



(86) Date de dépôt PCT/PCT Filing Date: 1994/10/24  
(87) Date publication PCT/PCT Publication Date: 1995/05/04  
(45) Date de délivrance/Issue Date: 2011/05/03  
(85) Entrée phase nationale/National Entry: 1996/04/24  
(86) N° demande PCT/PCT Application No.: US 1994/012333  
(87) N° publication PCT/PCT Publication No.: 1995/011690  
(30) Priorités/Priorities: 1993/10/26 (US08/143,571);  
1993/12/16 (US08/168,776)

(51) Cl.Int./Int.Cl. *A61K 38/28* (2006.01),  
*A61K 31/16* (2006.01), *A61K 31/28* (2006.01),  
*A61K 31/35* (2006.01), *A61K 38/23* (2006.01),  
*A61K 47/16* (2006.01), *A61K 9/16* (2006.01),  
*C07C 229/42* (2006.01), *C07C 233/55* (2006.01),  
*C07C 233/63* (2006.01), *C07C 233/87* (2006.01),  
*C07C 235/38* (2006.01), *C07C 235/64* (2006.01),  
*C07C 235/84* (2006.01), *C07C 237/22* (2006.01)

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(54) Titre : SYSTÈME D'ADMINISTRATION ORALE DE DEFEROXAMINE  
(54) Title: DESFERRIOXAMINE ORAL DELIVERY SYSTEM

(57) **Abrégé/Abstract:**

Modified amino acids and methods for their preparation and use as oral delivery systems for pharmaceutical agents are described. The modified amino acids are preparable by reacting single amino acids or mixtures of two or more kinds of amino acids with an amino modifying agent such as benzene sulfonyl chloride, benzoyl chloride, and hippuryl chloride. The modified amino acids may form encapsulating microspheres in the presence of the active agent under sphere-forming conditions. Alternatively, the modified amino acids may be used as a carrier by simply mixing the amino acids with the active agent. The preferred acylated amino acid carrier is salicyloyl-phenylalanine. The modified amino acids are particularly useful in delivering biologically active agents, e.g., desferrioxamine, insulin or cromolyn sodium, or other agents which are sensitive to the denaturing conditions of the gastrointestinal tract.



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A61K 37/00, A01N 43/16</b>		<b>A1</b>	(11) International Publication Number: <b>WO 95/11690</b>
			(43) International Publication Date: 4 May 1995 (04.05.95) <b>2174961</b>
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(22) International Filing Date: 24 October 1994 (24.10.94)			
(30) Priority Data: 143,571 26 October 1993 (26.10.93) US 168,776 16 December 1993 (16.12.93) US		(81) Designated States: AM, AT, AU, BB, BG, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(60) Parent Applications or Grants (63) Related by Continuation US 143,571 (CIP) Filed on 26 October 1993 (26.10.93) US 186,776 (CIP) Filed on 16 December 1993 (16.12.93)		Published With international search report.	
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(54) Title: DESFERRIOXAMINE ORAL DELIVERY SYSTEM			
(57) Abstract <p>Modified amino acids and methods for their preparation and use as oral delivery systems for pharmaceutical agents are described. The modified amino acids are preparable by reacting single amino acids or mixtures of two or more kinds of amino acids with an amino modifying agent such as benzene sulfonyl chloride, benzoyl chloride, and hippuryl chloride. The modified amino acids may form encapsulating microspheres in the presence of the active agent under sphere-forming conditions. Alternatively, the modified amino acids may be used as a carrier by simply mixing the amino acids with the active agent. The preferred acylated amino acid carrier is salicyloyl-phenylalanine. The modified amino acids are particularly useful in delivering biologically active agents, e.g., desferrioxamine, insulin or cromolyn sodium, or other agents which are sensitive to the denaturing conditions of the gastrointestinal tract.</p>			

## DEFERRIOXAMINE ORAL DELIVERY SYSTEM

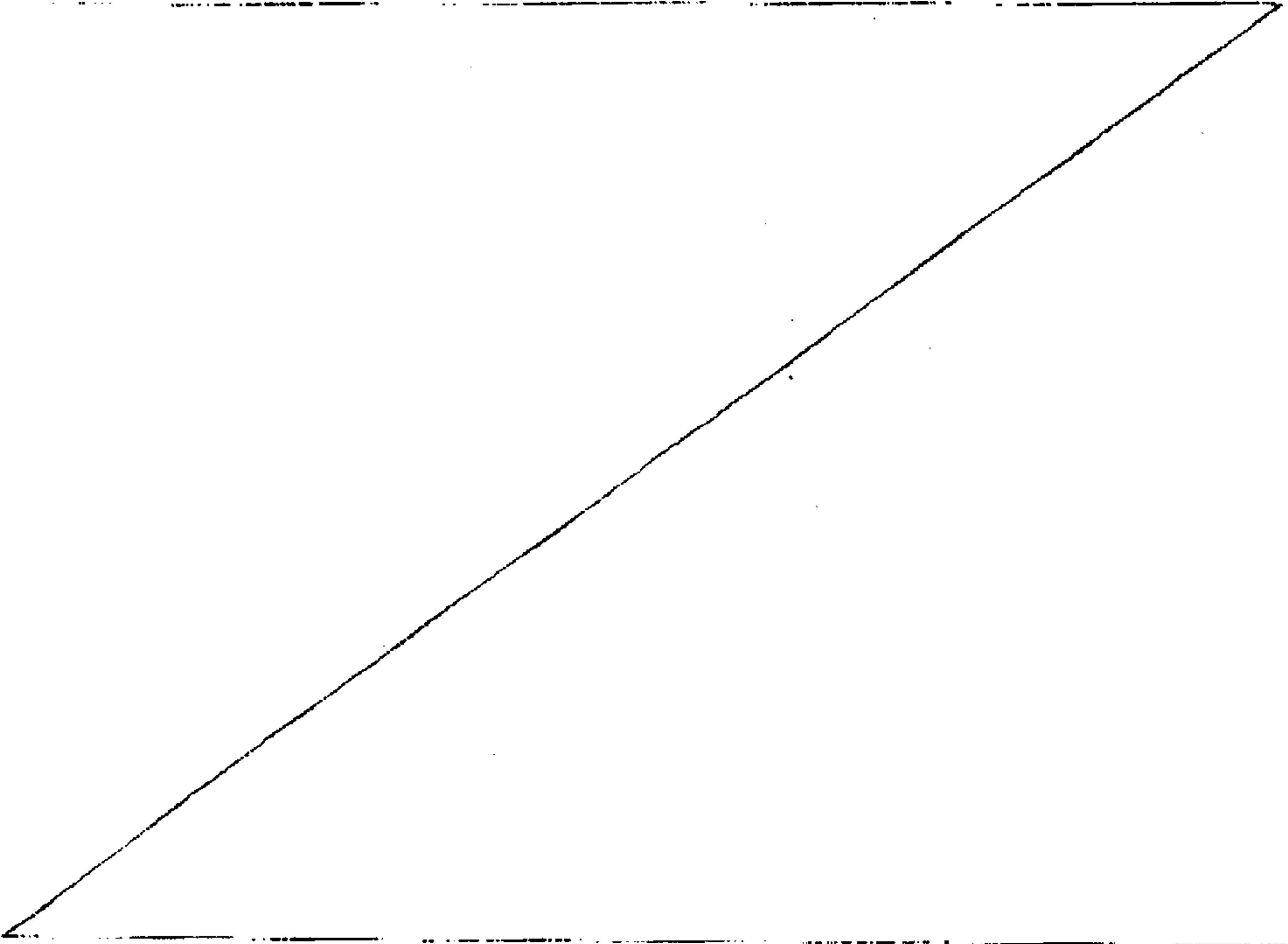
### Field of the Invention

This invention relates to an oral delivery system, and in particular to modified amino acids for use as a delivery system for desferrioxamine, insulin and cromolyn sodium. The modified amino acids releasably encapsulate active agents and are suitable for oral administration to mammals. Methods for the preparation of such amino acids are also disclosed.

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### Background of the Invention

Conventional means for delivering pharmaceutical and therapeutic agents to mammals often are severely limited by chemical or physical barriers or both, which are imposed by the body. Oral delivery of many biologically-active agents would be the route of choice if not for the presence of chemical and physicochemical barriers such as extreme pH in the gut, exposure to powerful digestive enzymes, and



impermeability of gastrointestinal membranes to the active ingredient. Among the numerous pharmacological agents which are known to be unsuitable for oral administration are biologically active peptides and proteins, such as insulin.

5 These agents are rapidly destroyed in the gut by acid hydrolysis and/or by proteolytic enzymes.

Prior methods for orally administering vulnerable pharmacological agents have relied on co-administration of adjuvants (e.g. resorcinols and non-ionic surfactants such

10 as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether) to artificially increase the permeability of the intestinal walls; and co-administration of enzymatic inhibitors (e.g. pancreatic trypsin inhibitor, diisopropylfluorophosphate (DFF) and trasylol) to avoid

15 enzymatic degradation. Liposomes as drug delivery systems for insulin and heparin have also been described. See, for instance, U.S. Patent No. 4,239,754; Patel et al. (1976) FEBS Letters Vol. 62, page 60; and Hashimoto et al. (1979) Endocrinol. Japan, Vol. 26, page 337. The broader use of

20 the aforementioned methods, however, as drug delivery systems are precluded for reasons which include: (1) the use of toxic amounts of adjuvants or inhibitors; (2) narrow range of low MW cargoes; (3) poor stability of the system and inadequate shelf life; (4) difficulty in manufacturing;

25 and (5) the failure of the method to adequately protect the active ingredient or promote its absorption.

More recently, artificial amino acid polymers or proteinoids forming microspheres have been described for encapsulating pharmaceuticals. For example, U.S. Patent No.

30 4,925,673 (the '673 patent), the disclosure of which is hereby incorporated by reference in its entirety, describes such microsphere constructs as well as methods for their preparation and use. The proteinoid microspheres of the '673 patent are useful for encapsulating a number of active

35 agents, however the preparation methods result in a complex mixture of high molecular weight (MW) (> 1000 daltons) and low MW ( $\leq$  1000 daltons) peptide-like polymers which are difficult to separate and yield relatively small amounts of the low MW microsphere-forming fraction. Thus, there is a



need in the art for a simple and inexpensive delivery system which is simple to prepare and which can encapsulate a broad range of active agents such as proteinaceous drugs.

#### Summary of the Invention

Compositions for orally delivering biologically-active agents incorporating modified amino acids, amino acid derivatives, peptides and peptide derivatives as carriers are provided.

10 The composition of the invention comprises:

a) a biologically active agent selected from the group consisting of desferrioxamine, insulin and cromolyn sodium (sodium or disodium cromoglycate); and

(b) an acylated amino acid carrier comprising salicyloyl-phenylalanine.

The invention also concerns a pharmaceutical composition for an oral administration comprising:

(a) a biologically active agent selected from the group consisting of desferrioxamine, insulin and cromolyn sodium;

20 (b) an acylated amino acid carrier comprising salicyloyl-phenylalanine ; and

(c) a pharmaceutically acceptable carrier or diluent.

Also contemplated is a method for preparing these compositions which comprises mixing at least one biologically active agent with at least one carrier as described above and, optionally, a dosing vehicle.

In an alternative embodiment, these non-toxic carriers are orally administered to animals as part of a delivery system by blending or mixing the carriers with the biologically active agent prior to administration. The carriers may also form microspheres in the presence of the active agent. The microspheres containing

the active agent are then orally administered. Also contemplated by the present invention are dosage unit forms that include these compositions.

According to the invention, modified amino acids are prepared by reacting single amino acids or mixture of two or more kinds of amino acids with an acylating or sulfonating agent which reacts with free amino moieties present in the amino acids to form amides or sulfonamides, respectively. The modified amino acids are then recovered from the mixture.

- 10 The modified amino acids are non-toxic and can be orally administered to mammals as a drug delivery system by simply mixing the modified amino acids with an active agent prior to administration. Alternatively, the modified amino acid may be converted into microspheres in the presence of the active agent. The microspheres, containing encapsulated active agent, are then orally administered.

When the biologically active agent (a) is respectively desferrioxamine, insulin or cromolyn sodium, the composition of the invention is used in a mammal respectively for lowering iron concentration, treating insulin or treating respiratory affections.

20

The invention also concerns dosage unit form comprising:

- (A) a pharmacological composition for an oral administration as defined above; and
- (B) (a) an excipient,
  - (b) a diluent,
  - (c) a disintegrant,
  - (d) a lubricant,
  - (e) a plasticizer,
  - (f) a colorant,

4a

- (g) a dosing vehicle, or
- (h) any combination thereof.

#### Description of the Drawings

Figure 1 illustrates rat serum calcium levels after oral administration of two dosage levels of a modified amino acid microsphere preparation containing calcitonin encapsulate and soluble preparation containing modified amino acid carrier and calcitonin after pre-dosing with a sodium bicarbonate solution as described in Example 5.

Figure 2 illustrates the induction of iron excretion in Cebus monkeys following oral administration of encapsulated versus unencapsulated desferrioxamine (DFO).

Figure 3 shows the iron excretion in rats following oral administration of encapsulated DFO.

Figure 4 to Figure 8 are graphic illustrations of the results of oral gavage testing in rats with insulin and salicyloyl phenylalanine carrier.

Figure 9 is a graphic illustration of the results of intraduodenal injection testing in rats with insulin and salicyloyl phenylalanine carrier.

Figures 10 and 11 are graphic illustrations of the results of oral gavage testing in rats with disodium cromoglycate and salicyloyl phenylalanine carrier.

#### Detailed Description of the Invention

The present invention arose from the discovery that amino acids, in modified form, may be used to orally deliver sensitive bioactive agents, e.g. peptide hormones such as insulin and polysaccharides such as heparin, which would not be considered orally administrable due to their sensitivity to the denaturing conditions of the



gastrointestinal (GI) tract. The modified amino acids may be used as a carrier by mixing it with the active agent or may be converted into microspheres containing encapsulated bioactive agent. In contrast to the modified amino acids of the invention, unmodified free amino acids provide inadequate protective effect for labile bioactive agents against degradation in the GI tract.

Pharmaceutical compositions containing insulin are useful for mammals suffering with diabetes.

Pharmaceutical compositions containing sodium cromolyn, an antiallergic, are useful for mammals suffering from respiratory afflictions, such as asthma or hay fever.

Other advantages provided by the present invention include the use of readily available and inexpensive starting materials and a cost-effective method for preparing and isolating modified amino acids which is simple to perform and is amenable to industrial scale-up production.

The modified amino acids of the present invention may be prepared by reacting single amino acids, mixtures of two or more kinds of amino acids, or amino acid esters with an amine modifying agent which reacts with free amino moieties present in the amino acids to form amides or sulfonamides. Amino acids and amino acid esters are readily available from a number of commercial sources such as Aldrich Chemical Co. (Milwaukee, WI, USA); Sigma Chemical Co. (St. Louis, MO, USA); and Fluka Chemical Corp. (Ronkonkoma, NY, USA).

In practicing the invention, the amino acids are dissolved in aqueous alkaline solution of a metal hydroxide, e.g., sodium or potassium hydroxide, and heated at a temperature ranging between about 5°C and about 70°C, preferably between about 10°C and about 40°C, for a period ranging between about 1 hour and about 4 hours, preferably about 2.5 hours. The amount of alkali employed per equivalent of  $\text{NH}_2$  groups in the amino acids generally ranges between about 1.25 and about 3 mmole, preferably between about 1.5 and about 2.25 mmole per equivalent of  $\text{NH}_2$ . The pH



of the solution generally ranges between about 8 and about 13, preferably ranging between about 10 and about 12.

Thereafter, an amino modifying agent is then added to the amino acid solution while stirring. The temperature of the mixture is maintained at a temperature generally ranging between about 5°C and about 70°C, preferably between about 10°C and about 40°C, for a period ranging between about 1 and about 4 hours. The amount of amino modifying agent employed in relation to the quantity of amino acids is based on the moles of total free  $\text{NH}_2$  in the amino acids. In general, the amino modifying agent is employed in an amount ranging between about 0.5 and about 2.5 mole equivalents, preferably between about 0.75 and about 1.25 equivalents, per molar equivalent of total  $\text{NH}_2$  groups in the amino acids.

Suitable, but non-limiting, examples of amino modifying agents useful in practicing the present invention include sulfonating agents such as benzene sulfonyl chloride and acylating agents such as benzoyl chloride, hippuryl chloride, salicyloyl chloride and carbodiimide derivatives of amino acids, particularly hydrophobic amino acids such as phenylalanine, tryptophan, and tyrosine.

The reaction is quenched by adjusting the pH of the mixture with a suitable acid, e.g., concentrated hydrochloric acid, until the pH reaches between about 2 and about 3. The mixture separates on standing at room temperature to form a transparent upper layer and a white or off-white precipitate. The upper layer is discarded and modified amino acids are collected from the lower layer by filtration or decantation. The crude modified amino acids are then dissolved in water at a pH ranging between about 9 and about 13, preferably between about 11 and about 13. Insoluble materials are removed by filtration and the filtrate is dried in vacuo. The yield of modified amino acids generally ranges between about 30 and about 60%, and usually about 45%.

If desired, amino acid esters, e.g. methyl or ethyl esters of amino acids, may be used to prepare the modified amino acids of the invention. The amino acid

esters, dissolved in a suitable organic solvent such as dimethylformamide or pyridine, are reacted with the amino modifying agent at a temperature ranging between about 5°C and about 70°C, preferably about 25°C, for a period ranging 5 between about 7 and about 24 hours. The amount of amino modifying agents used relative to the amino acid esters are the same as described above for amino acids.

Thereafter, the reaction solvent is removed under negative pressure and the ester functionality is removed by 10 hydrolyzing the modified amino acid ester with a suitable alkaline solution, e.g. 1N sodium hydroxide, at a temperature ranging between about 50°C and about 80°C, preferably about 70°C, for a period of time sufficient to hydrolyze off the ester group and form the modified amino 15 acid having a free carboxyl group. The hydrolysis mixture is then cooled to room temperature and acidified, e.g. aqueous 25% hydrochloric acid solution, to a pH ranging between about 2 and about 2.5. The modified amino acid precipitates out of solution and is recovered by 20 conventional means such as filtration or decantation.

The modified amino acids may be purified by recrystallization or by fractionation on solid column supports. Suitable recrystallization solvent systems include acetonitrile, methanol and tetrahydrofuran. 25 Fractionation may be performed on a suitable solid column supports such as alumina, using methanol/n-propanol mixtures as the mobile phase; reverse phase column supports using trifluoroacetic acid/acetonitrile mixtures as the mobile phase; and ion exchange chromatography using water as the 30 mobile phase. When anion exchange chromatography is performed, a subsequent 0-500 mM sodium chloride gradient is employed. The modified amino acids may also be purified by extraction with a lower alcohol such as methanol, butanol, or isopropanol to remove low molecular weight non-sphere 35 making material.

The modified amino acids of the present invention are soluble in alkaline aqueous solution ( $\text{pH} \geq 9.0$ ); partially soluble in ethanol, n-butanol and 1:1 (v/v)

toluene/ethanol solution and insoluble in neutral water. The titratable functional groups remaining in the modified amino acids are as follows: carboxylic acid groups (COOH) generally ranging between about 1.5 and about 3.5

5 milliequivalents/g, preferably about 2.3 milliequivalents/g; and amino groups ( $\text{NH}_2$ ) generally ranging between about 0.3 and about 0.9 milliequivalents/g, preferably about 0.5 milliequivalents/g.

If desired, mixtures of amino acids may be used in practicing the invention. Suitable amino acid mixtures include synthetic mixtures of two or more kinds of amino acids and readily available acid or enzyme hydrolyzed vegetable proteins. Sources of hydrolyzed liquid vegetable protein include Ajinomoto USA, Inc. (Teaneck, NJ 07666, USA); Central Soya Co., Inc. (Fort Wayne, IN, USA); and Champlain Industries, Inc. (Clifton, NJ, USA) and additional companies listed in "Food Engineering Master", an annual publication of Chilton Co., Radnor, PA 19089, USA. Prior to modification, the hydrolyzed protein solution is dried and the amino acid mixture is extracted from dried residue with a suitable organic solvent, e.g., methanol or tetrahydrofuran, followed by evaporating the solvent extract.

A particularly preferred hydrolyzed vegetable protein for use in practicing this invention is available from Ajinomoto USA under the tradename AJI-EKI. This product is an acid hydrolyzed liquid soybean protein which is derived from defatted soybean meal and generally contains titratable carboxylic acid groups (COOH) ranging between about 3 and about 8 milliequivalents/g preferably between about 4 and about 6 milliequivalents/g, total free amino groups (NH<sub>2</sub>) ranging between about 3 and about 9 milliequivalents/g, preferably ranging between about 4 and about 6 milliequivalents/g NH<sub>2</sub>. The molecular weight of the vegetable protein ranges between about 100 D and about 2000 D, preferably between about 100 and about 500 D. The modified amino acids of the present invention are stable and can stored for future use.



The modified amino acids may be used as a drug delivery carrier by simply mixing the modified amino acids with the active ingredient prior to administration.

Alternatively, the modified amino acids may be used to form encapsulating microspheres containing the active agent. The modified amino acids of the invention are particularly useful for the oral administration of certain pharmacological agents, e.g., small peptide hormones, which, by themselves, pass slowly or not at all through the gastrointestinal mucosa and/or are susceptible to chemical cleavage by acids and enzymes in the gastrointestinal tract. Non-limiting examples of such agents include human or bovine growth hormone, interferon and interleukin-II, calcitonin, atrial natriuretic factor, antigens and monoclonal antibodies.

If the modified amino acids are used as a carrier for an active ingredient, an aqueous solution of modified amino acids is mixed with an aqueous solution of the active ingredient just prior to administration. The solutions may contain additives such as phosphate buffer salts, citric acid, acetic acid, gelatin and gum acacia.

A solution of the modified amino acids is prepared by mixing the amino acids in aqueous solution in an amount ranging between about 1 mg and about 2000 mg, preferably ranging between about 300 mg and about 800 mg per mL of solution. The mixture is then heated to a temperature ranging between about 20 and about 50 C, preferably about 40 C, until the modified amino acid is dissolved. The final solution contains between about 1 mg and about 2000 mg of modified amino acids per mL of solution, preferably between about 300 and about 800 mg per mL. The concentration of active agent in the final solution varies and is dependent on the required dosage for treatment.

The modified amino acids may be used to prepare microspheres for encapsulating active agents. A useful procedure is as follows: Modified amino acids are dissolved in deionized water at a concentration ranging between about 75 and about 200 mg/ml, preferably about 100 mg/ml at a temperature between about 25 C and about 60 C, preferably

about 40 C. Particulate matter remaining in the solution may be removed by conventional means such as gravity filtration over filter paper.

Thereafter, the amino acid solution, maintained at  
5 a temperature of about 40 C, is mixed 1:1 (V/V) with an aqueous acid solution (also at about 40 C) having an acid concentration ranging between about 0.05 N and about 2 N, preferably about 1.7 N. The resulting mixture is further  
10 incubated at 40 C for a period of time effective for microsphere formation as observed by light microscopy. In practicing this invention, the preferred order of addition is to add the amino acid solution to the aqueous acid solution.

Suitable acids include any acid which does not (a)  
15 adversely effect the modified amino acids, e.g., chemical decomposition; (b) interfere with microsphere formation; (c) interfere with microsphere encapsulation of cargo; and (d) adversely interact with the cargo. Preferred acids for use in this invention include acetic acid, citric acid,  
20 hydrochloric acid, phosphoric acid, malic acid and maleic acid.

In practicing the invention, a microsphere stabilizing additive preferably incorporated into the aqueous acid solution or into the amino acid solution, prior  
25 to the microsphere formation process. The presence of such additives promotes the stability and dispersibility of the microspheres in solution.

The additives may be employed at a concentration ranging between about 0.1 and 5 % (W/V), preferably about  
30 0.5 % (W/V). Suitable, but non-limiting, examples of microsphere stabilizing additives include gum acacia, gelatin, polyethylene glycol, and polylysine.

Under these conditions, the modified amino acid molecules form hollow microspheres of less than 10 microns  
35 in diameter. If the modified amino acid microspheres are formed in the presence of a soluble material, e.g., a pharmaceutical agent in the aforementioned aqueous acid solution, this material will be encapsulated in the hollows

of the microspheres and confined within the amino acid wall defined by the spherical structure. In this way, one can encapsulate pharmacologically active materials such as peptides, proteins, and polysaccharides as well as charged organic molecules, e.g., antimicrobial agents, having poor bioavailability by the oral route. The amount of pharmaceutical agent which may be encapsulated by the microsphere is dependent on a number of factors which include the concentration of agent in the encapsulating solution, as well as the affinity of the cargo for the carrier.

The modified amino acid microspheres of the invention are pharmacologically harmless and do not alter the physiological and biological properties of the active agent. Furthermore, the encapsulation process does not alter the pharmacological properties of the active agent. While any pharmacological agent can be encapsulated within the amino acid microspheres, it is particularly valuable for delivering chemical or biological agents which otherwise would be destroyed or rendered less effective by conditions encountered within the body of the mammal to which it is administered, before the microsphere reaches its target zone (i.e., the area in which the contents of the microsphere are to be released) and which are poorly absorbed in the gastrointestinal tract.

The particle size of the microsphere plays an important role in determining release of the active agent in the targeted area of the gastrointestinal tract. Microspheres having diameters between about  $\leq 0.1$  microns and about 10 microns, preferably between about 5.0 microns and about 0.1 microns, and encapsulating active agents are sufficiently small to effectively release the active agent at the targeted area within the gastrointestinal tract. Small microspheres can also be administered parenterally by being suspended in an appropriate carrier fluid (e.g., isotonic saline) and injected into the circulatory system or subcutaneously. The mode of administration selected will, of course, vary, depending upon the requirement of the



active agent being administered. Large amino acid microspheres (>10 microns) tend to be less effective as oral delivery systems.

The size of the microspheres formed by contacting modified amino acid with water or an aqueous solution containing active agents can be controlled by manipulating a variety of physical or chemical parameters, such as the pH, osmolarity or ionic strength of the encapsulating solution, and by the choice of acid used in the encapsulating process.

The amino acid microspheres of the invention may be orally administered alone as solids in the form of tablets, pellets, capsules, and granulates suitable for suspension in liquids such as water or edible oils. Similarly, the microspheres can be formulated into a composition containing one or more physiologically compatible carriers or excipients, and which can be administered via the oral route. These compositions may contain conventional ingredients such as gelatin, polyvinylpyrrolidone and fillers such as starch and methyl cellulose. Alternatively, small microspheres (size less than 10  $\mu\text{m}$ ) can be administered via the parenteral route.

The amount of active agent in the composition typically is a pharmacologically or biologically effective amount. However, the amount can be less than a pharmacologically or biologically effective amount when the composition is used in a dosage unit form, such as a capsule, a tablet or a liquid, because the dosage unit form may contain a multiplicity of carrier/biologically-active agent compositions or may contain a divided pharmacologically or biologically effective amount. The total effective amounts will be administered by cumulative units containing in total pharmacologically or biologically active amounts of biologically-active agent.

The total amount of biologically-active agent to be used can be determined by those skilled in the art. However, it has surprisingly been found that with certain biologically-active agents, the use of the presently disclosed carriers provides extremely efficient delivery.

Therefore, lower amounts of biologically-active agent than those used in prior dosage unit forms or delivery systems can be administered to the subject, while still achieving the same blood levels and therapeutic effects.

5. The amount of carrier in the present composition is a delivery effective amount and can be determined for any particular carrier or biologically-active agent by methods known to those skilled in the art.

Dosage unit forms can also include any of  
10 excipients; diluents; disintegrants; lubricants; plasticizers; colorants; and dosing vehicles, including, but not limited to water, 1,2-propane diol, ethanol, olive oil, or any combination thereof.

Administration of the present compositions or  
15 dosage unit forms is oral or by intraduodenal injection.

The following examples are illustrative of the invention but are not intended to limit the scope of the invention.

20 **EXAMPLE 1: Modification of Amino Acids**  
**with Benzene Sulfonylchloride**

A mixture of sixteen amino acids were prepared prior to chemical modification. The constituents of the mixture are summarized in Table 1. 65 grams of the amino  
25 acid mixture (total concentration of [-NH<sub>2</sub>] groups = 0.61 moles) was dissolved in 760 mL of 1N sodium hydroxide solution (0.7625 equivalents) at room temperature. After stirring for 20 minutes, benzene sulfonylchloride (78 ml, 1 equivalent) was added over a 20 minute period. The reaction  
30 mixture was then stirred for 2.5 hours, without heating. As some precipitation had occurred, additional NaOH solution (2N) was added to the solution until it reached pH 9.3. The reaction mixture stirred overnight at room temperature. Thereafter, the mixture was acidified using dilute  
35 hydrochloric acid (38%, 1:4) and a cream colored material precipitated out. The resulting precipitate was isolated by decantation and dissolved in sodium hydroxide (2N). This

solution was then reduced *in vacuo* to give a yellow solid, which was dried on the lyophilizer (34.5 g).



TABLE 1: Amino Acid Composition

Amino Acid	Weight (g)	% of Total Weight	No. of moles of each Amino Acid ( $\times 10^{-2}$ )	No. of Moles of $[-NH_2]$
Thr	2.47	3.8	2.07	2.07
Ser	2.25	3.46	2.1	2.1
Ala	4.61	7.1	5.17	5.17
Val	4.39	6.76	3.75	3.75
Met	0.53	0.82	0.35	0.35
Ile	2.47	3.8	0.36	0.36
Leu	3.86	5.94	2.95	2.95
Tyr	1.03	1.58	0.56	0.56
Phe	4.39	6.76	0.27	0.27
His	2.47	3.8	1.6	3.2
Lys	4.94	7.6	3.4	6.8
Arg	5.13	7.9	2.95	5.90
Glutamine	9.87	15.18	6.76	13.42
Glutamic Acid	9.87	15.18	6.70	6.70
Asparagine	3.32	5.11	2.51	5.02
Aspartic Acid	3.32	5.11	2.50	2.50

**EXAMPLE 2: Preparation Modified Amino Acid/ Salmon Calcitonin**

**Compositions**

(a) Preparation of Modified Amino acid microspheres containing encapsulated Salmon Calcitonin

The modified amino acid mixture, prepared in accordance with Example 1, was dissolved at 40°C in distilled water (pH 7.2) at a concentration of 100 mg/ml. The solution was then filtered with a 0.2 micron filter and the temperature was maintained at 40°C. Salmon calcitonin (Sandoz Corp., Basel, Switzerland) was dissolved in an aqueous solution of

to 40°C. The two heated solutions were then mixed 1:1 (v/v). The resulting microsphere suspension was then filtered with glass wool and centrifuged for 50 minutes at 1000 g. The pellet was resuspended with 0.85N citric acid to a volume 5 to 7 fold less than the original volume. Salmon calcitonin concentration of the resuspended pellet was determined by HPLC. Additional microspheres were made according to the above procedure without salmon calcitonin. These "empty microspheres" were used to dilute the encapsulated salmon calcitonin microsphere preparation to a final dosing suspension for animal testing.

(b) Preparation of a Soluble Modified Amino acid carrier/Salmon Calcitonin system

A soluble amino acid dosing preparation containing salmon calcitonin was prepared by dissolving the modified amino acid material in distilled water (pH 8) to an appropriate concentration. The solution was heated to 40°C and then filtered with a 0.2 micron filter. Salmon calcitonin, also dissolved in distilled water, was then added to the modified amino acid solution prior to oral administration.

EXAMPLE 3: In Vivo Evaluation of Calcitonin Preparations in Rats

In vivo evaluation of modified amino acid microspheres containing encapsulated calcitonin and soluble modified amino acid carrier/calcitonin system, prepared as described in Example 2, were evaluated in rats. Rats were gavaged with the oral dosing preparations and blood samples were withdrawn at various time intervals for serum calcium concentration determinations.

Nine rats are divided into three groups as follows:

1. calcitonin microspheres: 10 ug calcitonin/kg body weight by oral gavage (3 rats);
2. calcitonin microspheres: 30 ug calcitonin/kg body weight by oral gavage (3 rats); and

3. soluble (unencapsulated) modified amino acid/calcitonin system: 30 ug calcitonin/kg body weight by oral gavage (3 rats). The rats were pre-dosed with 0.7 meq of aqueous sodium bicarbonate solution prior to administration of the soluble system.

Oral gavage dosing of rats is performed. Calcitonin microspheres are prepared immediately prior to dosing and Group 1 rats and Group 2 rats each receive an appropriate dosage of the microsphere suspension. Group 3 rats receives the unencapsulated calcitonin/modified amino acid system. Approximately 0.5 ml of blood is withdrawn from each rat just prior to dosing ("0" time) and 1 h, 2 h and 3 h post-dosing. Serum from the blood samples are stored at -20°C.

The calcium levels of thawed serum taken from group 1-3 rats are analyzed by conventional methods. Experimental results in rats have demonstrated a significant increase in pharmacological activity (i.e., decreasing serum calcium levels) when calcitonin is orally administered either as a encapsulate in modified amino acid microspheres or a mixture with modified amino acids as compared to basal levels. As shown in Figure 1, soluble modified amino acid solution containing salmon calcitonin demonstrated a significant increase in pharmacological activity (i.e., decreasing serum calcium levels) when compared to basal levels after oral administration. The results show that orally administered calcitonin exerted a relatively greater biological effect when administered in its unencapsulated form with modified amino acid as carrier.

**EXAMPLE 4: Modification of a Mixture of Five Amino Acids Using Benzene Sulfonyl Chloride**

An 86.1 g (0.85 moles of  $\text{NH}_2$ ) mixture of amino acids (see Table 1) was dissolved in 643 mL (1.5 equiv.) of aqueous 2N sodium hydroxide solution. After stirring for 30 minutes at room temperature, benzene sulfonyl chloride (108 mL, 0.86 moles) was added portionwise into the amino acid solution over a 15 minute period. After stirring for 2.5



hours at room temperature, the pH of the reaction mixture (pH 5) was adjusted to pH 9 with additional 2N sodium hydroxide solution. The reaction mixture stirred overnight at room temperature. Thereafter, the pH of the reaction mixture was adjusted to pH 2.5 by addition of aqueous hydrochloric acid solution (4:1, H<sub>2</sub>O:HCl) and a precipitate of modified amino acids formed. The upper layer was discarded and the resulting yellow precipitate was isolated by decantation, washed with water and dissolved in 2N sodium hydroxide (2N). The solution was reduced in vacuo to give a yellow solid which was lyophilized overnight. The yield of crude modified amino acid was 137.9 g.

Table 2

Amino Acid	Moles of Amino Acid (x 10 <sup>-2</sup> )	Moles of [-NH <sub>2</sub> ] x 10 <sup>-2</sup>
Valine	7.5	7.5
Leucine	10.7	10.5
Phenylalanine	13.4	13.4
Lysine	21.0	42.0
Arginine	6.0	12.0

**EXAMPLE 5: Modification of a Mixture of Five Amino Acids Using Benzoyl Chloride**

An 86 g (0.85 moles of NH<sub>2</sub>) mixture of amino acids (see Table 2 in Example 4) was dissolved in 637 mL (1.5 equiv.) of aqueous 2N sodium hydroxide solution. After stirring for 10 minutes at room temperature, benzoyl chloride (99 mL, 0.85 moles) was added portionwise into the amino acid solution over a 10 minute period. After stirring for 2.5 hours at room temperature, the pH of the reaction mixture (pH 12) was adjusted to pH 2.5 using dilute hydrochloric acid (4:1, H<sub>2</sub>O:HCl). The reaction mixture stirred overnight at room temperature. Thereafter, the pH of the reaction mixture was adjusted to pH 2.5 by addition of aqueous hydrochloric acid solution (4:1, H<sub>2</sub>O:HCl) and a precipitate of modified amino acids formed. After settling

for 1 hour, the resulting precipitate was isolated by decantation, washed with water and dissolved in sodium hydroxide (2N). This solution was then reduced in vacuo to give crude modified amino acids as a white solid (220.5 g).

5

**EXAMPLE 6:      Modification of L-Valine Using Benzene Sulfonyl Chloride**

L-Valine (50 g, 0.43 mol) was dissolved in 376 mL (1.75 equiv.) of aqueous 2N sodium hydroxide by stirring at room temperature for 10 minutes. Benzene sulfonyl chloride (48.7 mL, 0.38 mol, 1.25 equiv.) was then added to the amino acid solution over a 20 minute period at room temperature. After stirring for 2 hours at room temperature, a precipitate appeared. The precipitate was dissolved by adding 200 mL of additional 2N sodium hydroxide solution. After stirring for an additional 30 minutes, dilute aqueous hydrochloric acid solution (1: 4, H<sub>2</sub>O:HCl) was added until the pH of the reaction mixture reached 2.6. A precipitate of modified amino acids formed and was recovered by decantation. This material was dissolved in 2N sodium hydroxide and dried in vacuo to give a white solid. Yield of crude modified amino acids = 84.6 g, 77%).

**EXAMPLE 7:      Modification of Phenylalanine Methyl Ester Using Hippuryl Chloride**

L-Phenylalanine Methyl Ester Hydrochloride (15 g, 0.084 mole) was dissolved in dimethylformamide (DMF) (100 mL) and to this was added pyridine (30 mL). A solution of hippuryl chloride (16.6 g, 0.084 moles in 100 mL DMF) was immediately added to the amino acid ester solution in two portions. The reaction mixture was stirred at room temperature overnight. The reaction mixture was then reduced in vacuo and dissolved in 1N aqueous sodium hydroxide. The solution was heated at 70°C for 3 hours in order to hydrolyze the methyl ester to a free carboxyl group. Thereafter, the solution was acidified to pH 2.25 using dilute aqueous hydrochloric acid solution (1:3 HCl/H<sub>2</sub>O). A gum-like precipitate formed and this was recovered and dissolved in 1N sodium hydroxide. The

solution was reduced in vacuo to afford 18.6 g of crude modified amino acid product (Yield 18.6 g). After recrystallization from acetonitrile, pure modified phenylalanine (12 g) was recovered as a white powder. m.p. 223-225°C.

**Example 8: Modification of Phenylalanine Using Acetylsalicyloyl Chloride**

10 ml of pyridine were added to a mixture of acetylsalicyloyl chloride (10 g., 50.4 mmol) and phenylalanine benzyl ester toluene sulfonate (21.4 g., 50 mmol). The reaction was allowed to stir overnight and was monitored by thin layer chromatography (TLC). The reaction mixture was washed with 1N hydrochloric acid (100 ml). The organic layer was separated, dried with  $\text{MgSO}_4$ , filtered, and concentrated to provide an oil. The oil was purified by flash chromatography on silica gel (30% EtOAc/hexane) to provide 16.9 g (80.9%) of pure diester product, m.p. 56-57°C,  $R_f = 0.35$  (20% EtOAc/hexane).

The diester, acetylsalicyloyl phenylalanine benzyl ester (30 g. 71.9 mmol), was added to an aqueous saturated solution of  $\text{NaHCO}_3$  (50 ml), acetone (200 ml), and methanol (125 ml). This mixture was stirred at room temperature and was monitored by TLC until no starting diester remained. Removal of the solvents in vacuo provided an oily suspension which was extracted with methylene chloride (3 x 80 ml). The extracts were combined, washed with water, and dried with  $\text{MgSO}_4$ . The dried extracts were filtered and concentrated to provide the benzyl ester product which was purified by flash chromatography on silica gel (30% EtOAc/hexane).

The salicyloyl phenylalanine benzyl ester (16.9 g, 45 mmol) was placed in methanol (400 ml) in a reaction vessel, and a palladium/carbon catalyst was added. The vessel was flushed with nitrogen (3 times), and hydrogen gas (1.7 g) was introduced. The reaction was monitored by TLC and was complete in about 4 hours. The catalyst was removed



by filtration and washed with methanol. Removal of the solvent provided a quantitative yield of the pure salicyloyl phenylalanine, m.p. 52-53°C.

Properties are listed below:

5  $^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ )  $\delta$ : 13 (s, 1H), 12 (d, 1H), 9 (d, 1H), 8 (dd, 1H), 7.4 (m, 1H), 7.2 (q, 5H), 6.9 (t, 2H), 4.7 (m, 1H), 3.1 (m, 2H).

Analysis: calculated for  $\text{C}_{16}\text{H}_{15}\text{NO}_4$ : C, 67.36; H, 5.30; N, 4.91;  
Found: C, 67.15; H, 5.27; N, 4.84;

10

**Example 9: Preparation and Evaluation of Desferrioxamine (DFO) -containing Microspheres**

In this Example, a study was undertaken to evaluate the relative efficacy of the iron chelator  
15 desferrioxamine B (DFO) when administered orally with modified amino acid carriers. Currently, DFO must be administered parenterally in order to treat iron overload in mammals. The currently accepted mode of treatment (subcutaneous infusion of DFO) raises problems of  
20 compliance, especially in patients who must be maintained on chelation therapy for long periods of time, and is also difficult to achieve in Third World countries with suboptimal medical facilities. Thus, there is a need for a convenient orally administrable form of DFO. Effective  
25 amounts of DFO are well known in the art and range between about 10 and about 50 mg/kg/day (or between about 0.4 to about 2.0 g/day total.)

Preparation of DFO in modified amino acid carriers was performed in the same manner as described above. DFO  
30 was obtained from Ciba-Geigy (Basel, Switzerland) under the trade name DESFERAL. DFO modified amino acid carriers were prepared with salicyloyl-Phe modified amino acid, and the final modified amino acid carrier suspension contained 125 mg/ml DFO.

35 The efficacy of modified amino acid-carried DFO was evaluated in both the Cebus monkey iron clearance model system (Bergeron et al., Sixth Cooley's Anemia Symposium,

Ann.N.Y.Acad.Sci. 612:378 (1991)), and in bile duct-cannulated rats (Bergeron et al., Blood 79:1882 (1992)).

#### Experimental Procedure

##### 5 A. Cebus monkeys:

Eight days prior to drug administration, Cebus monkeys were anesthetized with Ketamine (7-10 mg/kg intramuscularly), after which a baseline blood sample was obtained. The animals were then transferred to metabolic  
10 cages and started on a low-iron liquid diet. One day prior to administration, the animals were fasted. On day 0, the animals were anesthetized and the drug was administered at a dose of 200 mg/kg using a pill gun. An additional blood sample was obtained on day 5.

15 Fecal and urine samples were collected at 24-h intervals between day -3 and day +5. The volume of the urine and the wet weight of the feces were determined, after which the samples were autoclaved. The urine samples were then acidified with low-iron nitric acid. The feces samples were  
20 freeze-dried for 48h, after which their dry weight was measured; they were then refluxed for 48h with low-iron nitric acid. The iron content of the acidified urine and feces samples was then measured in triplicate on an atomic absorption spectrophotometer. The iron clearance rates were  
25 calculated on the basis of a 1:1 DFO-iron complex. The iron output for 4 days prior to administration of the drug was averaged and subtracted from the iron clearance after administration; this value was then divided by the theoretical output to calculate the efficiency of clearance.

30

Oral administration of DFO in the absence of modified amino acid carriers induced little if any clearance of iron in a 48-h period following administration (Figure 2). In contrast, DFO prepared with the modified amino acid  
35 carriers of the present invention prior to administration induced a rapid peak of iron secretion in both urine and feces, corresponding to a 4% clearance rate during that interval. Subcutaneously administered DFO induced a 5%

clearance rate. These results demonstrate that a single dose of DFO modified amino acid carrier was substantially as effective as parenterally administered DFO in delivering a therapeutically active bioavailable dose of DFO.

5

#### B. Rats

Male Sprague-Dawley rats were anesthetized, after which their bile ducts were cannulated, such that continuous  
10 bile samples could be collected while the animals were free to move around their cages. The rats were fasted for 24h, after which the drug was administered by gavage at a dose of 45 mg/kg. Bile samples were collected at 3-h intervals, and urine samples were taken every 24 h. The iron content of  
15 the bile and urine samples were measured by atomic absorption spectrometry essentially as described in part A.

Figure 3 shows the biliary and urinary excretion of iron in rats after oral administration of DFO with modified amino acid carriers. The stimulation of iron  
20 excretion by orally administered modified amino acid-carried DFO is equivalent to that observed when free DFO was administered via a subcutaneous route (Bergeron et al., Blood, *ibid.*).

#### 25 Example 10: Preparation of Insulin/Delivery System

In a test tube, 1800 mg of salicyloyl phenylalanine carrier were added to 4 ml of water. The solution was stirred, and the pH was adjusted to 8.0-8.5 with NaOH (1.0N) or HCl (1.0N).

30 Insulin was prepared by adding 20 mg of insulin to 2 mL of NaHCO<sub>3</sub> solution (40 mg/ml NaHCO<sub>3</sub>). The concentration of insulin was 10 mg/ml.

0.900 ml of insulin solution was added to the carrier solution. Water was added to bring the total volume  
35 to 6.0 mL. The sample had a carrier concentration of 300 mg/mL. The total insulin concentration was 1.5 mg/mL.



**Example 11      Insulin In Vivo Experiments in Rats**

Following the procedure in Example 10, insulin/carrier preparations of 3 mg/kg of insulin mixed with 60 mg/kg of salicyloyl-Phe carrier, 600 mg/kg of salicyloyl-Phe carrier and 1200 mg/kg of salicyloyl-Phe carrier, respectively were prepared. Fasted rats were anesthetized with Ketamine (14 mg/kg). The fasted rats were administered, by oral gavage, a dosing volume of two (2) mL/kg of one of the preparations. Blood samples were collected by cutting the tip of the tail, and one drop of blood was analyzed with a ONE TOUCH II Glucose Analyzer (available from Life Scans, Inc., Mitpitsas, CA, USA).

Results are illustrated in Figure 4.

**Example 12      Insulin In Vivo Experiment**

Following the procedure in Example 10, insulin/carrier preparations were prepared, and a first group of fasted rats was administered, by oral gavage, 0.5 mg/kg of insulin mixed with 600 mg/kg of salicyloyl-Phe carrier. A second group of fasted rats was administered 600 mg/kg of salicyloyl-Phe carrier without insulin. Blood samples were collected and analyzed as described in Example 11.

Results are illustrated in Figure 5.

**Example 13      Insulin In Vivo Experiment**

Following the procedure in Example 10, insulin/carrier preparations were prepared, and a first group of fasted rats was administered, by oral gavage, 0.5 mg/kg of insulin mixed with 600 mg/kg of salicyloyl-Phe carrier. A second group of fasted rats was administered 3 mg/kg of insulin without a carrier. Blood samples were collected and analyzed as described Example 11.

Results are illustrated in Figure 6.

**Example 14     Insulin In Vivo Experiment**

Following the procedure in Example 10, insulin carrier preparations were prepared, and a first group of fasted rats was administered, by oral gavage, 1.0 mg/kg of insulin mixed with 600 mg/kg of salicyloyl-Phe carrier. A second group of fasted rats was administered 600 mg/kg of salicyloyl-Phe carrier without insulin. Blood samples were collected and analyzed as described in Example 11.

Results are illustrated in Figure 7.

10

**Example 15     Insulin In Vivo Experiment**

Following the procedure in Example 10, insulin/carrier preparations were prepared, and a first group of fasted rats was administered, by oral gavage, 3.0 mg/kg of insulin mixed with 1200 mg/kg of salicyloyl-Phe carrier. A second group of fasted rats was administered, by oral gavage, doses of 1200 mg/kg of salicyloyl-Phe carrier without insulin. Blood samples were collected and analyzed as described in Example 11.

20

Results are illustrated in Figure 8.

**Example 16     In Vivo Insulin Experiment**

Following the procedure in Example 10, insulin/carrier preparations were prepared, and a first group of fasted rats was administered, by intraduodenal injection, 1.0 mg/kg of insulin mixed with 300 mg/kg of salicyloyl-Phe carrier. A second group of rats was administered, by intraduodenal injection, 1.0 mg/kg of insulin mixed with 300 mg/kg of salicyloyl-Phe carrier and 6 mg/kg of urea. Blood samples were collected and analyzed as described in Example 11.

30

Results are illustrated in Figure 9.

**Example 17****35 In Vivo Evaluation of Cromoglycate Preparations in Rats**

A preparation of 100 mg/ml salicyloyl-Phe solution in 0.85N citric acid and 0.5% acacia and 25 mg/ml of disodium cromoglycate was prepared.

Fasted rats were administered, by oral gavage, dosages of the preparation containing 50 mg/kg of disodium cromoglycate mixed with 200 mg/kg of carrier, at a dosing volume of two (2) mL/kg. The delivery was evaluated by using the procedure described by A. Yoshimi in *Pharmacobio-Dyn.*, 15, pages 681-686, (1992).

Results are illustrated in Figure 10.

#### Example 18

##### 10 In Vivo Evaluation of Cromoglycate Preparations in Rats

Following the procedure in Example 17, a disodium cromoglycate preparation was prepared, fasted rats were administered, by oral gavage, a mixture of 50 mg/kg of disodium cromoglycate and 400 mg/kg of salicyloyl-Phe carrier, at a dosing volume of two (2) mL/kg.

Results are illustrated in Figure 11.

As clearly illustrated by the data in the Examples and Figures the use of compositions of the subject invention show significant advantages for the delivery of biologically active agents.



**WHAT IS CLAIMED IS:**

1. A composition for oral administration comprising:
  - (a) a biologically active agent selected from the group consisting of desferrioxamine, insulin and cromolyn sodium; and
  - (b) an acylated amino acid carrier comprising salicyloyl-phenylalanine.
2. The composition as defined in claim 1, wherein said biologically active agent is desferrioxamine.
3. The composition as defined in claim 1, wherein said biologically active agent is insulin.
- 10 4. The composition as defined in claim 1, wherein said biologically active agent is cromolyn sodium.
5. A pharmaceutical composition for oral administration comprising:
  - (a) a biologically active agent selected from the group consisting of desferrioxamine, insulin and cromolyn sodium;
  - (b) an acylated amino acid carrier comprising salicyloyl-phenylalanine; and
  - (c) a pharmaceutically acceptable carrier or diluent.
6. The pharmaceutical composition as defined in claim 5, wherein said biologically active agent is desferrioxamine.
7. The pharmaceutical composition as defined in claim 5, wherein said  
20 biologically active agent is insulin.
8. The pharmaceutical composition as defined in claim 5, wherein said biologically active agent is cromolyn sodium.

9. Use of a composition as defined in claim 2, for lowering iron concentration in a mammal.

10. Use of a composition as defined in claim 3, for treating diabetes in a mammal in need of such treatment.

11. Use of a composition as defined in claim 4, for treating respiratory afflictions in a mammal in need of such treatment.

12. Use of a composition as defined in claim 2, for the making of a medicament for lowering iron concentration in a mammal.

10 13. Use of a composition as defined in claim 3, for the making of a medicament for treating diabetes in a mammal.

14. Use of a composition as defined in claim 4, for the making of a medicament for treating respiratory afflictions in a mammal.

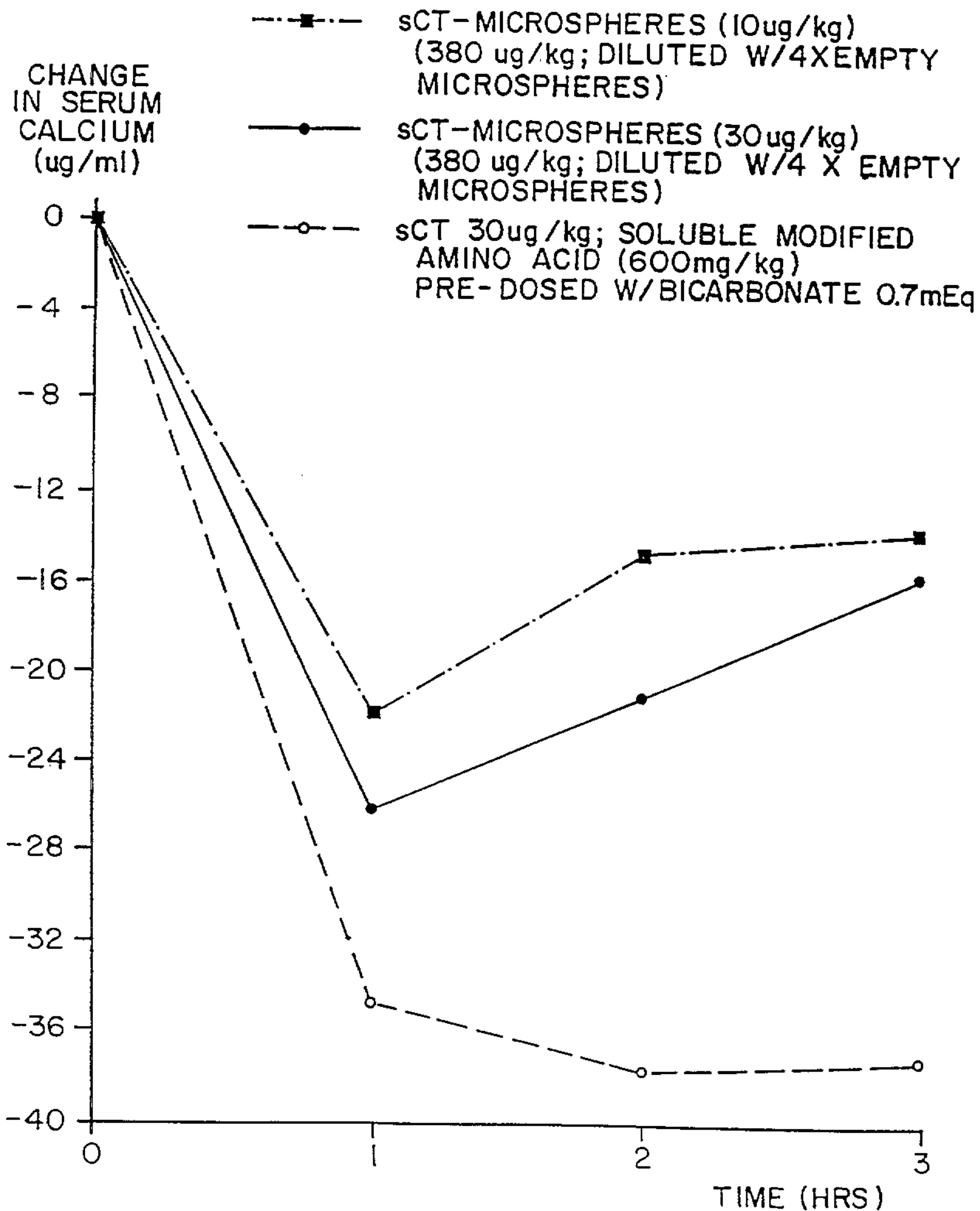
15. A dosage unit form comprising:

- (A) a pharmacological composition for an oral administration as defined in claim 5; and
- (B)
  - (a) an excipient,
  - (b) a diluent,
  - (c) a disintegrant,
  - (d) a lubricant,
  - (e) a plasticizer,
  - (f) a colorant,
  - (g) a dosing vehicle, or
  - (h) any combination thereof.

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FIG. 1





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FIG. 2

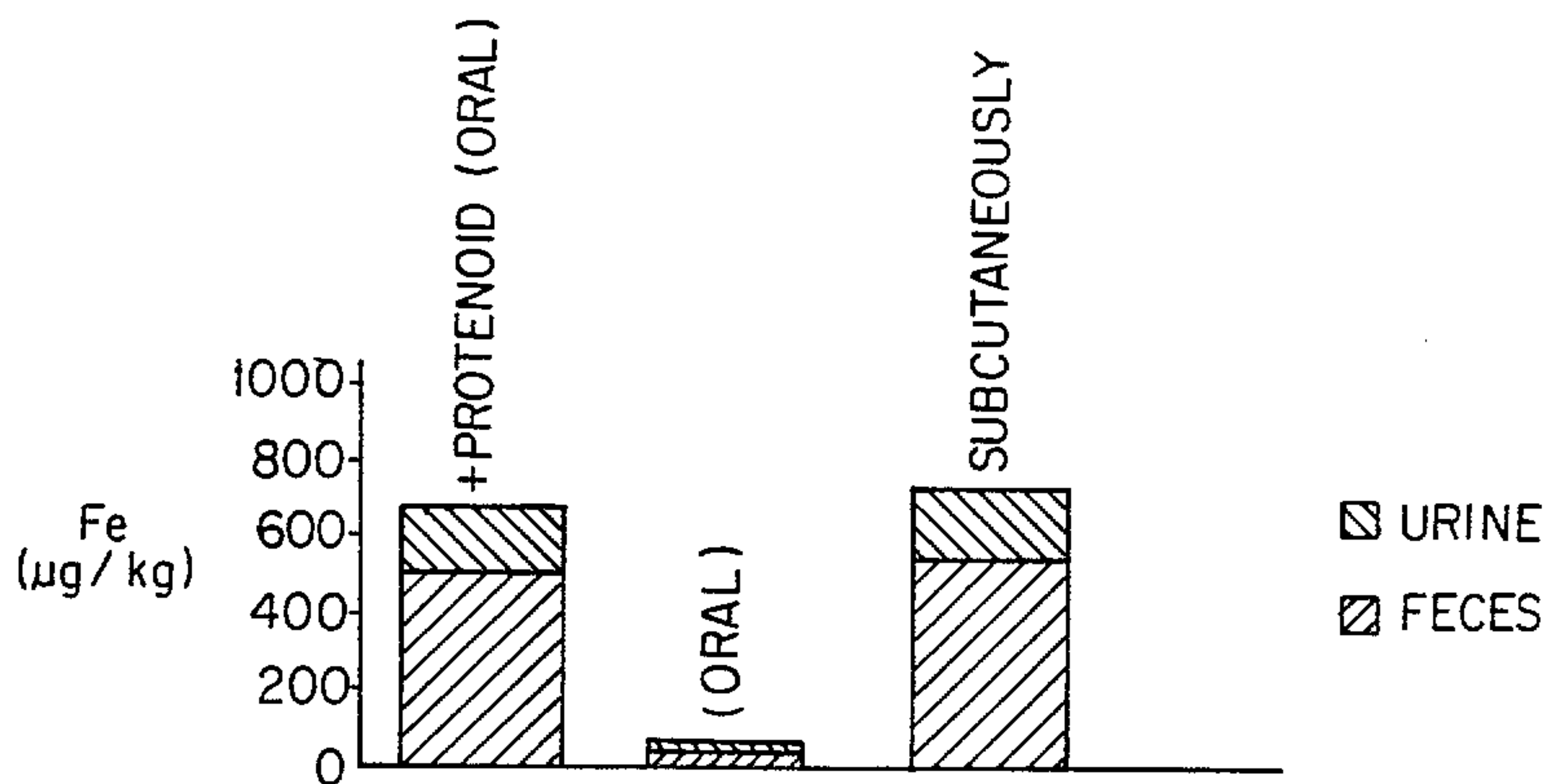
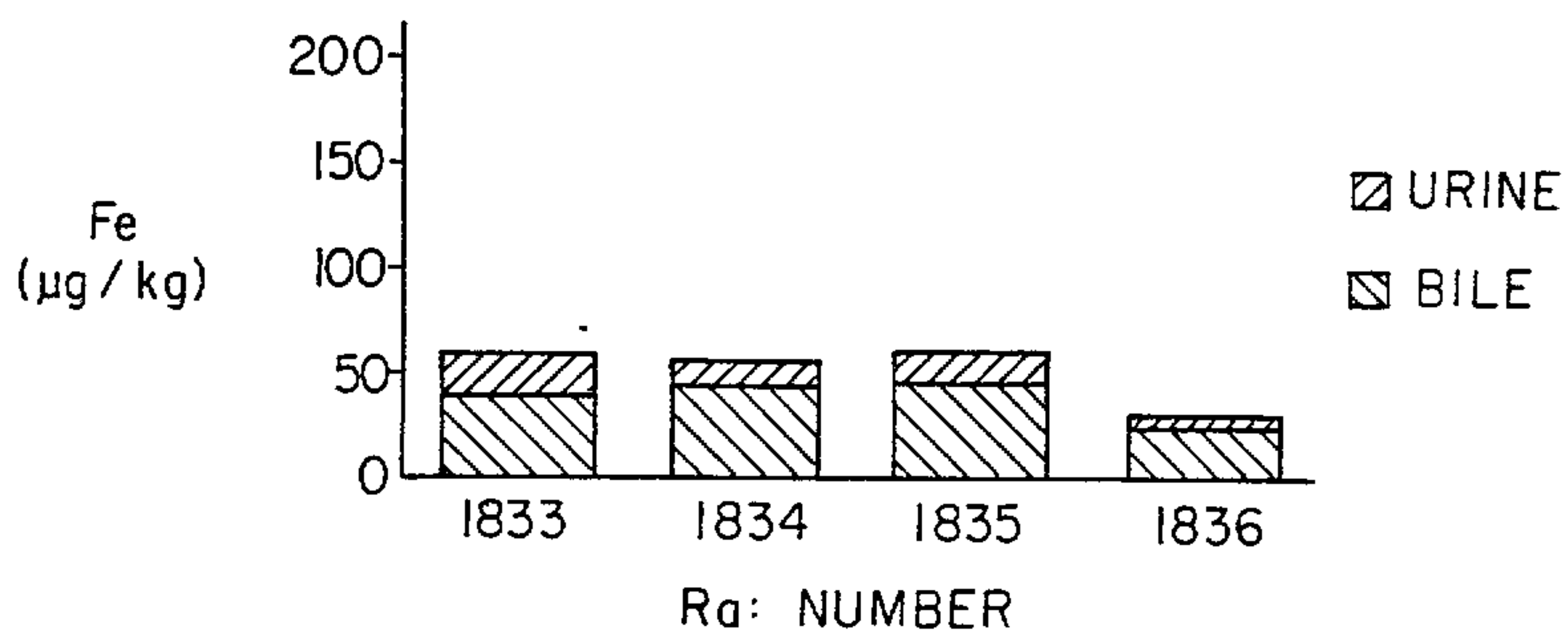
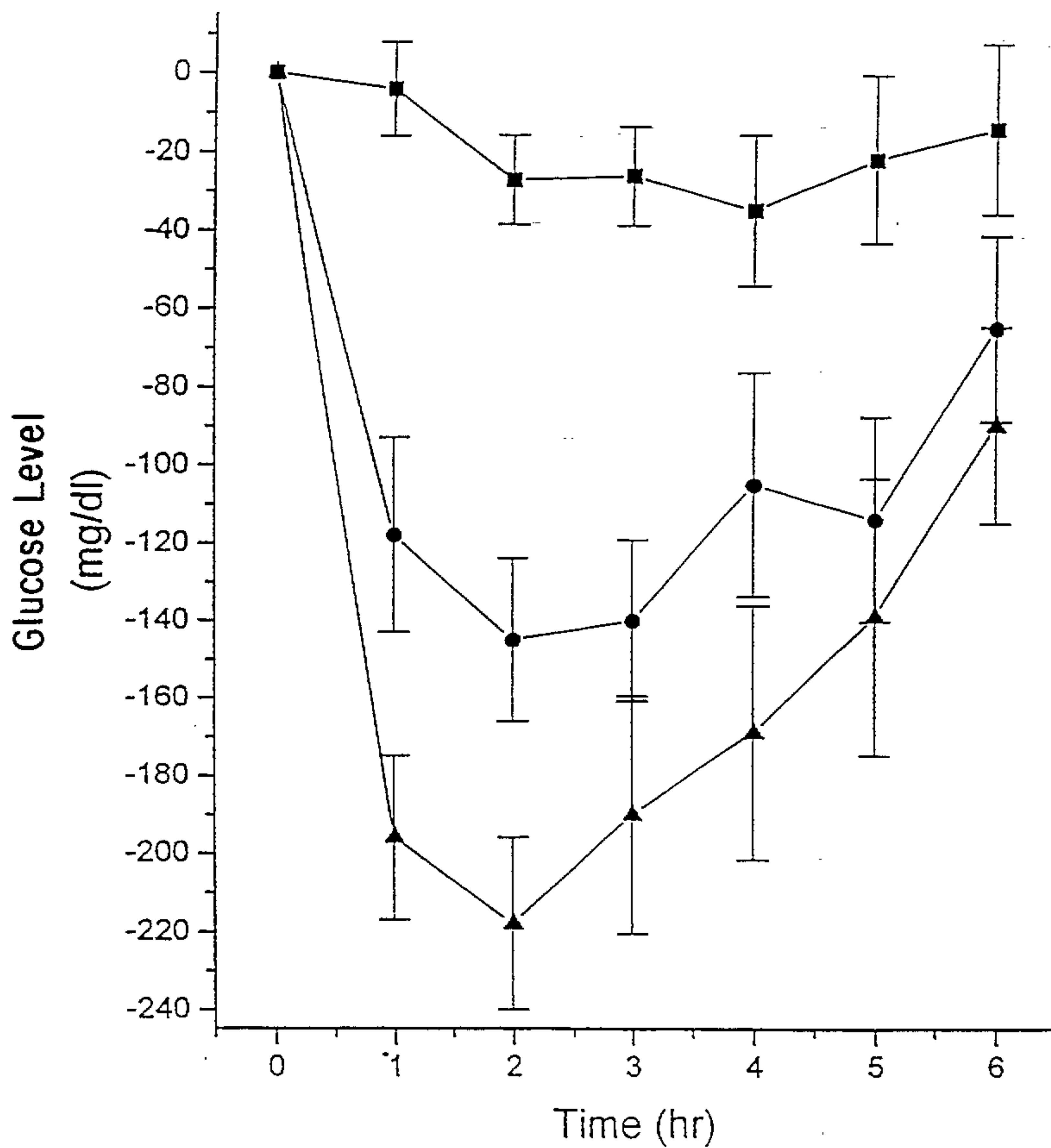


FIG. 3



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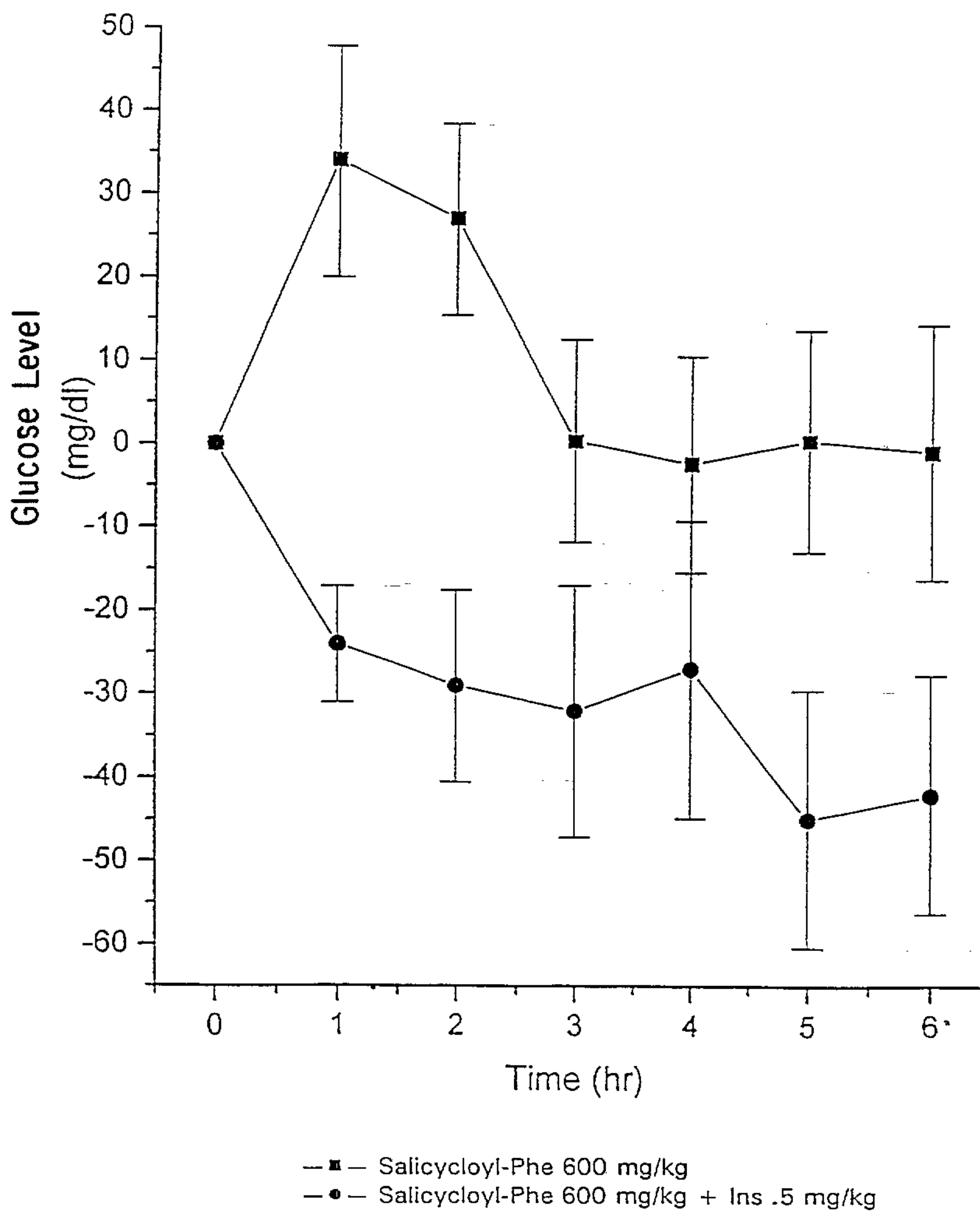
FIG. 4



- Salicycloyl-Phe 60 mg/kg + INS 3 mg/kg
- Salicycloyl-Phe 600 mg/kg + INS 3 mg/kg
- ▲— Salicycloyl-Phe 1200 mg/kg + INS 3 mg/kg

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FIG. 5



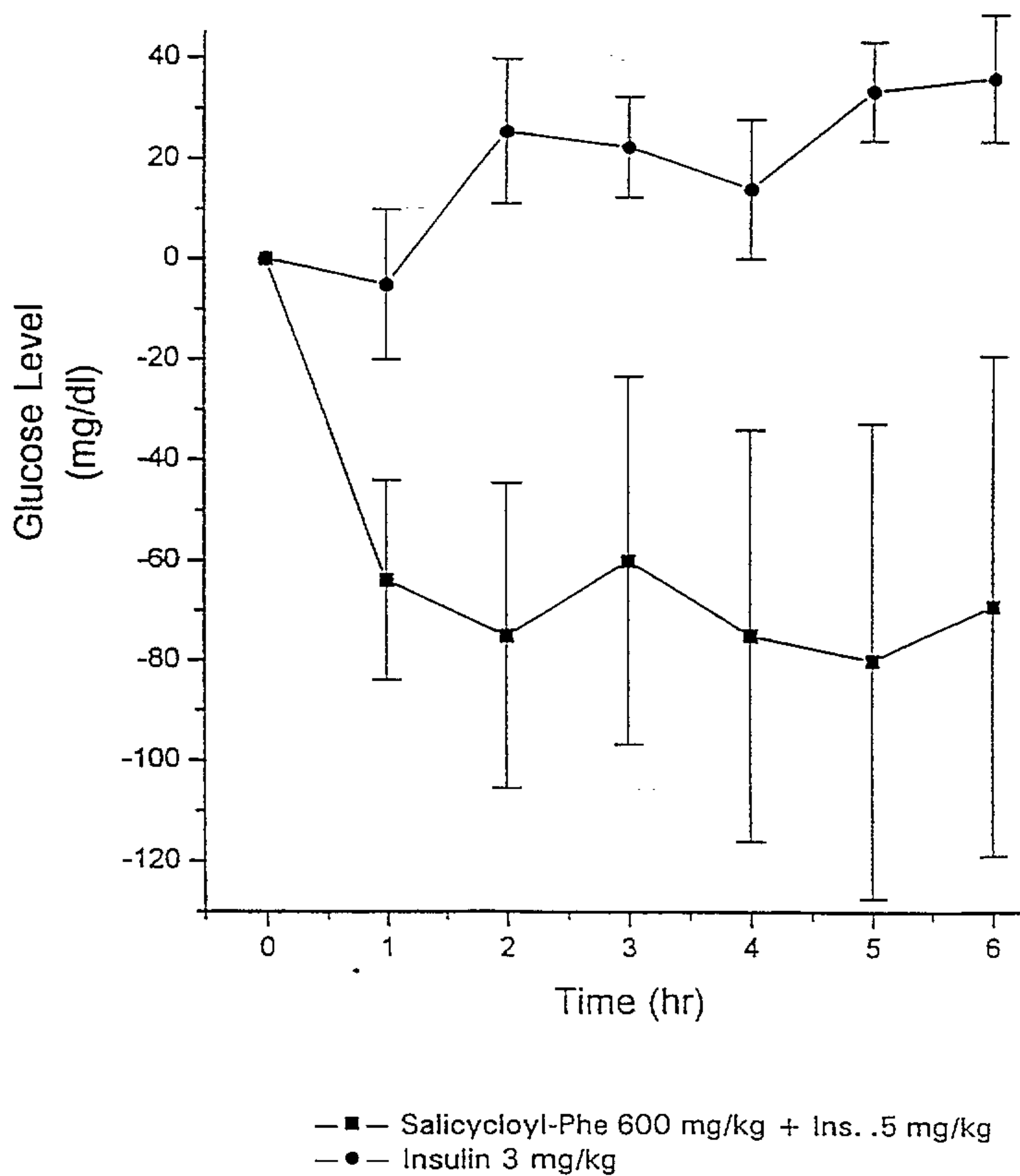
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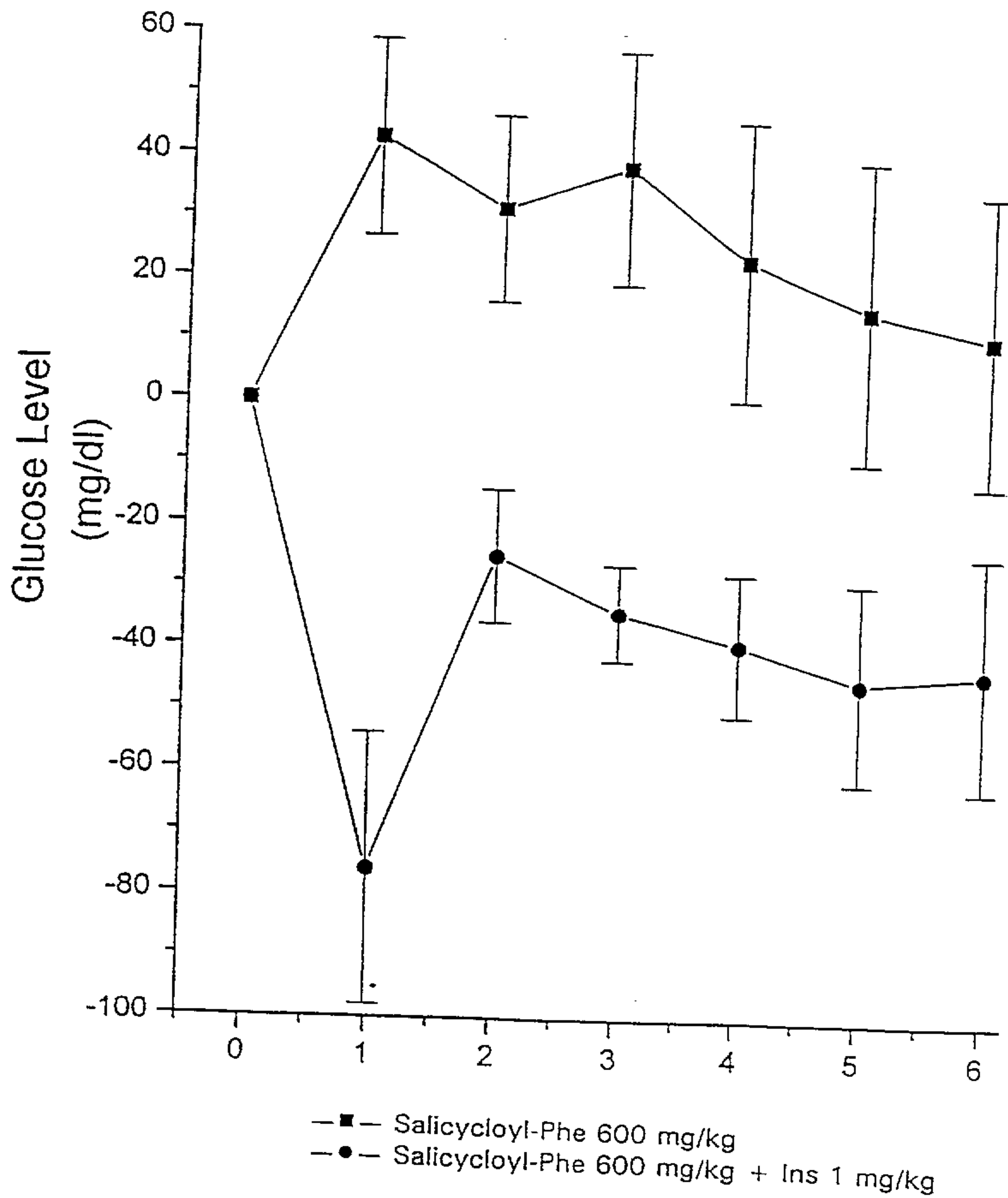
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FIG. 6



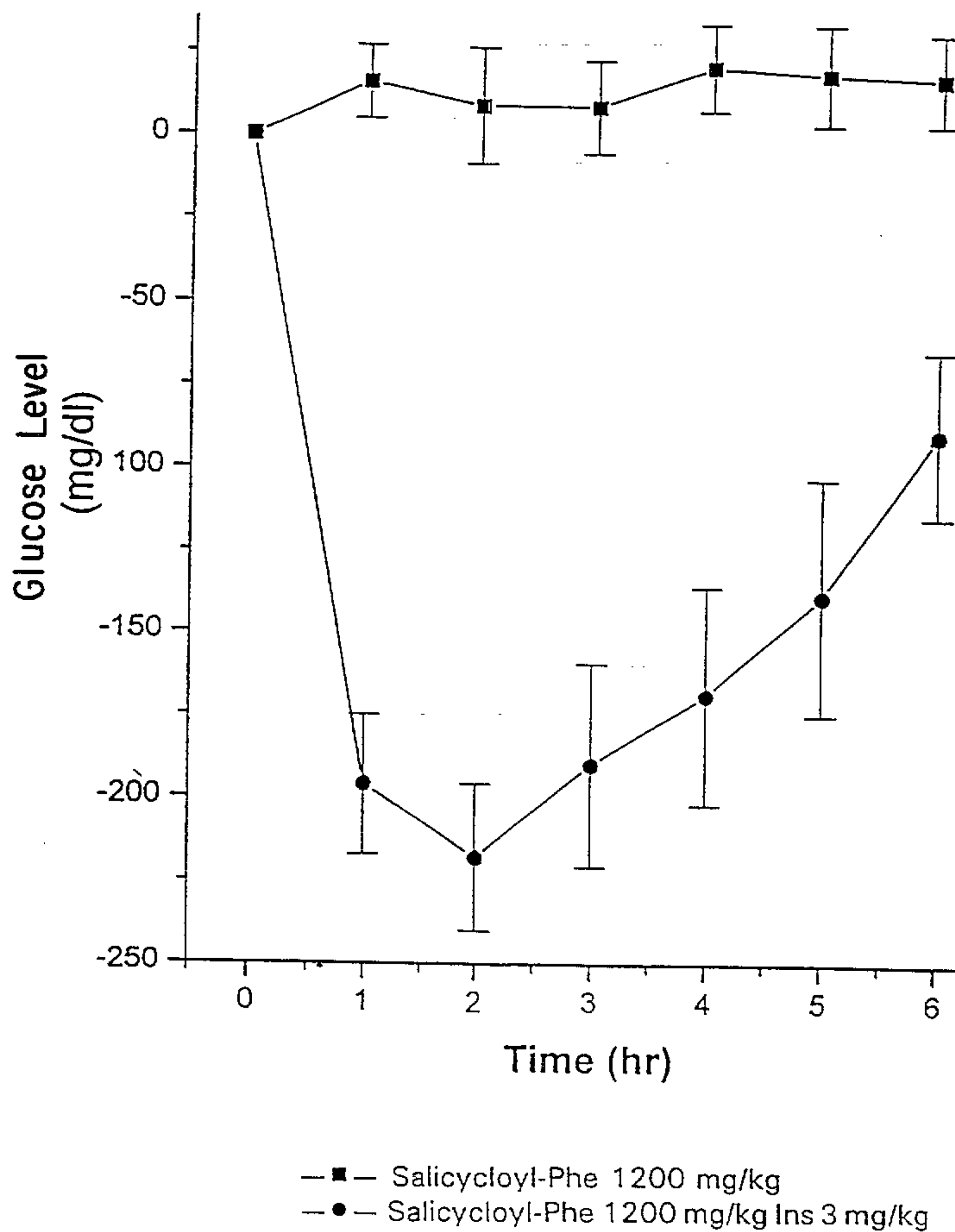
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FIG. 7



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FIG. 8

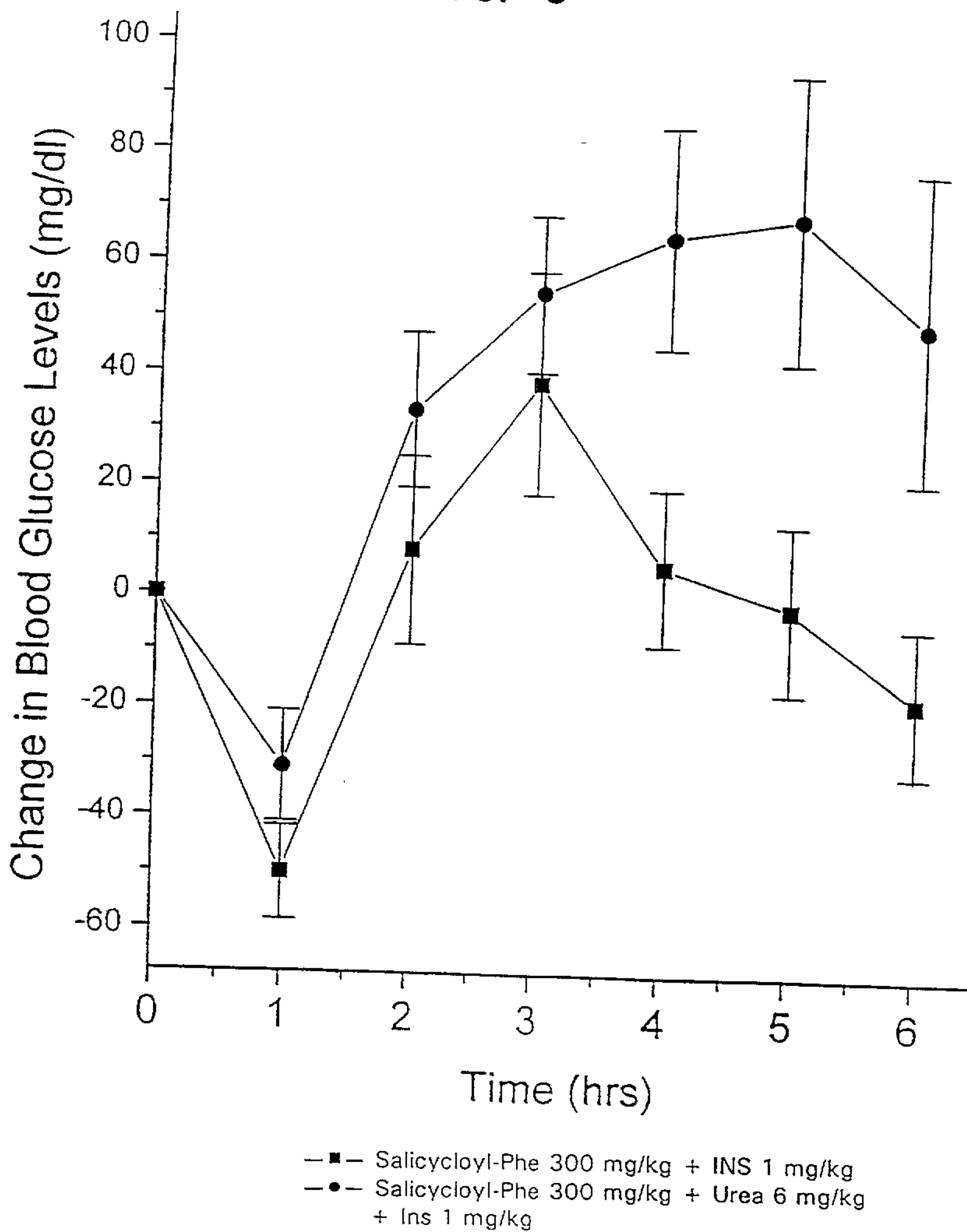




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FIG. 9



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FIG. 10

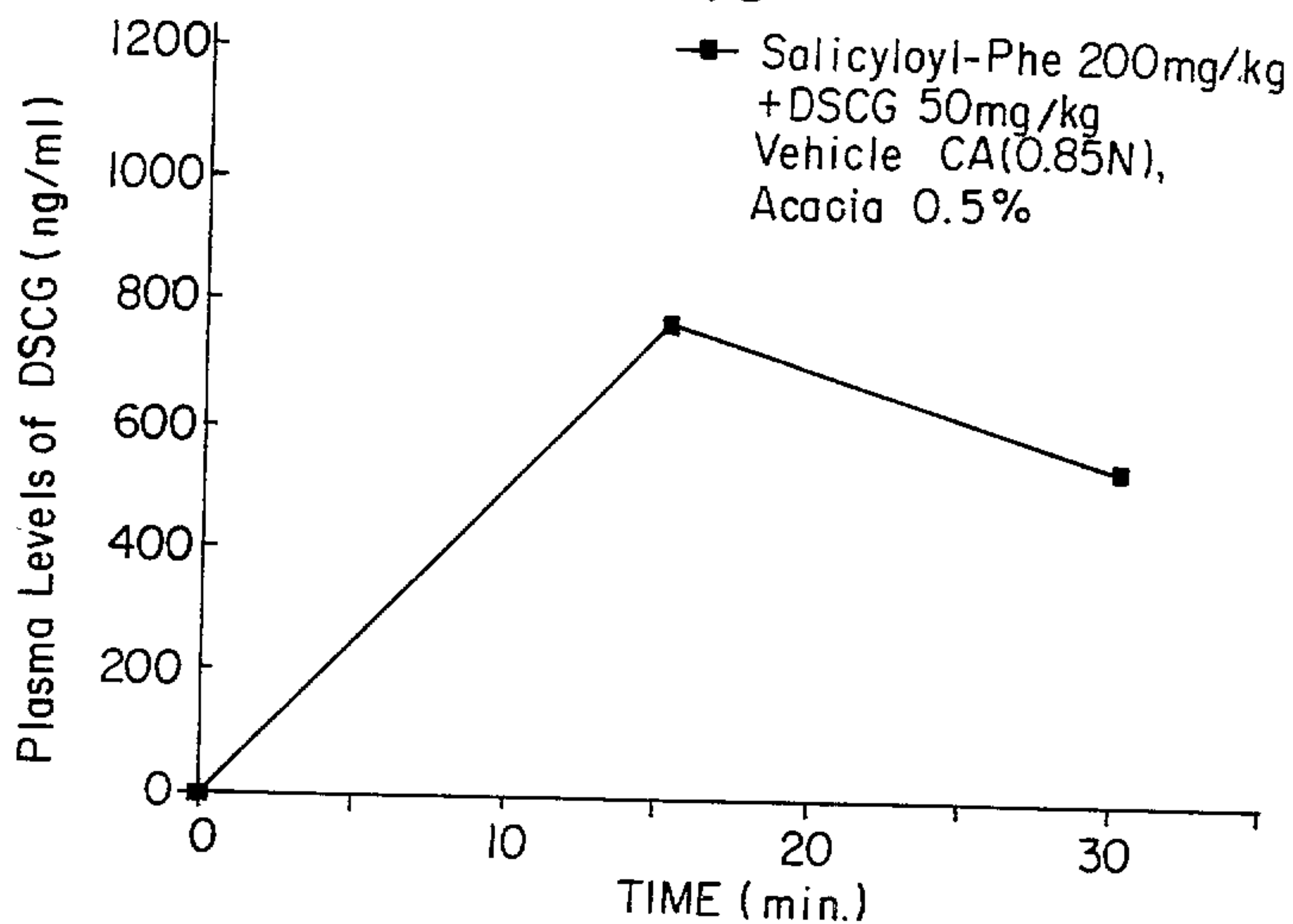


FIG. 11

