to follow the course of antibody formation or to obtain a supply of antibodies. About 1 week after the last injection, the animal was bled, approximately 500 cc of blood being mixed with 10 ml of 25% sodium citrate. The mixture was then centrifuged at 5,000 rpm for 20 minutes. The plasma was aspirated off and mixed with 10 ml of 25% calcium chloride. In order to enhance clotting, 2 NIH units of thrombin per ml of plasma was added and the mixture allowed to stand overnight at about 35°C.

The resulting clot was chopped up and the mixture centrifuged at 5,000 rpm for about 30–45 minutes at 5°C. The serum was then filtered through glass wool and isolated. To the serum was then added dropwise an equal volume of saturated ammonium sulfate in water with constant stirring at 4°C. After allowing the mixture to stand for one hour at that temperature, the mixture was centrifuged at 10,000 rpm for 30 minutes. The supernatant was decanted, and the precipitate (γ-globulin) was resuspended in 0.4M, pH8, borate buffer, containing 1 g/l. of sodium azide and 0.1 g/l. of Thimerosal. Initially, buffer is added at one half the original serum volume and addition is continued until the precipitate is dissolved. The solution is then dialyzed continuously against 4 liters of the same buffer, after which it is filtered through a 22μ millipore filter. The product is then ready for use.

The antibody solution was found to have a binding constant with morphine of 6.1×10^6 after the first booster shot.

The antibodies prepared in response to the antigens of this invention are selective for the indicated opiate, and the conjugate of N-imidoylmethyl opiate to an antigenic protein, e.g. bovine serum albumin.

EXAMPLE I

A. In a 1 l. round-bottomed flask fitted with a reflux condenser, drying tube and magnetic stirrer was placed morphine hydrate (10.0 g, 0.033 mole). This was dissolved in 70 ml DMF (dimethyl formamide, dried over 4A molecular sieves) and 500 ml acetone (dried over sieves) added. Freshly pulverized potassium carbonate

(40 g) was added, followed by chloroacetonitrile (10.0 ml, 0.16 mole). The mixture was refluxed with rapid stirring for 20 hours. The brown reaction mixture was cooled and filtered. The filter cake was washed thoroughly with a 20:1 acetone-DMF mixture and then discarded. The filtrate was concentrated in vacuo and the residual DMF removed at about 1 mm on the rotary evaporator. The residue was dried at about 50° under vacuum to remove residual chloroacetonitrile and DMF, then dissolved in 750 ml methylene chloride and insoluble material removed by filtration. The filtrate was evaporated and the resultant oil cyrstallized from ethyl acetate. After cooling in ice, filtering and washing with cold ethyl acetate, 8.2 g of yellow plates were obtained. Concentration of the mother liquor afforded another 0.2 g. The combined solids were recrystallized from 80 ml acetonitrile to give 7.5 slightly yellow plates, mp 187°–8°. The mother liquor afforded another 0.8 g. Total 8.3 g (78%).

B. To a suspension of O^3-cyanomethyl morphine (1.00 g, 3.1 mmoles) in 43 ml methanol (dried over 3A molecular sieves) was added a freshly prepared solution of sodium methoxide in methanol (7 ml of 0.0435M NaOMe, 0.3 mmole). After stirring in a dry atmosphere for 24 hours, glacial acetic acid (18 μl, 0.3 mmole) was added and the solvent evaporated. The residue was dissolved in methylene chloride and the precipitated inorganic salts removed by filtration.

The filtrate was concentrated at reduced pressure producing a hygroscopic foam which was dried under high vacuum at room temperature for one hour. The desired product, O^3-(imidomethoxy)methyl morphine was obtained in quantitative yield (1.1 g).

EXAMPLE II

A solution of O^3-(imidomethoxy(methyl morphine (54 mg, 0.15 mmole) in 1 ml DMF was added in one portion to an ice-cold solution of bovine serum albumin (BSA) (340 mg, 0.3 mmole lysine) in 30 ml water which had been previously adjusted to pH 8.7. The pH, which initially rose at 8.8 upon addition of the imidate solution, fell slowly, and was maintained at about 8.7 by the addition of 0.05 N NaOH. The pH was not allowed to rise above 9.0 or fall below 8.5. After stirring for 3 hours in an ice bath, it was allowed to stir overnite in the cold room (4°) in a covered beaker. The pH was adjusted to 7.0 with 0.05 N NCl and the slightly cloudy mixture transferred to dialysis tubes and dialyzed against water. After four changes totaling 16 l. over a 2-day period, the mixture was centrifuged (12,000 RPM, 20 min.) and lyophylized to give 250 mg protein conjugate. The UV spectrum was taken in 0.05 N NaOH (1 mg/ml solution, A= 1.01, λ_{max} = 285 nm). Assuming ϵ_{285} = 1,030 for the hapten, a conjugation number of 27 was claculated.

EXAMPLE III

A. To a solution of 5 g of morphine monohydrate in 40 ml of dry dimethyl formamide (DMF) and 300 ml of acetone, 25 g of finely powdered potassium carbonate and 5 ml of chloroacetonitrile was added. The solution was allowed to reflux for 20 hours, cooled to room temperature and filtered. The filtrate was washed three times with acetone-DMF in a 20:1 mixture and then evaporated to dryness in vacuo. Methylene chloride (500 ml) was added and the mixture heated to reflux, filtered while hot and the filtrate evaporated to give a brown oil. Addition of 200 ml of ethyl acetate led to crystallization by cooling overnight in the icebox. Total yield 4.4 g of cyanomethylmorphine. m.p. 186°–188°.

B. To a solution of 1.0 g (3.09 mmoles) of cyanomethylmorphine in 50 ml of dry methanol was added 7 ml of a 0.0435 M sodium methoxide solution in methanol. The reaction was allowed to stir at room temperature for 48 hours. After this time, 18 μl of glacial acetic acid was added, the reaction was stirred and then evaparated to dryness. The residue was dissolved with ethylene chloride and filtered, to remove sodium acetate. After filtration, the organic phase was evaporated to give 1.2 g of a light yellow salt.

C. A solution of 18 mg (0.5 mmoles) of O^3-methoxyimidoylmethylmorphine in 0.5 ml of dry DMF was added dropwise to a cold (0°) solution of 60 mg of lysozyme in 6 ml of water. The aqueous solution was first adjusted to pH 7.5 with 0.05 M sodium hydroxide. The solution was then stirred at 0° overnight. The pH was adjusted to 7 and the aqueous solutuion was dialyzed against water for 48 hours. The resulting dialysate was suitable for enzyme immunoassay. Lysozyme activity could be inhibited by morphine antibodies, and full recovery of activity could be achieved upon the addition of an aqueous solution of morphine.

EXAMLE IV

To 4 mg of glucose 6-phosphate dehydrogenase (L. mesenteroides) in 0.05M sodium phosphate, pH 8.5, was added 75μl of a solution containing 73 mg of O^3-methoxyimodylmethyl morphine per ml of DMF (200 μmole per ml) at 4°. The pH was maintained at 8.5 by addition of dilute HCl as needed. The reaction was allowed to proceed for 4 hours, after which the solution was exhaustively dialyzed against 0.055M Tris HCl-0.033M. magnesium chloride, pH 7.8. The solution was diluted to 8 cc with the same buffer. The enzyme was found to contain 10.5 morphine groups per molecule.

The activity of 10μl of a 1:50 dilution of this solution was found to 0.099 OD/min. With addition of 5μl of an opiate antibody, 1.3×10^{-4} M binding sites, the rate was 0.019 OD/min. When 20μl of 1.7×10^{-3} M codeine was added prior to adding the antibody, the rate was 0.102 OD.min.

EXAMPLE V

Malate dehydrogenase (2.8ml, 10 mg/ml) in 70% ammonium sulfate was centrifuged and the pellet dissolved in 1.3 ml of 0.01M phosphate buffer (ph 7.5). The solution was dialyzed against the same buffer and then transferred with washing with 0.15M phosphate-0.075 carbonate buffer, pH 9, to provide a total volume of 5.6ml. The solution was adjusted to pH 8.5, with aqueous hydrochloric acid and five one ml aliguots withdrawn. The fifth aliquot was acidified to a pH 7.5. A dimethyl formamide solution (35.6 mg/ml) of O^3-(imidomethoxy)methyl morphine (100 μmole/ml) was added varying amounts to the different vials. After allowing the reaction to occur for three hours, the resulting solutions were dialyzed against 0.05M phosphate, pH 7.5, five changes over 40 hours. The residues were then diluted to 2 ml with the above-indicated phosphate buffer. The following table indicates the varying amounts of material in the different vials and the pH.

Sample	pH	Enzyme Moles $\times 10^{-8}$	Morphine Imidoester vol, μl	Morphine Imidoester Moles$\times 10^{-6}$	No. of Morphines per Enzyme
1	8.5	6.76	6	.6	2.3
2	8.5	6.76	12	1.2	3.9
3	8.5	6.76	24	2.4	7.2
4	8.5	6.76	48	4.8	9.0
5	7.5	6.76	24	2.4	4.5

reduced the activity by 78% (0.035 OD/min). When 50μl of 10^{-4}M morphine in water was added to the substrate prior to the addition of the antibody and enzymes, the total enzyme activity was recovered.

Following the above procedure, a number of preparations were carried out so as to determine the effect of varying parameters. The following table indicates the parameters which were changed and the results.

TABLE A

Example	Buffer[a]	pH	G6PDH Moles $\times 10^{-8}$	Morphine Imidate Moles $\times 10^{-5}$	Deactivation[b] %	Inhibition[c] %	Morphine[d] G6PDH
i	P	8.5	3.85[g]	0.5	41	48	4.7
ii	P	8.5	3.85	1.5	66	80	10.5
iii	P	8.5	3.85	4.5	88	94	21.2
iv[e]	T	8.5	3.85	1.5	45	75	9.5
v[f]	T	8.5	3.85	1.5	48	82	11.5
vi	T	8.5	3.85	1.5	63	87	12.0
vii	P	6.0	1.92[h]	0.75	16	9	1.3
viii	P	7.0	1.92	0.75	52	78	13.0
ix	P	8.0	1.92	0.75	67	91	17.4

[a]P - 0.05M sodium phosphate T - 0.055M tris HCl-0.003 M $MgCl_2$
[b]% of original enzyme specific activity remaining after conjugation and dialysis
[c]maximum inhibition by excess antibody
[d]determined by liquid scintillation counting of aliquots of products, employing radioactive morphineimidate
[e]prior to conjugation, added glucose 6-phosphate to 50mM and NAD to 40mM and adjusted pH to 8.5 upon dropping to 6
[f]prior to conjugation, added glucose 6-phosphate to 50mM and NADH to 40mM
[g]460 IU/mg i-vi
[h]561 IU/mg vii-ix

EXAMPLE VI

Glucose-6-phosphate dehydrogenase was employed as a 4.8 mg/l. solution of 30% glycerol, having a specific activity of 561 IU/mg protein for NAD reduction at 30°. The glycerol solution was dialyzed against 0.05 M phosphate, pH 7.5 and diluted with that buffer to a concentration of 2 mg/ml. After adjusting the pH to 7.0 with one M HCl, a one ml aliquot was cooled to 40° and was added with stirring in five portions during 5 minutes to 37.5μl of a 0.2M solution of O^3-(imidomethoxy)-methyl morphine in dimethyl formamide (DMF). After each addition, the pH rose slightly and was readjusted to 7 with 1 M HCl. The solution was maintained for 5 hours at 4° and then dialyzed exhaustively against 0.055 M tris-HCl, pH 7.9. The enzyme solution was then diluted to 2 ml with dialysis buffer and the incorporation of haptens was determined, showing 13 haptens per enzyme molecule.

The morphine modified enzyme was employed in an enzyme immunoassay technique. The assay mixture had a total volume of 1 ml and was prepared from 20μl 0.1M NAD in water (pH 5-6), 50μl of 0.066M glucose-6-phosphate in acetate buffer, and the enzyme solution. The remaining volume was made up by the assay buffer, which was 0.055M tris-HCl, pH 7.9. After incubating the mixture 60 seconds in a spectrometer flow cell at 30°, the increase in absorbance at 340nm was then read over a one minute interval. Ten μl of the above solution diluted 1:100 in assay buffer containing 0.1% rabbit serum albumin gave a rate of 0.160 optical density units per minute (OD/min). This corresponded to 52% of the activity of the native enzyme. The presence of excess anti-opiate α-globulin solution (5μl of a solution that was 8×10^{-5}M in binding sites) prior to addition of the enzyme solution to the assay mixture,

Employing the imidate has numerous advanages over the use of the mixed anhydride. The conditions under which the reaction is carried out are comparatively mild and, therefore, any denaturation of the polypeptide or protein is minimized. In addition, the charge type of the polypeptide or protein is retained, since the imidate forms a basic amidine group as contrasted to carboxy, which forms a substantially neutral carboxamide group. Thus, modification of the charge distribution on the surface of the protein is minimized.

One of the advantages of maintaining the charge nature of the protein is improved water solubility. Also, modification of the conformation of the protein due to changes in hydrogen bonding, dipolar affects, and ionic attractions is also minimized.

It is found in the conjugation with enzyme proteins that, deactivation of the enzyme with increasing numbers of hapten is reduced, as compared with conjugation with the carboxy group. Following the procedure of Example V, malate dehydrogenase was conjugated with different numbers of the O^3-(imidomethoxy)-methyl morphine, and the amount of deactivation determined by assaying the enzyme after conjugation and dividing the result by the original activity of the enzyme.

In the following experiment, malate dehydrogenase was conjugated with O^3-carboxymethyl morphine.

Malate dehydrogenase (4 ml, 40.0 mg Calbiochem lot 101089) was centrifuged (17,500 rpm, 20 minutes). The resulting pellet was dissolved in 1 ml of distilled water and dialyzed against 250 ml of 0.01M phosphate buffer, pH 7.5, at 3° with 2 × 250 ml buffer changes in 4 hours. The resulting dialyzate was diluted to 5 ml with 0.15M phosphate — 0.075M carbonate buffer, pH 9.0 to give a solution of approximately 0.1M phosphate and 0.05 carbonate.

An aliquot of one-half of the above solution was cooled to 0° in an ice bath, and 0.1 M radioactive O^3-carboxymethyl morphine (0.243×10^{-5} counts/min/-μmole) was added with rapid stirring in 4—5 μl increments. Four aliquots of from 0.4 to 0.5 ml were withdrawn at appropriate times, the additions and withdrawals being carried out while maintaining the pH of the solution between 8.8 and 9.0. The samples were withdrawn when the absence of ph charges indicated reaction had occurred.

The following table indicates the results obtained employing carboxy conjugation and imidate conjugation:

TABLE B

Conjugation of Malate Dehydrogenase			
Carboxy		Imidoyl	
No. of Haptens	% Deactivation	No. of Haptens	% Deactivation
3	22	4	9
6	30	7	21
10	55	9	37

The product of the conjugation of a hapten to an enzyme finds particular use in an enzyme immunoassay which is sold by Syva Corp. under the trademark EMIT. A description of the method may be fouond in copending application Ser. No. 143,609, filed May 14, 1971. Oversimplified, the method is dependent upon substantial reduction of activity when the hapten conjugated enzyme is bound to antibody, and the amount of antibody bound to the hapten conjugated to enzyme is proportional to the amount of hapten present in solution. By assaying for the enzyme activity, the amount of hapten can be determined.

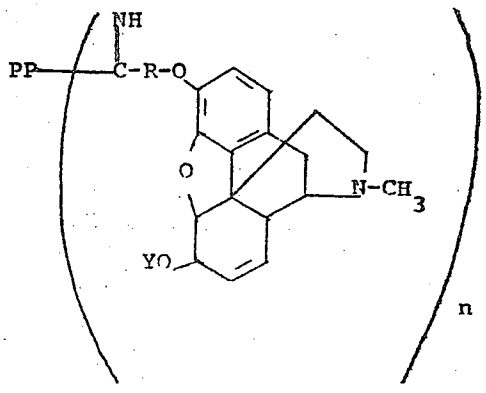
Independent studies have shown that with an increasing number of haptens randomly bound to enzyme, up to a certain percentage, increasing deactivation of the enzyme results upon binding to antibody. Therefore, it is important to be able to retain the greatest amount of enzymatic activity with increasing numbers of haptens conjugated to the enzyme.

It is also found that when the hapten is conjugated to an antigenic protein, the aqueous solubility is retained up to a fairly large number of haptens for unit molecular weight of antigen. Thus, ease of handling is achieved and solubility retained, both in the preparation and the handling, as well as the injection into animals for obtaining antibodies to the hapten.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the invention, as limited only by the scope of the appended claims.

What is claimed is:

1. A conjugate of the formula:



wherein:

y is hydrogen or acetyl;

R is alkylene of from 1–6 carbon atoms, wherein the linkage is through an amidine group;

PP is antigenic poly(amino acid); and

n is at least one and not greater than the molecular weight of PP divided by 500.

2. A conjugate according to claim **1**, wherein Y is hydrogen, R is alkylene of from 1–4 carbon atoms, and *n* is in the range of 10 to 200.

3. An antibody prepared in response to a conjugate according to claim **2.**

4. An antibody prepared in response to a conjugate according to claim **1.**

5. A conjugate of bovine serum albumin and 2-(O^3-morphinyl)acetimidoyl, wherein the linkage is through an amidine group.

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