(100–200 μl), instead of the previous requirements of 1–3 ml for II and III (8, 10–13) and 4–5 ml for IV (15, 17), without increasing the lowest detectable serum concentration. The high resolution of the capillary column (23) reduced the extraction stage to a single step instead of the three steps often suggested to eliminate the interfering substances (12, 13, 15, 17). Furthermore, derivatization steps such as silylation (10) and methylation (12) were unnecessary. The rapid elution time (24) shortened the retention times. These conditions led to shorter extraction and chromatographic stages. Nevertheless, the accuracy and sensitivity were usually improvements over previous assays (3–5, 9, 11, 12, 15, 17).

The determination in serum of four drugs useful in toxicology (25–29) or therapeutic monitoring (30–32) demonstrated the versatility and potential of this chromatographic system and its possible routine use in view of the growing importance of serum level determinations of an increasing number of diverse drugs in clinical practice.

The method was demonstrated by the determination of serum concentrations of diazepam in five epileptic patients (Table IV), of meprobamate in four cases of voluntary overdose (Table V), and of phenylbutazone in a nursing mother given a 250-mg suppository twice daily for 10 days (Table VI).

REFERENCES

(1) J. P. Cano, L. Vignoli, and A. Viala, *Ann. Pharm. Fr.*, **25**, 697 (1967).

(2) J. A. F. De Silva and C. V. Puglisi, *Anal. Chem.*, **42**, 1725 (1970).

(3) H. P. Gelbke, H. J. Schlicht, and G. Schmidt, *Arch. Toxicol.*, **38**, 295 (1977).

(4) M. Linnoila and F. Dorrity, Jr., *Acta Pharmacol. Toxicol.*, **41**, 458 (1977).

(5) E. Rey, J. M. Turquais, and G. Olive, *Clin. Chem.*, **23**, 1338 (1977).

(6) L. B. Foster and C. S. Frings, *ibid.*, **16**, 177 (1970).

(7) F. Marcussi, R. Fonelli, and E. Mussini, *J. Chromatogr.*, **37**, 318 (1968).

(8) L. Martis and R. H. Levy, *J. Pharm. Sci.*, **63**, 834 (1974).

(9) M. P. Rabinowitz, P. Reisberg, and J. I. Bodin, *ibid.*, **61**, 1974 (1972).

(10) R. B. Bruce, W. R. Maynard, and L. K. Dunning, *ibid.*, **63**, 446 (1974).

(11) I. J. McGilveray, K. K. Midha, R. Brien, and L. Wilson, *J. Chromatogr.*, **89**, 17 (1974).

(12) K. K. Midha, I. J. McGilveray, and C. Charette, *J. Pharm. Sci.*, **63**, 1234 (1974).

(13) J. A. Bogan, *J. Pharm. Pharmacol.*, **29**, 125 (1977).

(14) A. Sioufi, F. Caudal, and F. Marfil, *J. Pharm. Sci.*, **67**, 243 (1978).

(15) F. A. J. Vanderheeren, D. J. C. J. Theunis, and M.-T. Rosseel, *J. Chromatogr.*, **120**, 123 (1976).

(16) S. H. Curry and G. P. Mould, *J. Pharm. Pharmacol.*, **21**, 674 (1969).

(17) E. C. Dinovo, L. A. Gottschalk, B. R. Nandi, and P. G. Geddes, *J. Pharm. Sci.*, **65**, 667 (1976).

(18) J. de Graeve and J. Vanroy, *J. Chromatogr.*, **129**, 171 (1976).

(19) F. Berthou, D. Picart, L. Bardou, and H. H. Floch, *ibid.*, **118**, 135 (1976).

(20) J. H. Goudie and D. Burnett, *Clin. Chim. Acta*, **43**, 423 (1973).

(21) R. C. Baselt and R. H. Cravey, *J. Anal. Toxicol.*, **1**, 81 (1977).

(22) C. L. Winek, *Clin. Chem.*, **22**, 832 (1976).

(23) D. Debruyne, H. Kinsun, M. A. Moulin, and M. C. Bigot, *J. Pharm. Sci.*, **68**, 511 (1979).

(24) J. Macloue, M. Rigaud, J. Durand, and X. Chebrou, *Prostaglandins*, **6**, 999 (1976).

(25) R. K. Maddock and H. A. Bloomer, *J. Am. Med. Assoc.*, **201**, 999 (1967).

(26) J. Kanto, R. Sellman, M. Haataja, and P. Hurme, *Int. J. Clin. Pharmacol. Biopharm.*, **16**, 258 (1978).

(27) J. E. Cree, J. Meyer, and D. M. Hailey, *Br. Med. J.*, **4**, 251 (1973).

(28) A. Gaucher, R.-J. Royer, P. Netter, M. J. Royer-Morrot, G. Faure, and J. Pourel, *Sem. Hôp.*, **52**, 1723 (1976).

(29) L. A. Gottschalk, E. Dinovo, R. Biener, and B. R. Nandi, *J. Pharm. Sci.*, **67**, 155 (1978).

(30) M. M. Reidenberg, M. Levy, H. Warner, C. B. Coutinho, M. A. Schwartz, G. Yu, and J. Cheripko, *Clin. Pharmacol. Ther.*, **23**, 371 (1978).

(31) R. J. Royer, M. Meyruey, A. Maillard, C. Monnod, M. J. Morrot, and P. Netter, *Ann. Med. Nancy*, **4**, 1317 (1975).

(32) R. Axelsson and E. Martensson, *Curr. Ther. Res.*, **19**, 242 (1976).

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Immobilization of Proteins in Microspheres of Biodegradable Polyacryldextran

PETER EDMAN, BO EKMAN, and INGVAR SJÖHOLM ˣ

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Abstract □ Macromolecules were immobilized by an emulsion polymerization technique in biodegradable microspheres of polyacryldextran, prepared by copolymerizing bisacrylamide with acryldextran. Such particles can be characterized by D–T–C expressions, where D denotes the concentration of derivatized dextran, T is the total concentration of acrylic compounds in the monomeric solution, and C denotes the fraction of cross-linker. In microparticles based on dextran T40 with a D–T–C of 11–1–75, the yield of immobilized protein was greater than in polyacrylamide particles. The properties of the immobilized proteins, *e.g.*, K_m and V_{max}, were retained. The heat stability of the proteins was improved so that 5–10% of carbonic anhydrase still was active after 30 min at 100°. However, the leakage of proteins from the polyacryldextran particles was greater than from polyacrylamide particles.

Keyphrases □ Microspheres—biodegradable polyacryldextran particles, immobilization of proteins □ Polyacryldextran—microspheres, immobilization of proteins □ Proteins—immobilization in polyacryldextran microspheres

The therapeutic use of proteins *in vivo* usually is hampered by rapid protein inactivation or by immunological reactions when the proteins are immunogenic. For instance, asparaginase is not active for long periods in the treatment of acute leukemias (1). However, the noncovalent immobilization of the proteins in microspheres of

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highly cross-linked polyacrylamide significantly increases stability against denaturation by heat or proteolytic enzymes (2, 3).

BACKGROUND

The proteins are immobilized by two mechanisms: they are partially bound in the polymeric threads and partially entrapped in the polymeric network if they are sufficiently large (2). The leakage is negligible and the biological properties essentially are retained when the particles are small and macroporous (3–5). However, the yield of protein immobilized in the microparticles generally is low (5–10%). After being cleared from the circulation by the reticuloendothelial system, the particles are metabolized slowly, mainly in the liver and spleen (6). The half-life of microspheres injected intravenously or intraperitoneally in the mouse is 10–14 weeks in the liver (6), which in most cases is an inconveniently long half-life.

Preliminary experiments indicated that the yield of proteins in the microspheres can be increased by adding hydrophilic polymers (*e.g.*, dextrans, starch, and polyvinylpyrrolidones) to the monomeric solution during polymerization. In addition if dextran comprises a part of the hydrocarbon chain in the polyacrylamide and forms an intimate part of the microsphere, the polymer should be metabolized more easily. This result can be achieved if the polymeric microspheres are formed from a dextran substituted with acrylic groups and suitable cross-linking agents, *e.g.*, bisacrylamide.

The present paper describes the synthesis of an acryl ether of dextran, the preparation and characteristics of polyacryldextran microparticles, and the properties of the immobilized proteins.

EXPERIMENTAL

Materials—Human serum albumin[1], immunoglobulin G[1], bovine carbonic anhydrase[2] (EC 4.2.1.1), and catalase[3] (EC 1.11.1.6) were used without further purification. Acrylamide[4], *N,N'*-methylenebisacrylamide[4] (I), *p*-nitrophenyl acetate[2], *N,N,N',N'*-tetramethylethylenediamine[2], tris(hydroxymethyl)aminomethane[2], acrylic acid glycidyl ester[3], and other chemicals were analytical grade.

Synthesis of Acrylic Dextrans—Acrylic derivatives of dextran[5] were prepared by reacting acrylic acid glycidyl ester with aqueous alkaline solutions of dextrans of different molecular weights (10,000–2,000,000).

In a typical experiment, dextran (30 g) was dissolved in 250 ml of pH 11, 0.01 *M* carbonate buffer. Acrylic acid glycidyl ester (15 ml) was added, and the two-phase system was stirred magnetically for 5 days at room temperature. The dextran was precipitated with 1000 ml of ethanol and washed with ~300 ml of ethanol. The precipitated dextran was dissolved in 100 ml of distilled water, precipitated again, and washed with ethanol. The procedure was repeated at least four times to free the product from excess glycidyl ester.

Determination of Acrylic Groups—The content of acrylic groups in the dextran derivatives was measured by a method based on the halogenation of the double bonds (7). Aqueous bromine solution was added to the unsaturated acrylic groups, and the subsequent color reduction at 480 nm was measured. The number of acrylic groups was determined from a standard curve obtained with acrylamide as a standard.

Preparation of Microparticles—Microparticles with immobilized proteins were prepared according to a reported method (2, 3). The protein was dissolved in the acrylic monomer solution. In a typical example, the aqueous phase was composed of 3.75 ml of a 2% aqueous solution of I and 6.25 ml of an acryldextran solution (containing 177 mg of derivatized dextran T40/ml with 4.28 mg of acrylic groups/ml).

The water solution was homogenized together with 500 ml of chloroform–toluene (1:4) to produce a water-in-oil emulsion. By addition of catalysts, the water-phase droplets were polymerized to microparticles, which were freed from the organic phase by repeated washings with water and buffer. The microparticles were isolated easily by centrifugation.

^{14}C-Labeled particles were prepared as described by Sjöholm and Edman (6) with [^{14}C]I.

The polyacryldextran gels can be characterized by D–T–C expressions by analogy with the T–C nomenclature suggested by Hjertén (8) for homogeneous polyacrylamide. The first value (D) denotes the concentration of derivatized macromolecules (in this case, dextran) participating in particle formation (grams per 100 ml of solvent). The second value (T) denotes the total concentration of acrylic monomers (grams per 100 ml of solvent). The third value (C) denotes the relative amount of the cross-linker, I, expressed as the percentage (w/w) of the total amount of acrylic monomers. The example given for microparticle preparation yielded particles having a D–T–C of 11–1–75.

Protein Determination—The protein content of the microparticles was determined by amino acid analysis with an automatic amino acid analyzer after hydrolysis in 6 *M* HCl at 105° for 20 hr. The amount of proteins leaking out from the microparticles was determined according to the method of Lowry *et al.* (9). In each case, the protein studied was used as the standard.

Determination of Microparticle Density—The density of the microparticles was determined by density gradient centrifugation using a self-generating gradient medium[6] as reported earlier (2).

Determination of Carbonic Anhydrase Activity—The activity of the carbonic anhydrase was determined using *p*-nitrophenyl acetate as the substrate (10). The enzyme in free form or in microparticles was added to 4.7 ml of 0.05 *M* tris(hydroxymethyl)aminomethane hydrochloride buffer at pH 7.4. The hydrolysis of *p*-nitrophenyl acetate was followed spectrophotometrically at 400 nm at room temperature (22°). From the change of the absorbance, the velocity of the reaction was calculated [$\epsilon = 2.1 \times 10^4$ (10)] and expressed as micromoles hydrolyzed per minute. Correction was made for the spontaneous hydrolysis of the substrate. The microparticles of polyacryldextran did not cause any light scattering at the wavelength used.

Radioactivity—Radioactivity was measured by liquid scintillation counting.

Functional Capacity of Albumin—The functional capacity of albumin was determined as described previously (11). Microparticles with albumin were incubated with [^{14}C]salicylic acid. The amount of albumin binding salicylic acid was calculated from the binding degree and from a standard curve derived by equilibrium dialysis with different albumin concentrations.

Iodination of Human Serum Albumin—Human serum albumin (5 mg) in 0.3 ml of 0.01 *M* phosphate buffer at pH 7.5 was labeled with sodium [^{125}I]iodide[7] (1 mCi) in the presence of lactoperoxidase[2] (EC 1.11.1.7) (40 μg) and hydrogen peroxide (20 μl, 0.005 *M*) according to a literature method (12). The reaction was stopped after 10 min by dilution of the sample with buffer. Separation of ^{125}I-labeled human serum albumin from the reaction mixture was carried out by gel filtration on a column (43 cm^3) of Sephadex G-25[8].

RESULTS

Preparation of Acryldextrans—Acrylic acid glycidyl ester reacts with a hydroxyl group of a glucose residue in the dextran to form a 3-acryloyl 2-hydroxypropyl ether. The molecular weight of the dextrans used (T10, T40, T70, T500, and T2000) ranged from 10,000 to 2,000,000. All dextrans followed a zero-order reaction with the same rate. After 5 days at room temperature (22–24°), one acrylic group was introduced per 20 glucose residues, regardless of the molecular weight of the dextrans.

The best dextran derivative for preparation of the microparticles was T40 (mol. wt. 40,000). Dextrans of higher molecular weight (T70, T500, and T2000) had a solubility too limited for the particle preparation and gave too high a viscosity of the water phase. The lower molecular weight dextran derivative (T10) did not significantly increase the yield of proteins in the particles.

Immobilization of Proteins in Polyacryldextran—The amount of proteins immobilized in polyacrylamide microparticles depends on the size of the protein and the composition of the polymer, which is characterized by the concentration of monomers (T) and the fraction of cross-linking agent (C) in the water phase. The pore radius of the polymer formed is dependent on both T and C. In addition, the radius of the polymeric threads forming the polymeric network is directly proportional to C (2). This relationship generally means that the incorporation of proteins increases with C, but, at the same time, the functional availability of the immobilized proteins is impaired when the pore radius decreases. An increased yield of immobilized proteins also is achieved by increasing

[1] KABI AB, Stockholm, Sweden.
[2] Sigma Chemical Co.
[3] Fluka AG.
[4] Eastman Kodak Co.
[5] Dextrans of different molecular weights (T10, T40, T70, T500, and T2000) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.
[6] Percoll, Pharmacia Fine Chemicals, Uppsala, Sweden.
[7] AB Atomenergi, Studsvik, Sweden.
[8] Pharmacia Fine Chemicals, Uppsala, Sweden.

Table I—Amount of Albumin Immobilized in Microparticles of Different Gel Compositions [a]

Particle Composition (D-T-C)	Total Amount of Protein Immobilized, mg	Total Amount of Active Protein in Particles, mg
0-8-35	46.5	17.8
(10)[b]-8-35	59.8	33.2
10-8-35	92.8	56.8
0-4-100	8.0	2.7
4-2-96	22.6	15.0
0-5-80	16.4	3.8
11-1-75	83.5	66.8

[a] The study was carried out with 5 ml of the water phase with 500 mg of ^{125}I-labeled human serum albumin. After the preparation of the particles, their content of protein was measured in a γ-counter. The amount of functionally active protein in the microparticles was determined by estimating the capacity of the albumin to bind [^{14}C]salicylic acid (11). [b] Underivatized dextran.

the concentration of hydrophilic polymers in the water phase, *e.g.*, by adding starch or dextran. Table I summarizes some results from studies on the incorporation of ^{125}I-labeled human serum albumin in different microspheres of highly cross-linked polyacrylamide and polyacryldextran based on dextran T40.

It is evident from Table I that addition of 10% dextran to microparticles with T–C values of 8–35 increased the total yield of protein from 46.5 to 60 mg under the conditions used. The yield was improved further when the dextran was bound covalently in the polymer, as in acryldextran. However, the functional availability measured by the amount of salicylic acid bound was still low. The effect of the acryldextran is illustrated further by the results from other particles. In particles with D–T–C values of 0–4–100 (*i.e.*, those using only I at a concentration of 4%), 8 mg of albumin was incorporated. By adding acryldextran (0.4 g/10 ml) containing 2% acrylic groups, which constitutes only 4% of the total number of monomeric acrylic groups in the water phase, the immobilization of protein was increased significantly. Not even by adding acrylamide (to 20% of the total amount) to D–T–C values of 0–5–80 could the same increase be achieved.

In the subsequent work, polyacryldextran microparticles with D–T–C values of 11–1–75 were used. Dextran T40 containing 2.3% acrylic side chains (w/w) was employed. The amount of I added thus corresponded to 0.075 g/10 ml. The total yield of albumin in such particles was ~83.5 mg, *i.e.*, 16.7% of the total amount present in the water phase, and 80% of this amount was functionally active, *i.e.*, it bound salicylic acid.

Density of Polyacryldextran Microspheres—The density of polyacrylamide microparticles is largely determined by the porosity of the gel (2, 3). Linear polyacrylamide has a density of 1.15 g/ml, which decreases initially when the fraction of the cross-linker increases up to 5–10%. Above this limit, the characteristics of the polymer change to a more macroporous structure, so that the density increases again with C.

Proteins immobilized in the gel decrease the density of the particles (2). As expected, the same phenomenon was seen when dextran was included in the gel. This result most probably was due to two effects: a decreased porosity of the gel and the lower density of dextran compared to that of polyacrylamide. Thus, microparticles with D–T–C values of 11–1–75 have a density of 1.030 g/ml as determined in the colloidal silica density gradient by centrifugation.

Properties of Immobilized Carbonic Anhydrase—The enzymatic properties of carbonic anhydrase in the free state and in polyacryldextran microparticles are compared in Fig. 1*a*. In the two series of experiments, the amounts of enzyme were the same, based on amino acid analysis. From this figure, the Michaelis constant (K_m) for free and immobilized carbonic anhydrase was calculated to be 18 and 22 mM, respectively. The maximal velocity (V_{max}) as calculated from the y axis intercepts was 15% lower with the immobilized enzyme, which means that the enzyme was not utilized efficiently in the microparticles.

Figure 1*b* shows the differences in the enzymatic properties of carbonic anhydrase immobilized in microspheres and after solubilization of the same spheres with dextranase[2] (EC 3.2.1.11) (4 IU/ml of particle suspension incubated for 3 hr at 22°). The Michaelis constant (K_m) was 21 mM for the immobilized enzyme compared to 18 mM for the enzyme released after solubilization of the spheres. This value is the same as that found with the free enzyme. In addition, the dextranase treatment of the microspheres resulted in a 10% increase in V_{max}.

Leakage of Proteins from Polyacryldextran Microspheres—Microparticles (based on dextran T40 with D–T–C values of 11–1–75) were prepared with proteins of various sizes, carbonic anhydrase [mol. wt. 31,000 (13)], human serum albumin [mol. wt. 66,500 (14)], human immunoglobulin G [mol. wt. 150,000 (15)], and catalase [mol. wt. 240,000 (16)]. The microspheres were suspended in pH 7.4 physiological saline so that the protein concentration was 0.2, 4.91, 0.9, and 0.21 mg/ml, respectively. Sodium azide (0.1%) was added as a preservative, and the samples were left at room temperature for 80 days. After centrifugation, the leakage of protein from the spheres was determined in the supernate. Figure 2 summarizes the results. Thus, 65% of carbonic anhydrase was lost from the particles after 80 days, while 33% of human serum albumin, 44% of immunoglobulin G, and 30% of catalase were lost.

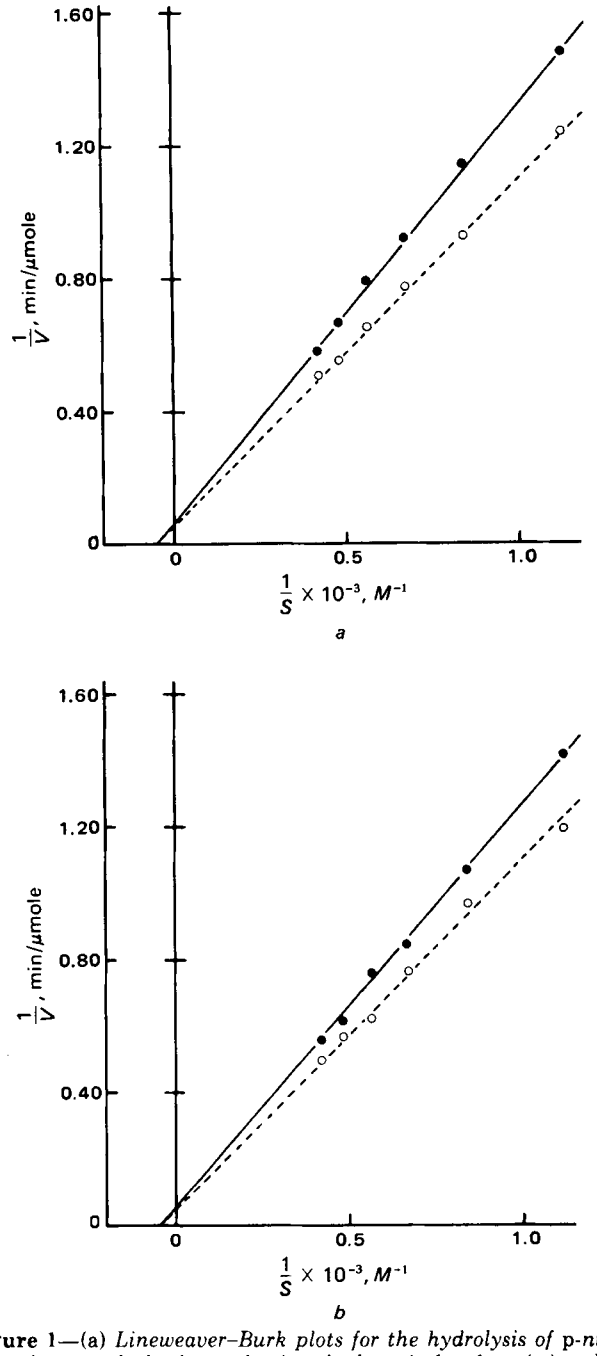

Figure 1—(a) *Lineweaver–Burk plots for the hydrolysis of* p*-nitrophenyl acetate by bovine carbonic anhydrase in free form* (○) *and immobilized* (●) *in microparticles of polyacryldextran.* (b) *Lineweaver–Burk plots obtained with carbonic anhydrase in microparticles before* (●) *and after* (○) *treatment with dextranase.*

Thermostability of Immobilized Carbonic Anhydrase—To investigate the heat stability of immobilized proteins, carbonic anhydrase was incorporated into microspheres of polyacryldextran (based on dextran T40). The spheres were exposed to elevated temperatures for 5 and 30 min. After treatment, the microspheres were cooled in an ice bath for

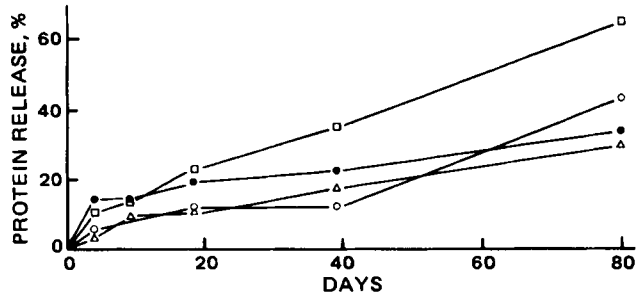

Figure 2—*Leakage of proteins from microspheres during storage at room temperature at pH 7.4. The immobilized proteins were carbonic anhydrase (□), human serum albumin (●), immunoglobulin (○), and catalase (△).*

5 min. After preincubation of the samples at room temperature for 30 min, the remaining activity was measured. Figure 3 shows that the immobilized enzyme was more resistant to heat treatment. All of the free enzyme and 50% of the immobilized enzyme were denatured at 65°. Approximately 5–10% of the enzyme activity was retained even after 30 min at 100°.

Hydrolytic Stability of Polyacryldextran Microspheres—The hydrolytic stability of the polymer, polyacryldextran, was investigated with particles with ^{14}C-labeled I as a cross-linking agent. The labeled microparticles then were incubated at different pH values (2–12) for 2, 4, 8, 12, and 24 hr at 37°. After centrifugation for 15 min at 4°, the supernate was withdrawn and the radioactivity content was measured in the scintillation counter. Figure 4 summarizes the results. The microspheres were not affected significantly at pH 2–9. At pH 11, ~20% of the particles were hydrolyzed in 24 hr. Essentially all of the particles were lost at pH 12 when stored at 37° for 24 hr.

DISCUSSION

The immobilization of proteins in acrylic polymers is influenced strongly by the composition of the polymer, *i.e.*, the total concentration of monomers and the relative fraction of the cross-linker (T and C) in the polymerization mixture. In the present study, the presence of polymers in the mixture, *e.g.*, dextrans or acrylic dextran derivatives, significantly affected the incorporation of protein in the microparticles formed by emulsion polymerization. The yield of protein in the microparticles was increased substantially, even when the total concentration of acrylic monomeric groups in the water phase was decreased substantially. To specify the composition of the polyacryldextran formed, it is suggested that the content of derivatized polymer present is given in grams per 100 ml by adding a D value to the existing nomenclature for characterizing polyacrylamide gels.

The yield of entrapped protein in polyacryldextran may be increased by two mechanisms. First, addition of dextran decreases the gel pore radius. Second, the dextran sterically retains the protein inside the gel during polymerization. The result is increased protein entrapment, implying that the fraction of protein immobilized in the polymeric threads is decreased. This result is evident from the heat stability of incorporated carbonic anhydrase and the leakage of proteins from the microparticles. The fraction of the enzyme that resists treatment at 100° for 30 min is smaller in polyacryldextran (10%) than in polyacrylamide [30% (3)], and the fraction leaking out from polyacryldextran when stored for prolonged periods is substantially larger.

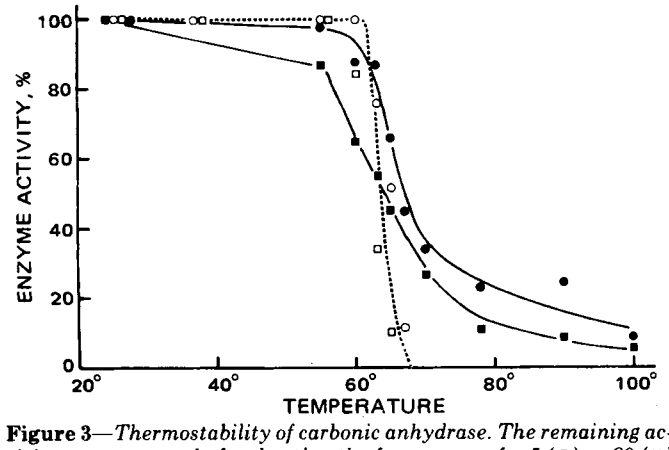

Figure 3—*Thermostability of carbonic anhydrase. The remaining activity was measured after heating the free enzyme for 5 (○) or 30 (□) min and the immobilized enzyme in polyacryldextran for 5 (●) or 30 (■) min.*

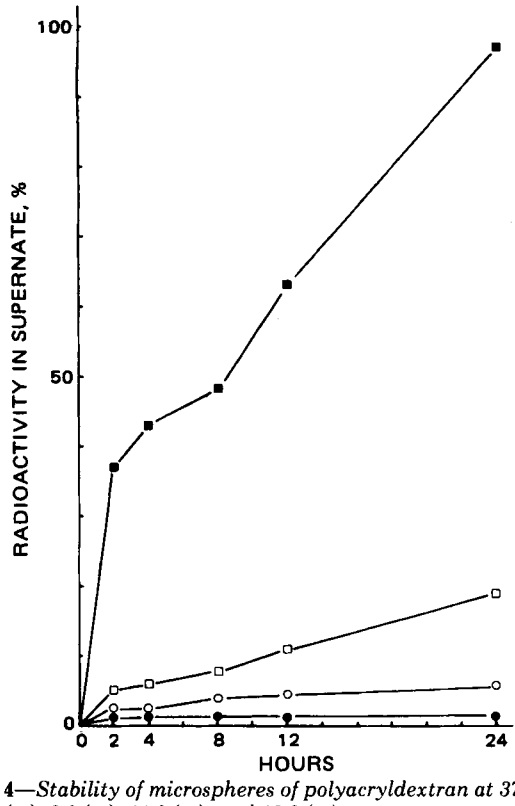

Figure 4—*Stability of microspheres of polyacryldextran at 37° and at pH 2–7 (●), 9.0 (○), 11.0 (□), and 12.0 (■).*

However, in other respects, immobilization in polyacryldextran offers the same advantages as in normal polyacrylamide. For example, the proteins retain their biological properties; K_m and V_{max} of carbonic anhydrase are affected only insignificantly, indicating that the equilibrium between enzyme and substrate is not diffusion dependent, which means that the substrate is not hindered sterically providing the particles are small. Moreover, the incorporation is a mild process as shown by the finding that the small loss in V_{max} and the increase in K_m are recovered almost completely after digestion of the polymer with dextranase.

In addition, other experiments[9] with 11–1–75 particles showed that polyacryldextran was metabolized more rapidly *in vitro* with rat liver homogenates and also was eliminated faster *in vivo* after intraperitoneal injection in mice than in the cases involving polyacrylamide (T–C value of 8–25). These findings were expected since dextran should be degraded easier *in vivo* than hydrocarbon chains, the content of which is comparatively small in polyacryldextran particles with D–T–C values of 11–1–75. The use of dextran thus offers a rich variation in the design of polymeric particles having different biological half-lives. The duration of the enzymatic effects *in vivo* can be controlled adequately without unwanted intracellular accumulation of polymers.

The results presented here were obtained with microparticles of polyacryldextran with D–T–C values of 11–1–75, *i.e.*, those with low T. The total yield of albumin immobilized in the particles was ~15% but could be increased up to ~20% by increasing T, *i.e.*, the concentration of the acrylic monomers in the polymerization mixture. However, the functional availability of the immobilized protein was impaired simultaneously, probably due to the decreased pore radius of the polymer. Furthermore, the leakage of protein from the microspheres was decreased.

In some applications, microparticles with larger amounts of proteins have advantages, even if such particles will be metabolized more slowly. Therefore, for each situation, a compromise must be made between total yield and functional availability and will depend on the desired rate of degradation of the gel, the cost of protein, the acceptable or desired rate of leakage of proteins, and the type of effect aimed at *in vivo*.

[9] P. Edman and I. Sjöholm, manuscript in preparation.

REFERENCES

(1) D. Killander, A. Dohlwitz, L. Engstedt, S. Franzén, G. Gahrton, B. Gullbring, G. Holm, A. Holmberg, S. Höglund, A. Killander, D. Lockner, H. Mellstedt, P. J. Moe, J. Palmblad, P. Reizenstein, K. O. Skårberg, B. Swedberg, A.-M. Udén, B. Wadman, L. Wide, and L. Åhström, *Cancer*, **37**, 220 (1976).

(2) B. Ekman, C. Lofter, and I. Sjöholm, *Biochemistry*, **15**, 5115 (1976).

(3) B. Ekman and I. Sjöholm, *J. Pharm. Sci.*, **67**, 693 (1978).

(4) P. Edman and I. Sjöholm, *J. Pharmacol. Exp. Ther.*, **211**, 663 (1979).

(5) A. Kober, B. Ekman, and I. Sjöholm, *J. Pharm. Sci.*, **67**, 107 (1978).

(6) I. Sjöholm and P. Edman, *J. Pharmacol. Exp. Ther.*, **211**, 656 (1979).

(7) H. Hoppe, J. Koppe, and F. Winkler, *Plaste Kautsch.*, **24**, 105 (1977).

(8) S. Hjertén, *Arch. Biochem. Biophys. Suppl. 1*, 147 (1962).

(9) O. Lowry, N. Rosebrough, L. Farr, and R. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(10) Y. Pocker and J. Stone, *J. Am. Chem. Soc.*, **87**, 5497 (1965).

(11) I. Sjöholm, B. Ekman, A. Kober, I. Ljungstedt-Påhlman, B. Seiving, and T. Sjödin, *Mol. Pharmacol.*, **16**, 767 (1979).

(12) J. Marchalonis, *Biochem. J.*, **113**, 299 (1969).

(13) S. Lindskog and B. Malmström, *J. Biol. Chem.*, **237**, 1129 (1962).

(14) B. Meloun, L. Morávek, and V. Kostka, *FEBS Lett.*, **58**, 134 (1975).

(15) G. Edelman, B. Cunningham, W. Gall, P. Gottlieb, U. Rutishauser, and M. Waxdal, *Proc. Natl. Acad. Sci. USA*, **63**, 78 (1969).

(16) T. Samejima, W. McCabe, and J. Yang, *Arch. Biochem. Biophys.*, **127**, 354 (1968).

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Isolation, Identification, and Synthesis of 4-Amino-6,7-dimethoxy-3-quinolinol, the Major Metabolite of Amiquinsin Hydrochloride in Rats and Humans

GEORGE C. WRIGHT *x, RONALD J. HERRETT ‡, JAMES P. HEOTIS ‡, and JAMES L. BUTTERFIELD ‡

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Abstract □ The metabolism of amiquinsin hydrochloride (4-amino-6,7-dimethoxyquinoline hydrochloride monohydrate, I) was studied in rats and humans. The major metabolite isolated from human urine was identified through synthesis as 4-amino-6,7-dimethoxy-3-quinolinol hydrochloride hydrate (II). Acid hydrolysis of the major metabolite from rat urine also gave II. The structure of the rat metabolite subsequently was tentatively identified as the potassium salt of the 3-*O*-sulfate of II. The pharmacological and toxicological properties of I and II are discussed.

Keyphrases □ Amiquinsin hydrochloride—rat and human metabolites, isolation and identification □ Antihypertensive agents—amiquinsin hydrochloride, rat and human metabolites, isolation and identification □ 4-Amino-6,7-dimethoxy-3-quinolinol—isolation and identification as metabolite of amiquinsin hydrochloride, rats and humans

Wright *et al.* (1) reported that amiquinsin hydrochloride (4-amino-6,7-dimethoxyquinoline hydrochloride monohydrate, I) (Scheme I) demonstrated hypotensive activity. Early biological studies with I included the pharmacological mechanism of action (2) and the absorption, distribution, and elimination of I in dogs (3, 4).

In the present investigation, the metabolism of I was studied in rats and humans. Williams (5) reported that 4-aminoquinoline administered to rabbits was metabolized to 4-amino-3-hydroxyquinoline, which was excreted mainly as an ether sulfate, and that glucuronic acid conjugation was low. Although 4-amino-7-chloroquinoline also was believed to be hydroxylated in rabbits to the 3-hydroxy derivative (5), hydroxylation of this compound in humans was not observed (6).

RESULTS AND DISCUSSION

Isolation and Identification of Metabolites—Metabolite studies with I were done initially in rats. When the rats were dosed orally with [3-^{14}C]I, the major excreted material was parent I; TLC followed by estimation of the activity of the radioactive materials showed the presence of I (61%) and substances at R_f 0.6 (25%), 0.0 (12%), and 0.9 (2%). The materials at R_f 0.0 and 0.9 were not identified.

A preparative method was developed for the isolation of the major rat metabolite at R_f 0.6 that involved separation and purification by gel filtration.

Treatment of the purified R_f 0.6 metabolite with hot methanolic hydrogen chloride gave a hydrolyzed product (hydrochloride of R_f 0.6 metabolite) with physical properties similar to those of the starting R_f 0.6 compound but with significant differences in the NMR spectra and TLC values (Table I). However, a comparison of the physical properties of the hydrolyzed product with those of the synthetic 3-hydroxy derivative II (Table I) clearly demonstrates that the two compounds are identical with respect to their NMR and UV spectra and TLC values. Thus, the hydrochloride of the hydrolyzed R_f 0.6 metabolite was identified as 4-amino-6,7-dimethoxy-3-quinolinol hydrochloride.

The R_f 0.6 metabolite was characterized further through enzymatic hydrolysis with a combination of β-glucuronidase and aryl sulfatase. Isolation of the end-products of enzymatic treatment was attained by extraction and TLC, and each spot then was counted for carbon 14. In the absence of enzymes, or by treatment with β-glucuronidase alone, the structure of the R_f 0.6 metabolite was not altered (Table II). However, treatment with aryl sulfatase and β-glucuronidase together converted the ^{14}C-labeled R_f 0.6 metabolite to [^{14}C]II. Thus, the enzymatic data indicate that the R_f 0.6 metabolite may be the sulfate conjugate of II. The IR spectrum showed a strong S=O stretching band at 8.1 μm, also indicative of a sulfate group in the molecule. The NMR spectra showed that both the R_f 0.6 metabolite and II are substituted in the 3-position. Considering the specificity of the enzymes used, the IR data, and the substitution pattern from the NMR spectra, the R_f 0.6 metabolite probably

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