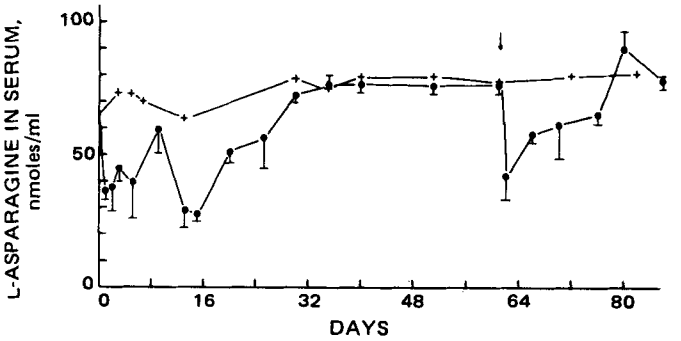

Figure 2—*L-Asparagine concentration in serum after subcutaneous implantation of 100 IU of L-asparaginase (700 IU/kg) in rats (●). The arrow shows the time when the implant was removed and a new gel tablet containing 100 IU of L-asparaginase (300 IU/kg) was inserted. Each point represents samples drawn from two rats and shows the mean and the range. The normal L-asparagine level (+) was obtained from an untreated rat from the same litter.*

The decrease of the serum L-asparagine concentration was of the same magnitude after the second implantation as that found initially, in spite of the lower dosage of L-asparaginase. This finding indicates that no significant amounts of antibodies were produced in the rats during the first 60 days of exposure to the implants. This conclusion is in accordance with earlier findings that proteins immobilized in highly cross-linked polyacrylamide are stably bound in the gel (6). Thus, it is feasible to assume that not enough of the immunogenic enzyme leaks out of the gel to activate the immune system.

By subcutaneous administration of L-asparaginase in implants of polyacrylamide, the effect can be substantially prolonged compared to the duration obtained by administration of soluble enzyme or by enzyme immobilized in microspheres. Although duration of the systemic effect *in vivo* was not limited by denaturation, the formation of a collagenous capsule around the implant effectively prevented the contact between the enzyme and the circulating L-asparagine. The encapsulation is a normal reaction to the presence of a foreign body. The same effects were detected after implantation of other acrylic polymers in rats (10, 11).

Updike *et al.* (12) also increased the duration of L-asparaginase by immobilizing the enzyme in resealed red blood cell ghosts. Undetected or very low levels of circulating L-asparagine lasted twice as long after the intravenous injection in monkeys of ghost-entrapped enzyme than after injection of the same amount of free enzyme. In this case, the duration of the enzymatic effect was limited by the survival of the red blood cell ghosts in the circulation.

Chang *et al.* (13) also showed that L-asparaginase entrapped in semipermeable microcapsules (~80 μm in diameter) can deamidate L-asparagine *in vitro*. When given intraperitoneally in mice, the entrapped enzyme (3.5 IU) was more efficient than native enzyme and cured 50% of the treated mice bearing an L-asparagine-dependent lymphoma cell line, 6C3HED (14).

The L-asparaginase implants thus were able to depress the systemic L-asparagine level significantly for prolonged periods. However, as is evident from the figures, the concentration of L-asparagine never reached zero. Therefore, it is not possible to predict whether the decreased level would influence the proliferation of L-asparagine-dependent tumors. Such tests may be performed with the 6C3HED cell line growing in mice.

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This paper is Number 3 in a series on Acrylic Microspheres *In Vivo*.

Fluorometric Determination of All-*trans* Retinol in Rat Serum

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Abstract □ A rapid, specific, and sensitive fluorometric assay for retinol in rat serum is reported. Serum retinol is extracted into cyclohexane after dilution of the serum sample with aqueous acetonitrile. Sample volumes as small as 0.2 ml can be used with a limiting detectable concentration of 2.5×10^{-8} *M* for retinol.

Keyphrases □ Vitamin A—fluorometric determination in rat serum □ Retinol, all-*trans*—fluorometric determination in rat serum □ Fluorometry—determination of all-*trans* retinol in rat serum

Vitamin A (all-*trans* retinol) is well known for its importance in general growth, the growth and differentiation of epithelial tissues, visual function, and reproduction (1–4). A number of retinoids prevent or inhibit the growth of epithelial tumors (5–9). As early as 1926, it had been noticed that vitamin A deficiency leads to carcinomas in

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the stomach of rats (10) and later to hyperkeratosis of the skin and to metaplastic changes in epithelia of the GI, respiratory, and urogenital tract (11–14).

In the late 1960's, several scientists showed that the induction of benign and malignant epithelial tumors in animals could be retarded or even prevented by systemically applied retinol or retinol palmitate (5, 6). The use of these compounds for cancer chemoprevention was reviewed previously (15, 16). These nutritional surveys and cancer prevention studies required a method for measuring micromolar to nanomolar amounts of retinol in small volumes of serum, a method whereby numerous analyses could be performed without undue analytical time. For these reasons, there is an intense search for exact, sensitive, and simple methods for determining retinol in biological material.

The innate sensitivity and speed of fluorescence measurements as an analytical technique have been demonstrated (17–26). However, current methodology is still not as sensitive as some recently reported high-pressure liquid chromatographic (HPLC) procedures (27–31). The present paper reports an improved fluorometric assay for retinol in rat serum that is more sensitive than reported methods and compares favorably with HPLC procedures.

EXPERIMENTAL

Apparatus—All fluorescence measurements were made with a fluorescence spectrophotometer[1] equipped with an x–y recorder. Quartz cells of a 1-cm light path were used. Daily variation in instrumental response was corrected with a dilute quinine bisulfate standard solution (5×10^{-5} M in 0.1 N H_2SO_4).

Since organic solvents were found to extract fluorescent substances from plastic caps, tubes, and bottles (25), a glass apparatus was used during all analytical manipulations. Glass culture tubes[2] were used to collect serum samples and extract retinol from serum.

A high-speed centrifuge[3] was used for centrifugation, and a vibrating mixer[4] was used for mixing and extraction.

Reagents and Solutions—Retinol[5] (all-*trans*, crystalline, synthetic Type X) and retinyl acetate[5] (all-*trans*, crystalline, synthetic Type I) were used as received. Solid samples were stored at −10° under nitrogen and protected from light. All solvents were the highest grade commercially available; they were checked for fluorescent impurities before use. A buffer solution (μ = 0.15, pH 7.4) was prepared from analytical grade monobasic sodium phosphate[6], dibasic sodium phosphate, and distilled, deionized water.

Standard Solutions—Sufficient retinyl acetate was weighed into a 10-ml amber volumetric flask and dissolved in 10 ml of acetonitrile to yield a standard stock solution (~1×10^{-3} M). Serial dilutions of this solution yielded working standard solutions with concentrations ranging from 1×10^{-4} to 1×10^{-6} M. All solutions were stored at −10° and protected from light. The purity of the solutions was checked by comparing their absorption spectra with their corrected fluorescence excitation spectra. Retinyl acetate was used as a standard because it has long been accepted as a standard for retinol determination by fluorometric analysis. The reported quantum efficiencies for each compound are identical, and the stability of retinol acetate solutions is much better (32–34). All experimental manipulations were conducted under yellow light and in amber glassware.

Solvent Pair Selections—A water-miscible–water-immiscible solvent pair was selected based on the following criteria.

1. The fluorescence of retinyl acetate in 14 nonpolar and nine polar solvents was examined.

2. The nonpolar solvents were used to extract blank rat serum to detect extractable, fluorescent serum components. The nonpolar solvents were carbon tetrachloride, chloroform, methyl chloride, methylene chloride, ethylene dichloride, tetrachloroethylene, n-pentane, n-hexane, n-heptane, cyclohexane, ethyl acetate, octanol, benzene, and toluene. The polar solvents were dimethyl sulfoxide, acetonitrile, dimethylformamide, formamide, ethylene glycol, water, methanol, ethanol, and dioxane.

3. Various ratios of polar solvent to water-diluted serum were examined with regard to decreasing the quantity of endogenous serum components extracted into the nonpolar solvent. For these comparisons, the serum was spiked with retinoid, diluted with water, and then diluted further with the polar solvent. The resulting aqueous solution was extracted with the nonpolar solvent.

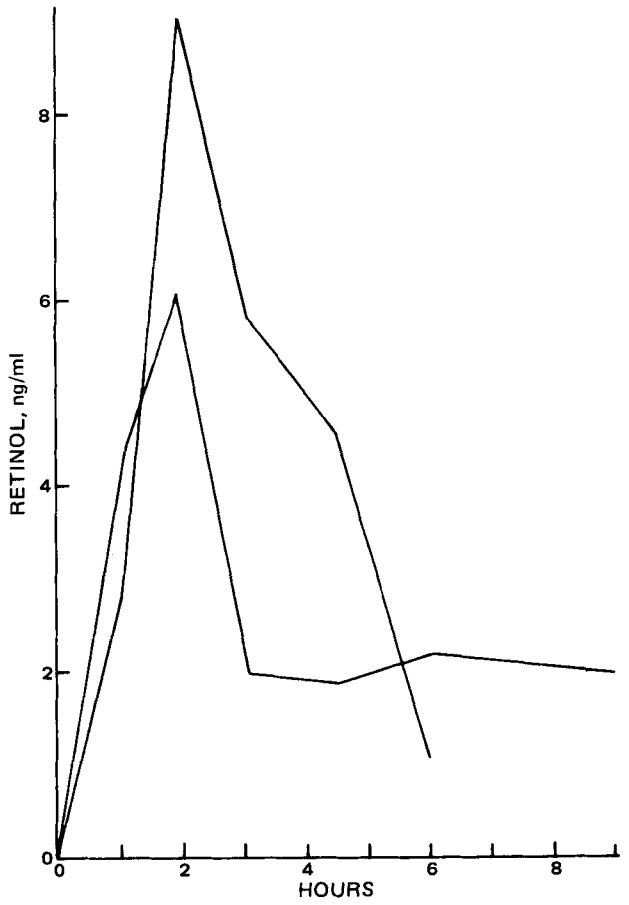

Figure 1—*Serum retinol blood profiles obtained from two rats after a single oral dose of 10 mg of retinyl acetate/kg. All points were corrected for blank fluorescence.*

Serum—Male and female rats[7] (300–400 g) were fasted for 19 hr before sacrifice. After the animal was anesthetized with ether, the rib cage was cut open to expose the heart. Total exsanguination from the left ventricle was accomplished with a 10-ml disposable plastic syringe. Approximately 10 ml of blood was obtained from each rat. The serum was obtained after centrifugation at 2500 rpm for 10 min in a glass culture tube.

The native fluorescence of the serum extracts was examined by mixing 0.2 ml of serum with 0.2 ml of phosphate buffer. To this mixture was added 2 ml of acetonitrile. After mixing, 2 ml of cyclohexane was added and shaken by a vibrating mixer for 30 sec. After this mixture was centrifuged at 2500 rpm for 5 min, the cyclohexane layer was removed for direct fluorometric measurement. The fluorescence intensity of each sample was measured at the excitation maximum of 327 nm and the emission maximum of 480 nm. This procedure was repeated with 15 different rat serum samples without drug. The average fluorescence intensity measured from these 15 blank serum samples was used as the blank.

Buffer and serum samples (0.2 ml) spiked with varying concentrations of retinyl acetate were extracted in the same manner as the blank serum extracts. Second extractions of the spiked samples were performed by adding 2 ml of cyclohexane to the aqueous mixture from which the first

[1] Model MPF-44A, Perkin-Elmer, Norwalk, CT 06856.
[2] Type 9825, 16 × 100 mm, Pyrex, Corning Glass Works, Corning, N.Y.
[3] Vari-HI Speed Centricone, Precision Scientific Co., Subsidiary of GCA Corp., Chicago, Ill.
[4] Super-Mixer, Lab-Line Instruments, Melrose Park, Ill.
[5] Sigma Chemical Co., St. Louis, MO 63178.
[6] J. T. Baker Chemical Co., Phillipsburg, NJ 08865.
[7] Sprague–Dawley strain, Hiram Davis Co., Stockbridge, Ga.

Table I—Comparison of Fluorescence Excitation Intensity as a Function of Different Acetonitrile–Aqueous Serum Sample Ratios of Retinyl Acetate Extracted by 2 ml of Cyclohexane from Spiked Rat Serum

Rat Serum (ml)–Water (ml) + Acetonitrile (ml)	Net Relative Fluorescence Excitation Intensity of Cyclohexane Layer (λ_{ex} 327; λ_{em} 480), arbitrary units	Acetonitrile–Aqueous Serum Sample
0.2:0.2 + 0	54.0	0
0.2:0 + 0.2	54.0	1:1
0.2:0 + 2	57.3	1:0.09
0.2:0 + 1	54.0	1:0.17
0.2:0.2 + 2	62.3	1:0.17[a]
0.4:0.2 + 2	61.4	1:0.23
0.6:0.2 + 2	60.3	1:0.29
0.8:0.2 + 2	57.0	1:0.33

[a] Optimum solution conditions.

cyclohexane layer had been removed. Since the fluorescence intensity of the second sample extract was the same as that of the blank, the fluorescence intensity of each sample recorded was from the first extract.

In vivo experiments were performed using three sets of four rats each, administered a 2-, 4-, or 10-mg/kg dose of retinyl acetate by stomach intubation. Each dose was prepared by dissolving an appropriate quantity of drug in ethanol. Before drug administration, each rat was fasted for 19 hr. The blood was withdrawn from the heart 2 hr postadministration.

Two more rats with body weights of 390 g were given 10 mg/kg of retinyl acetate orally. Blood samples of each rat were withdrawn from the tail at 0, 1, 2, 3, 4.5, 6, and 9 hr. All samples were centrifuged, and the serum was removed and kept frozen until assayed (Fig. 1).

RESULTS AND DISCUSSION

Cyclohexane–acetonitrile was the best solvent pair when acetonitrile was added to the water-diluted serum samples (1:1) so that the final ratio of acetonitrile to aqueous sample was 1:0.17. The validity of using these proportions was checked (Table I). Methanol and ethanol also can be used as the water-miscible component as reported previously (22, 25, 35). However, they did not decrease the cyclohexane extraction of endogenous fluorescent substances in the serum as well as did acetonitrile. The other water-miscible solvents did not demonstrate this effect, the mechanism of which is not known. However, it appears to be a specific solvation effect, dependent on the solvent concentration as well as on the water concentration in the final solution (Table I). Simple dilution of the serum sample with water, with no acetonitrile added, resulted in a lower fluorescence intensity than under optimum conditions. Dilution with acetonitrile produced the same result. Sample fluorescence was maximized only after dilution with both acetonitrile and water in the given proportions.

Standard curves were constructed at six concentration points for retinol in (*a*) cyclohexane, (*b*) cyclohexane-extracted phosphate buffer, and (*c*) cyclohexane-extracted serum. Curves *a–c* were linear from 2.5×10^{-8} to 1.0×10^{-4} *M*. For *a* and *b*, each point was the mean of three determinations; for *c*, each point was the mean of 15 determinations. The correlation coefficients were 0.9998, 0.9997, and 0.9995 for *a–c*, respectively.

The extraction efficiencies of retinyl acetate from buffer solution and serum were 97.1 ± 1.2 and 91.4 ± 2.9%, respectively (mean ± *SD*). The results clearly show that a standard curve of this agent in cyclohexane-extracted buffer should not be used for extracted serum samples without a correction factor. Therefore, fluorometric procedures that do not include a correction factor will consistently report lower concentration values than are really present (22–26).

The procedure reported in this study is at least 10-fold more sensitive than current fluorometric procedures (22–26). However, sample volume and precision are essentially the same with both fluorometric and HPLC procedures now in use (22–26, 28–31). The method is applicable to bioavailability and pharmacokinetic studies, as demonstrated by the data in Fig. 1 and Table II.

Table II—Rat Serum Concentrations of Retinol Obtained 2 hr after a Single Oral Dose of Retinyl Acetate

Dose, mg/kg	Number of Rats and Total Samples	Serum Concentration $\times 10^7$ *M* (mean ± *SD*)
2	4 and 16	4.1 ± 0.9
4	4 and 16	7.6 ± 1.1
10	4 and 20	7.3 ± 1.0

The mean serum retinol concentration in 12 rats (52 samples) 2 hr after a single oral dose of retinyl acetate is shown in Table II. The data indicate a nonlinear correlation between dose and serum concentration. A 4-mg/kg dose produced a serum concentration nearly equal to that of the 10-mg/kg dose. An analysis of variance on the mean serum concentrations in Table II, using a Student procedure, showed that if the mean values differed by 1.82×10^{-7} or more, then they were different ($p \leq 0.05$). Therefore, since oral doses of 4 and 10 mg/kg produced the same serum retinol concentration, a dose-dependent saturation of some part of the absorption or metabolic network may be occurring.

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