# Oral Hydrocortisone Pharmacokinetics: A Comparison of Fluorescence and Ultraviolet High-Pressure Liquid Chromatographic Assays for Hydrocortisone in Plasma

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**Abstract** □ Three fasted, male subjects received single 10-, 30-, and 50-mg oral doses of hydrocortisone tablets on separate occasions. Endogenous hydrocortisone was suppressed by giving 2 mg of dexamethasone 9 hr prior to dosing. Plasma samples obtained serially for 8 hr after hydrocortisone dosing were assayed by reversed-phase high-pressure liquid chromatography (HPLC) with UV detection and by normal-phase HPLC with fluorescence detection of the dansylhydrazine derivative of hydrocortisone. The two assay methods yielded equivalent plasma hydrocortisone concentrations. Metabolite interference was absent in both assay methods. Drug concentrations in plasma from all three doses of hydrocortisone were described by one-compartment open-model kinetics, with first-order absorption and elimination, and an absorption lag time. Mean $C_{max}$ values of 199, 393, and 419 ng/ml were obtained at 1.0, 1.0, and 1.7 hr following the 10-, 30-, and 50-mg doses, respectively. Hydrocortisone was cleared from plasma with an elimination half-life of ~1.5 hr. Within the dosage range studied, plasma levels of hydrocortisone were related, but not directly proportional, to dose size. This apparent lack of proportionality may be due to reduced drug availability or altered distribution with increasing dose.

**Keyphrases** □ High-pressure liquid chromatography—comparison of fluorescence and UV high-pressure liquid chromatographic assays for hydrocortisone in plasma, humans □ Hydrocortisone—comparison of fluorescence and UV high-pressure liquid chromatographic assays for hydrocortisone in plasma, humans □ Pharmacokinetics—comparison of fluorescence and UV high-pressure liquid chromatographic assays for hydrocortisone tablets in plasma, humans

A number of high-pressure liquid chromatographic (HPLC) assays for hydrocortisone in biological fluids have recently appeared in the literature. Most of these employ UV detection of underivatized hydrocortisone (1–7), while others are based on detection of a fluorescent hydrocortisone derivative (8, 9).

In this report two assay methods, one using normal phase HPLC with fluorescence detection of the dansyl derivative of hydrocortisone and the other using reversed-phase HPLC with UV detection of hydrocortisone, were compared by assaying plasma samples obtained during an 8-hr period from three healthy volunteers who had ingested hydrocortisone tablets. The plasma concentration *versus* time data permits preliminary description of the pharmacokinetics of exogenous hydrocortisone.

## EXPERIMENTAL

**Materials**—Materials used in the HPLC–fluorescence assay have been described previously (8). For the HPLC–UV assay, chemical standards of hydrocortisone[1] and internal standard $\Delta^4$-pregnene-17$\alpha$, 20$\beta$,21-triol-3,11-dione[1] were analytical grade. Reagent grade methylene chloride[2] was distilled prior to use. All other solvents and chemicals were reagent grade and were used as supplied. Plasma for construction of standard curves was obtained from healthy volunteers between 7 and 9 am subsequent to administration of 2 mg of dexamethasone at 11 pm the previous day (10).

**Subjects**—Three male volunteers (22–45 years old) underwent complete physical examinations including blood and urine analyses after giving informed consent. Vital signs and laboratory values for all subjects were normal. The subjects weighed 64–75 kg, and their heights ranged from 178 to 180 cm.

**Protocol**—Subjects were instructed to take no drugs for at least 1 week before the study, and no drugs other than the required doses of dexamethasone and hydrocortisone during the study. No caffeine-containing beverages were permitted for 1 day before or during the plasma sampling period following each dose of hydrocortisone. Each hydrocortisone dose was administered after overnight fast, and no food was permitted until 4 hr postdose.

Each subject received 10-, 30-, and 50-mg doses of hydrocortisone at least 1 week apart according to a randomized design. Each hydrocortisone dose was given as 1 or more 10-mg tablets[3], which were swallowed whole.

At 11 pm on the day before hydrocortisone administration, subjects received 20 ml (2 mg) of dexamethasone elixir[4] with 180 ml of water orally. Hydrocortisone was administered at 8 am the following morning with 180 ml of water.

Heparinized blood samples (10 ml) were taken from a forearm vein immediately before and then at 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, and 8 hr postdose Subjects were ambulatory during the sampling period. Plasma was obtained by centrifugation and divided into two portions. Both portions were frozen at −20°: one portion was packed in dry ice and sent by air freight to the Food and Drug Administration laboratories for fluorimetric analysis; the other was assayed in this laboratory by the HPLC–UV method.

**Fluorimetric Assay**—The fluorimetric HPLC assay of plasma samples was carried out using a previously described method (8).

**UV Assay**—The HPLC–UV assay used was a modified version of a previously described method (1). Plasma (1 ml) containing 200 ng internal standard, and 0.1 ml of 2 *N* aqueous sodium hydroxide was vortexed with 10 ml of methylene chloride for 1 min and then centrifuged for 15 min at 500×*g*. Plasma and the creamy interface were aspirated off, and the organic layer was transferred to a clean tube and evaporated to dryness at room temperature under nitrogen. The tube walls were rinsed with 1 ml of methylene chloride which was evaporated as described previously. The residue was reconstituted in 100 $\mu$l of the HPLC mobile phase (60% aqueous methanol) and a 20-$\mu$l aliquot was injected into the chromatograph. The liquid chromatograph consisted of a 2.1 × 70-mm precolumn[5] and a 4.6 × 250-mm reversed-phase analytical column[6], through which mobile phase, 60% aqueous methanol, was pumped at a rate of 1.0 ml/min. Column effluent was monitored at 254 nm with a fixed wavelength detector[7]. Elution times for hydrocortisone and internal standard were 10 and 8 min, respectively. Calibration was by the method of peak height ratios. For plasma samples that yielded hydrocortisone and internal standard peak height ratios of <0.1 (hydrocortisone concentrations <25 ng/ml), a second injection was performed and the peak height ratios

[1] Sigma Chemical Co., St. Louis, MO 63178.
[2] Aldrich Chemical Co., Milwaukee, WI 53233.
[3] Hydrocortisone 10-mg tablets, Lot V 2478, Merck Sharp and Dohme Labs., West Point, PA 19486.
[4] Decadron Elixir, Lot A 3240, Merck Sharp and Dohme Labs.
[5] CO:PELL ODS, 30–38 m, Whatman Inc., Clifton, NJ 07014.
[6] Lichrosorb C18, 10 m, Altex Scientific Inc., Berkeley, CA 94710.
[7] Model 440, Waters Associates, Milford, MA 01757.

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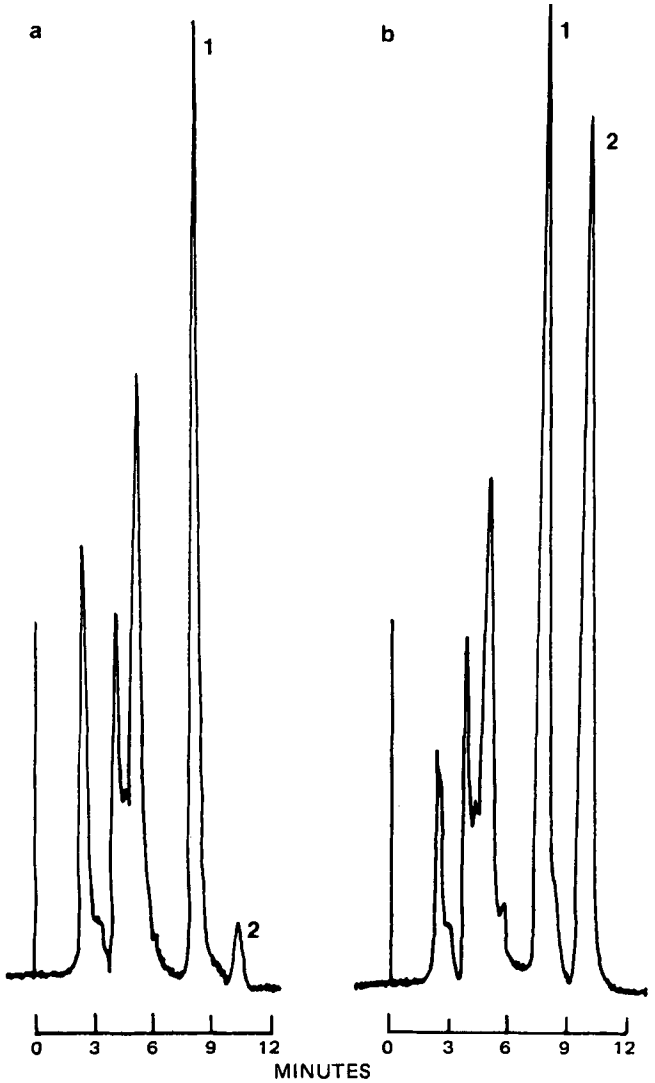


**Figure 1**—*Representative Chromatograms from HPLC–UV assay of hydrocortisone, peak 2, and internal standard, peak 1, extracted from human plasma.* a—*Dexamethasone—suppressed plasma containing 200 ng/ml internal standard and 11 ng/ml hydrocortisone (endogenous).* b—*Sample plasma containing 200 ng/ml internal standard and 149 ng/ml hydrocortisone (dosed + endogenous).*

obtained from the two injections were averaged. The endogenous hydrocortisone concentration in predose plasma was subtracted from all postdose values to obtain circulating levels of drug resulting from administered hydrocortisone. The assay was linearly sensitive to plasma hydrocortisone concentrations between 5 and 700 ng/ml. Coefficients of variation from multiple replicates ($n = 6$) were within 4% at the higher drug concentrations and within 8% at the lower drug concentrations. Assay recovery was 82 ± 2% for hydrocortisone and 83% for internal standard (single determination).

**Pharmacokinetic and Statistical Analysis**—Plasma hydrocortisone concentration data from all three doses were adequately described in terms of the one-compartment open pharmacokinetic model with first-order absorption and elimination and an absorption lag time. Thus, the hydrocortisone concentration $C$ in plasma at any time $t$ after dosing was shown by (11):

$$C = \frac{FD}{V}\left(\frac{k_a}{k_a - k_{el}}\right)\left[e^{-k_{el}(t-t_0)} - e^{-k_a(t-t_0)}\right] \qquad \text{(Eq. 1)}$$

where $k_a$ and $k_{el}$ are the first-order absorption and elimination rate constants, $t_0$ is the absorption lag time, $F$ is the fraction of dose ($D$) absorbed, and $V$ is the apparent distribution volume of drug in the body.

Equation parameters were obtained by nonlinear regression of individual hydrocortisone concentration data sets using the program NREG (12) on a digital computer[8].

[8] Univac Model 1100.

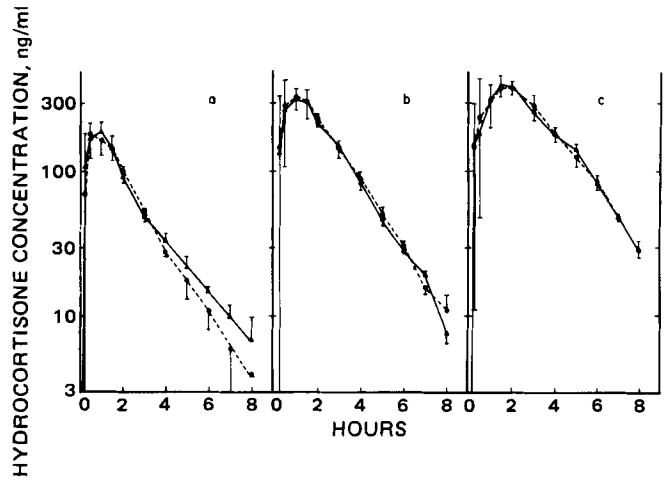


**Figure 2**—*Mean hydrocortisone plasma concentration* versus *time curves for three subjects (±1 SD) at dosages of* a, *10-mg;* b, *30-mg; and* c, *50-mg. Key:* ▲—▲, *UV;* ●---●, *fluorescence.*

Comparison of the hydrocortisone concentration values obtained by the two assay procedures was by paired $t$ test. Pharmacokinetic parameters were analyzed for dose and assay effects by ANOVA. Factors shown to have a significant effect by ANOVA were further analyzed using Tukey's significant difference test (13).

## RESULTS

Figure 1 shows two representative chromatograms from the HPLC–UV assay. Figure 1*a* illustrates the low level of endogenous hydrocortisone in suppressed plasma. The standard curve for hydrocortisone concentrations between 5 and 700 ng/ml in plasma was given by:

$$Y = -0.014\ (\pm 0.010) + 0.00497\ (\pm 0.00003)\ X \qquad \text{(Eq. 2)}$$

where $r = 0.999$ and $n = 6$.

**Comparisons of the UV and Fluorescence Assays**—Mean plasma hydrocortisone concentrations obtained using the two assay methods are shown in Fig. 2. Drug concentrations obtained by the different assays at each sampling time were compared by paired $t$ test, and a significant difference between assays was obtained at only 2 of the 33 sampling times (11 sampling times at each dose level). The linear regression of the results obtained at all sampling times with the UV assay *versus* those obtained with the fluorescence assay is shown in Fig. 3. The regression had a slope

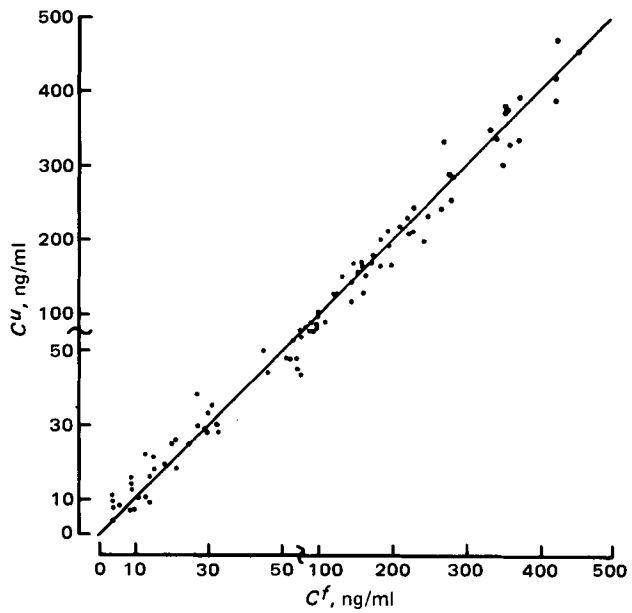


**Figure 3**—*Plasma hydrocortisone concentrations as obtained by UV detection* ($C^u$) versus *those obtained by fluorescence detection* ($C^f$). *Y = 0.992x + 1.59; n = 94, r = 0.987.*

**Table I—Mean Values of Pharmacokinetic Parameters Obtained Following 10-, 30-, and 50-mg Oral Doses of Hydrocortisone, with Analysis by HPLC–UV Assay**

| Parameter | Value | | | Statistic |
|---|---|---|---|---|
| | 10-mg dose | 30-mg dose | 50-mg dose | |
| $k_a$, $hr^{-1}$ | 7.7 ± 7.3[a] | 7.7 ± 6.3 | 1.4 ± 0.5 | AB > C[b] |
| $t_0$, hr | 0.18 ± 0.07 | 0.26 ± 0.14 | 0.13 ± 0.23 | B > A > C |
| $k_{el}$, $hr^{-1}$ | 0.47 ± 0.06 | 0.54 ± 0.04 | 0.43 ± 0.03 | NSD[c] |
| $t_{1/2el}$, hr | 1.50 ± 0.17 | 1.29 ± 0.09 | 1.62 ± 0.09 | NSD |
| $C_{max}$[d], ng/ml | 199 ± 29 | 393 ± 58 | 419 ± 45 | CB > A |
| $T_{max}$[e], hr | 1.0 ± 0.5 | 1.0 ± 0.5 | 1.7 ± 0.3 | C > AB |
| $C_{max}/D$[f], $10^{-6}$/ml | 19.9 ± 2.9 | 13.1 ± 2.0 | 8.4 ± 0.9 | A > B > C |
| $F/V$[g], $10^{-6}$/ml | 23.5 ± 3.6 | 17.0 ± 2.5 | 13.7 ± 1.5 | A > B > C |
| $AUC_{trap}$[h], ng hr/ml | 474 ± 48 | 958 ± 113 | 1559 ± 142 | C > B > A |
| $r^2$[i] | 0.98 ± 0.02 | 0.99 ± 0.02 | 0.99 ± 0.01 | NSD |

[a] Standard deviation ($n$ = 3). [b] A = 10-mg dose, B = 30-mg dose, C = 50-mg dose. [c] No significant differences ($p > 0.05$). [d] Maximum observed concentration of hydrocortisone in plasma. [e] Time of observed $C_{max}$. [f] Maximum concentration of hydrocortisone in plasma divided by dose. [g] The fraction of the dose which is adsorbed, expressed as a concentration in the apparent distribution volume; obtained by dividing the function $FD/V$, from Eq. 1, by the dose. [h] Area under plasma hydrocortisone concentration profile calculated by trapezoidal rule with end correction. [i] Coefficient of determination from nonlinear regression analysis of plasma data using Eq. 1 [$r^2 = (\Sigma obs^2 - \Sigma dev^2)/\Sigma obs^2$].

of 0.99, an intercept that was not significantly different from zero, and a correlation coefficient of 0.987 ($p < 0.001$).

**Pharmacokinetic Analysis**—No statistical differences were observed in the values of any pharmacokinetic parameters resulting from the two assay methods. Therefore, only those values obtained using the HPLC–UV assay are presented in Table I.

The high coefficients of determination that resulted from analysis of individual data sets indicated that hydrocortisone plasma profiles following oral doses are adequately described by a one-compartment kinetic model. Following the 10- and 30-mg doses, hydrocortisone was absorbed rapidly and peak drug levels in plasma were obtained 1 hr postdose. Following the 50-mg dose, hydrocortisone was absorbed at a slower rate, yielding peak drug levels in plasma at ~1.7 hr. For all three dosages, description of the absorption phase of drug profiles was improved by incorporation of a lag time. After achieving peak values, drug levels declined with a mean half-life of 1.3–1.6 hr; this value was independent of dose size.

Although the maximum concentrations of hydrocortisone in plasma, and also the areas under the drug concentration *versus* time curves, increased with increasing drug doses, they were not dose-proportional. Both $C_{max}$ and $AUC_{trap}$ increased twofold when hydrocortisone dose was increased threefold from 10 to 30 mg. When the dose was further increased 1.6-fold to 50 mg, the value of $AUC_{trap}$ increased in proportion to the dose, while $C_{max}$ increased by a factor of 1.1. When the values of $C_{max}$ and $FD/V$ were normalized for dose, there was a progressive decrease in both of these values as the dose was increased from 10 to 50 mg.

## DISCUSSION

HPLC assays for hydrocortisone in biological fluids have the advantage of greater compound specificity than previously used fluorescence (14, 15), competitive protein binding (16), enzyme immunoassay (17), and radioimmunoassay (18) procedures. HPLC assay with a fluorescence detector was claimed to be more sensitive than other analytical methods (8).

The results of this study show that an HPLC assay with UV detection, which requires no derivatization step, is equally specific and sensitive to the HPLC fluorescence assay. Both procedures are capable of measuring hydrocortisone concentrations in plasma following therapeutic doses, are free of interference from hydrocortisone metabolites and endogenous substances in plasma, and are also capable of measuring endogenous hydrocortisone levels following suppression by dexamethasone. The HPLC–UV assay is simpler and less time consuming than the HPLC fluorescence procedure and is therefore the method of choice.

The pharmacokinetic parameter values obtained in the clinical studies are largely in agreement with those published previously. While the elimination phase of plasma hydrocortisone levels was well defined, the short duration of the absorption phase, and also an apparent lag time, does not permit accurate description of this phase. Therefore, the use of first-order kinetics in the present interpretation does not preclude the possibility of alternative absorption mechanisms.

Mean elimination half-lives of hydrocortisone were 90, 77, and 97 min, respectively, following the 10-, 30-, and 50-mg doses. Previous studies using intravenous hydrocortisone have reported drug half-lives between 58 and 161 min (19–23). Circulating levels of hydrocortisone were quite variable at each dose level, which is consistent with previous observations (24, 25).

Additional studies are necessary to confirm the apparent lack of proportionality between circulating levels of hydrocortisone and the administered dose, although similar results to those obtained in this study were reported in subjects receiving oral cortisone acetate (26).

A number of possible factors might cause dose-nonproportionality in hydrocortisone plasma levels. Drug absorption efficiency may be reduced at higher doses due to limited tablet dissolution or to reduced transport across the GI epithelium. Hydrocortisone levels may also be influenced by changes in drug–protein binding. At low drug concentrations, hydrocortisone binds predominantly to the high affinity, low capacity protein transcortin (19, 27). At drug levels >200 ng/ml, binding sites on the transcortin molecule become saturated, and binding to the low affinity, high capacity binding sites of plasma albumin accounts for an increasing proportion of the bound drug. Due to the preponderance of low affinity binding at high drug levels, the percentage of circulating hydrocortisone in the bound form decreases. In the present study, hydrocortisone plasma concentrations >200 ng/ml were achieved with the 30- and 50-mg doses. Reduced overall binding of drug following the higher doses would permit a greater proportion of the drug to enter extravascular fluids, causing a reduction in plasma levels. The reduced binding of hydrocortisone to plasma proteins at high drug concentrations may also increase hepatic clearance during the first-pass, resulting in a smaller proportion of administered drug reaching the systemic circulation (28).

While circulating levels of endogenous hydrocortisone remain constant following dexamethasone suppression (10), further suppression of endogenous levels may result from the administered hydrocortisone by a feedback mechanism. However, suppressed endogenous hydrocortisone levels are low compared with the levels that resulted from administered compound, and further reduction in suppressed levels would not have noticeably affected the results.

Studies have been initiated to examine the dose-proportionality of hydrocortisone pharmacokinetics following parenteral doses.

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# Acrylic Microspheres *In Vivo* V: Immunological Properties of Immobilized Asparaginase in Microparticles

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Received February 2, 1981, from the *Department of Pharmaceutical Biochemistry, Biomedical Center, University of Uppsala, Box 578, 751 23 Uppsala, Sweden.* Accepted for publication September 3, 1981.

**Abstract** □ L-Asparaginase was immobilized in microparticles of polyacrylamide. Such particles were then injected by intramuscular/subcutaneous, intraperitoneal, or intravenous routes into mice to investigate the immunological consequences of the immobilization. Entrapment of L-asparaginase in microparticles did not prevent the formation of antibodies in intensively treated animals. Intraperitoneal and intravenous injections of particles produced significantly higher antibody levels than soluble L-asparaginase. Antigen administered intramuscularly/subcutaneously in microparticles elicited, however, a weak immune response. Dependent on the route of administration, the particles may thus function as an adjuvant. A modified Arthus reaction in the foot pads of immunized mice indicated that antigenicity decreased when L-asparaginase was immobilized in microparticles. Injection of free L-asparaginase, intramuscularly/subcutaneously (2 × 20 IU) in the preimmunized mice produced no effects on the serum level of L-asparagine, whereas intramuscular/subcutaneous injection of L-asparaginase in microparticles produced a depression of the serum concentration. It is concluded that the intramuscular/subcutaneous injection of L-asparaginase in microparticles is the choice route of administration with respect to duration and the immunological reaction.

**Keyphrases** □ L-Asparaginase—immunological properties of immobilized L-asparaginase in microparticles □ Microparticles—immunological properties of immobilized L-asparaginase □ Immunological properties—of immobilized L-asparaginase in microparticles

Exogenous enzymes have been used increasingly in biological systems to test their usefulness in treating genetic disorders, *e.g.*, lysosomal storage diseases (1, 2), or for therapeutic purposes, *e.g.*, L-asparaginase to depress circulating L-asparagine in the treatment of acute lymphatic leukemia (3). These enzymes are often used in the immobilized or polymerized form in order to prolong their duration (4–6). However, the desired prolonged effect is seemingly in conflict with the efforts to decrease their immunological properties manifested by the production of antibodies and hypersensitivity reactions, which is enhanced when exposure to the exogenous protein is prolonged. Thus, enzymes in liposomes have been shown to be immunogenic[1]. The liposomes have even, in some instances, been shown to exert adjuvant effects (7). This effect may be due partly to leakage of enzyme molecules out of the liposomes or lysis of the liposomes, but the findings that the adjuvant properties are related to the composition of the liposomes (7, 8) suggest that the adjuvant effects are inherently connected with the liposomes themselves. Likewise, polymethylmethacrylate has been shown to increase the immunogenic properties of simultaneously administered influenza virons (9). The adjuvant effect is strongly correlated to the route of administration, with intravenous or intraperitoneal injections of the immobilized systems generally producing relatively higher antibody titers (7, 10).

The present study was undertaken to investigate the immunological consequences of the utilization of microparticles of polyacrylamide as carrier of exogenous proteins *in vivo*. The polymer itself is not immunogenic (11), but immobilized proteins are partly localized on the surface of the microparticles during the preparation as evidenced by their interaction with cellular surface structures (12) or affinity chromatography material (13). Even if the major portion of the immobilized protein is secluded inside the microparticles, the fraction on the surface should exert immunological properties. The aim was to find the optimal route for the administration of immobilized L-asparaginase.

## EXPERIMENTAL

**Materials**—Aspartate aminotransferase[2] (83 IU/mg) isolated from porcine heart, and malic dehydrogenase[2] (2000 IU/ml) from pigeon breast

[1] In the present paper, the term immunogenic is used to describe the property of a macromolecular system to evoke an immunological response, *e.g.*, antibody production and T-cell stimulation, while the term antigenic is restricted to the property to react with the immunological effectors in a sensitized organism.
[2] Sigma Chemical Co.



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