Determination of Coumarin and Umbelliferone Mixtures in Whole Blood by Spectrophotofluorometry

HENRY S. I. TAN ˣ, WOLFGANG A. RITSCHEL, and PHYLLIS R. SANDERS *

Abstract □ A spectrophotofluorometric method is described for the quantitative analysis of coumarin, umbelliferone, and mixtures thereof in whole blood. The two drugs were selectively isolated from blood by solvent extraction. Analysis of the isolated coumarin was based on the measurement of the fluorophore at activation and emission wavelengths of 361 and 491 nm, respectively. The fluorophore was obtained by irradiating an alkaline methanolic solution of the drug with UV light. A linear relationship between fluorescence and concentration existed over the concentration range of 0.02–0.2 μg of coumarin/ml. A mean recovery value of 94.8% was obtained from whole blood. The isolated umbelliferone was determined according to established methods at activation and emission wavelengths of 370 and 450 nm, respectively, and the limit of detection was 10 times more sensitive than previously reported. A linearity response was obtained between 1 and 10 ng of umbelliferone/ml. Good recovery data for mixtures of coumarin and umbelliferone in whole blood were obtained.

Keyphrases □ Coumarin—spectrophotofluorometric analysis, alone and with umbelliferone, whole blood □ Umbelliferone—spectrophotofluorometric analysis, alone and with coumarin, whole blood □ Spectrophotofluorometry—analysis, coumarin and umbelliferone, alone and together, whole blood

Coumarin is the active principle in a number of European pharmaceutical preparations. It gains pharmaceutical importance due to its hemodynamic and lymphokinetic properties. Clinically, it has been used in postthrombotic syndromes, in varicose insufficiency, and in posttraumatic edema (1).

Recent pharmacokinetic studies on coumarin in this laboratory were hampered by the lack of suitable assay procedures for coumarin. Only one method has been reported for its determination in biological fluids. This method is based on an isotope dilution technique (2). Other published procedures (3–6) either dealt with coumarin in plant materials or involved methods that were not sufficiently sensitive for pharmacokinetic investigations, particularly since coumarin is administered in relatively low concentrations.

Coumarin is an acid–base fluorescence indicator. It fluoresces in alkaline solutions after UV irradiation, and this fluorescence behavior has been used for its detection (7). A fluorometric method for coumarin in clover was reported (8).

In test animals, the major metabolites of coumarin are 3-hydroxycoumarin and 7-hydroxycoumarin (umbelliferone) (2, 9). These hydroxycoumarins are known to fluoresce in alkaline solutions and are usually analyzed by fluorometry (9, 10).

Since current pharmacokinetic studies in this laboratory concern the evaluation of coumarin and free 7-hydroxycoumarin, an attempt was made to develop a sensitive assay procedure for coumarin, unconjugated umbelliferone, and mixtures thereof in whole blood. The methodology is based on the fluorescence characteristics of coumarin and umbelliferone.

EXPERIMENTAL

Apparatus—The following were used: a fluorescence spectrophotometer[1] with 1-cm cells and a fixed spectral bandwidth of 10 nm equipped with a 150-w, 7.5-amp, 20–75-v, dc xenon lamp[2] and a R212 photomultiplier tube; an analytical balance[3]; and a 250-w, 110–125 v, ac UV lamp[4]. The response of the fluorescence spectrophotometer was calibrated daily with a solution of quinine sulfate in dilute sulfuric acid (1 in 350) containing 0.25 μg/ml.

Materials and Reagents—The following were used: coumarin[5] (mp 69°); umbelliferone[6] (puriss. mp 231–233°); absolute methanol[7], containing 0.05% water; 2 *N* methanolic potassium hydroxide reagent, prepared by dissolving the required amount of the alkali in methanol, allowing the solution to settle for 24 hr in a closed container, and filtering rapidly the white precipitate of potassium carbonate through a glass wool filter; glycine[8] (ammonia free) buffer, pH 10.0, prepared according to Sørensen (11); and water, distilled from a glass distillation apparatus. All the other chemicals used were of analytical grade.

Preparation of Coumarin Standard Curve—A stock solution of coumarin was prepared by dissolving 25.0 mg of the drug in 250.0 ml of methanol. Further dilutions were made to obtain standard solutions containing 0.2–2.0 μg of coumarin/ml. One milliliter of each solution was transferred to a 10-ml volumetric flask for fluorophore development as described under *Assay Procedure for Coumarin in Whole Blood*, beginning with: "Add 5.0 ml of 2 *N* methanolic potassium hydroxide reagent ...." The stock and standard solutions should be freshly prepared.

Preparation of Umbelliferone Standard Curve—A stock solution of umbelliferone was prepared by dissolving 25.0 mg of the compound in 5.5 ml of methanol and diluting to 250.0 ml with water. Further dilutions were made to obtain standard solutions containing 0.01–2.0 μg of umbelliferone/ml. One milliliter of each solution was transferred to a 10-ml volumetric flask for fluorophore development as described under *Assay Procedure for Umbelliferone in Whole Blood*, beginning with: "... dilute to volume with pH 10.0 glycine buffer."

Assay Procedure for Coumarin in Whole Blood—Place 5.0 ml of normal saline solution and 0.1 ml of 15% (w/v) aqueous sodium citrate solution in a 50-ml glass-stoppered centrifuge tube. Swirl the contents of the tube, add 3.0 ml of the blood sample, and extract the blood two times with 5-ml portions of ether. Each time shake the mixture for 5 min and centrifuge for 5 min at 2000 rpm.

Transfer the ether layer to a beaker, and evaporate the combined ether fractions carefully at low heat to almost dryness with a gentle stream of nitrogen gas. Add approximately 1 ml of methanol and continue the evaporation until all ether is removed. Transfer quantitatively the remaining solution into a 10-ml volumetric flask with some methanol. Add 5.0 ml of 2 *N* methanolic potassium hydroxide reagent and dilute to volume with methanol.

Expose the flask to UV light, at a distance of 53.3 cm (21 in.) from the light source, for 40 min. Determine the fluorescence at activation and emission wavelengths of 361 and 491 nm, respectively, against a blank prepared similarly but without the coumarin. Read the concentration of coumarin present from a calibration curve.

Assay Procedure for Umbelliferone in Whole Blood—Place 5.0 ml of normal saline solution and 0.1 ml of 15% (w/v) aqueous sodium citrate solution in a 50-ml glass-stoppered centrifuge tube.

[1] Model 204, Perkin-Elmer Corp., Norwalk, Conn.
[2] Model XBO 150 W/1, Osram Gesellschaft, Berlin, Germany.
[3] Model H18, Mettler Instrument Corp., Princeton, N.J.
[4] General Electric Co., Cincinnati, Ohio.
[5] Schaper und Brümmer, Saltzgitter-Ringeheim, Germany.
[6] Fluka A. G., Buchs SG, Switzerland.
[7] Matheson, Coleman and Bell, Norwood, Ohio.
[8] Eastman Kodak Co., Rochester, N.Y.

Table I—Effect of Time on Fluorescence of Coumarin and Umbelliferone Fluorophores

Minutes	Coumarin[a], Relative Fluorescence	Umbelliferone[b], Relative Fluorescence
0	31.2	28.4
15	32.0	—
20	—	27.4
30	32.8	—
40	—	26.4
45	32.9	—
60	32.2	28.0
75	32.8	—
80	—	28.0
90	32.2	—
105	32.1	—

[a] Concentration, 0.08 μg/ml; selector, × 1; sensitivity 5 at λ_{act} 361 nm and $\lambda_{emission}$ 491 nm, after an irradiation time of 40 min with UV light. [b] Concentration, 0.08 μg/ml; selector, × 1/10; sensitivity 3 at λ_{act} 370 nm and $\lambda_{emission}$ 450 nm.

Swirl the contents of the tube and add 3.0 ml of the blood sample. Extract the blood two times with 5-ml portions of ether. Each time shake the mixture for 5 min and centrifuge for 5 min at 2000 rpm.

Transfer the ether layer into a clean glass-stoppered centrifuge tube. Extract the combined ether fraction two times with 3-ml portions of pH 10.0 glycine buffer. Each time shake the mixture vigorously for 5 min and centrifuge for 5 min at 2000 rpm. Transfer the glycine buffer fraction to a 10-ml volumetric flask and dilute to volume with pH 10.0 glycine buffer.

Measure the fluorescence at activation and emission wavelengths of 370 and 450 nm, respectively, against a blank prepared similarly but without the umbelliferone.

Assay Procedure for Mixtures of Coumarin and Umbelliferone in Whole Blood—Place 5.0 ml of normal saline solution and 0.1 ml of 15% (w/v) aqueous sodium citrate solution in a 50-ml glass-stoppered centrifuge tube. Swirl the contents and add 3.0 ml of the blood sample. Extract the blood two times with 5-ml portions of ether. Each time shake the mixture for 5 min and centrifuge for 5 min at 2000 rpm.

Transfer the ether fractions to a fresh glass-stoppered centrifuge tube and extract the ether two times with 3-ml portions of *cold* pH 10.0 glycine buffer. (*a*) Transfer the glycine buffer fractions to a fresh glass-stoppered centrifuge tube and extract once with 5 ml of ether. Combine the ether layer with the main ether solution in the beaker (see *b*). Transfer the glycine buffer solution to a 10-ml volumetric flask and proceed as described under *Assay Procedure for Umbelliferone in Whole Blood* beginning with: "... dilute to volume with pH 10.0 glycine buffer."

(*b*) Transfer the ether fractions to a beaker, evaporate the solvent cautiously at low heat with a gentle stream of nitrogen gas to almost dryness, and proceed as described under *Assay Procedure for Coumarin in Whole Blood* beginning with "Add approximately 1 ml of methanol...."

Table II—Recovery Data of Coumarin or Umbelliferone from Spiked Whole Blood Samples

Amount Added[a]	Analyzed at Concentration Level, ng/ml	Amount Found[a]	Recovery, %
Coumarin			
0.40	40	0.37	92.5
0.80	80	0.78	97.5
1.20	120	1.17	97.5
1.60	160	1.55	96.9
2.00	200	1.79	89.5
Overall percent recovery			94.8
SD			3.62
Standard error of the mean			1.62
Umbelliferone			
20.00	2	17.4	87.0
80.00	8	71.5	89.4
Overall percent recovery			88.2

[a] Coumarin: micrograms per 3 ml of blood; umbelliferone: nanograms per 3 ml of blood.

Table III—Reproducibility of Coumarin Determination from Replicate Whole Blood Samples Containing 0.4 μg of Coumarin/ml

Samples	Relative Fluorescence
1	37.9
2	38.6
3	43.0
4	41.0
5	37.0
Average	39.5
SD	2.46
Coefficient of variation, %	6.22

RESULTS AND DISCUSSION

Coumarin—Freshly prepared alkaline solutions of coumarin exhibit little fluorescence. However, when these solutions are irradiated with UV light, the solutions begin to fluoresce and the intensity increases to a maximum. It is assumed that when coumarin is dissolved in alkali, cleavage of the pyrone ring occurs whereby the anion of *cis*-coumaric acid is formed. Upon irradiation with UV light, the *cis*-form is converted to the *trans*-isomer (7). Apparently, in the *trans*-isomer the hydrogen atom of the phenol group forms a chelate with the sterically unhindered unsaturated carbon atom, giving rise to a six-membered ring which is the fluorophore.

The fluorophore showed excitation and emission wavelengths of 361 and 491 nm, respectively (Fig. 1). The fluorescence intensity depends upon the concentration of potassium hydroxide. Maximum fluorescence was obtained when the concentration of the potassium hydroxide was approximately 1 *N* in the final test solution. This finding is in agreement with previously reported data (8).

Since the fluorescence intensity increases to a maximum upon irradiation with UV light, the optimum exposure time was investigated. The distance of 53.3 cm (21 in.) between sample and light source was selected so that maximum irradiation could be achieved without heating the samples excessively. Figure 2 shows that maximum fluorescence was obtained in 20 min. However, for unknown reasons, samples irradiated for 20 min did not produce constant

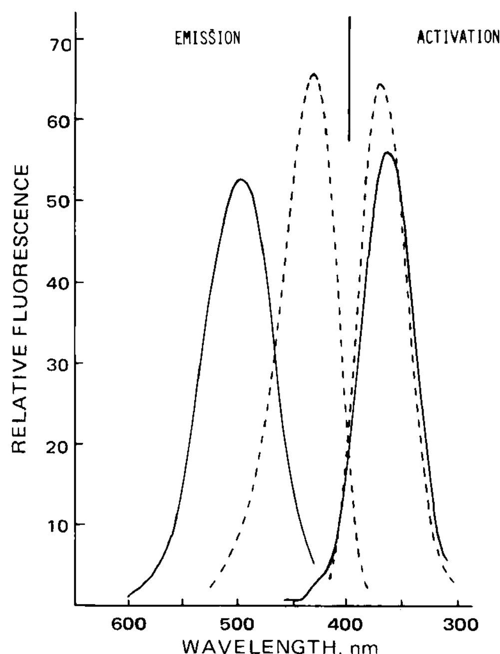

Figure 1—*Activation and emission spectra of coumarin (——) and umbelliferone (- - -) fluorophores.*

Table IV—Recovery Data of Mixtures of Coumarin and Umbelliferone from Whole Blood

Mixture	Amount Added, μg/3 ml of Blood		Amount Found, μg/3 ml of Blood		Recovery, %	
	Coumarin	Umbelliferone	Coumarin	Umbelliferone	Coumarin	Umbelliferone
A	0.400	0.020	0.361	0.0179	90.3	86.0
B	0.800	0.040	0.693	0.0339	86.6	84.8
C	1.600	0.060	1.36	0.0527	85.3	87.8
Overall percent recovery					87.4	86.2

fluorescence intensities, particularly if these samples were scanned repeatedly in the spectrophotofluorometer. An irradiation time of 40 min gave constant readings. The fluorescence intensities of samples so developed were stable for at least 90 min (Table I).

Under these experimental conditions, a linear relationship existed between fluorescence and concentration. A straight line was obtained over the concentration range of 0.02–0.2 μg of coumarin/ml. Statistical analysis shows a regression equation of $F_{361/491} = 292.21C + 1.37$ with a correlation coefficient of 0.9993, where F is expressed in relative fluorescence units and C in micrograms per milliliter[9]. The standard error of the estimate of F on C is 0.668.

Recovery experiments were performed by adding accurately known amounts of coumarin to freshly drawn whole blood. For best reproducibility, an anticoagulant must be present in the centrifuge tube into which the whole blood is transferred. In the absence of an anticoagulant, clotting commenced within a few minutes and the recovery of the drug was poor due to inclusion of the drug in the blood clot. For this purpose, sodium citrate was used at the concentration of 5 mg/ml of blood. The sodium citrate was mixed into the normal saline solution, which served as the diluting agent for the blood sample.

Care must be exercised upon evaporating the coumarin-containing ether extract. Because coumarin has a high vapor pressure and is steam distillable, the evaporation must be done at very low heat. Addition of methanol in the evaporation process greatly increased the recovery of drug. The results (Table II) indicated that recovery of coumarin from whole blood was essentially quantitative. The overall recovery was 94.8% with a standard deviation of 3.62% (n = 5).

The precision study on coumarin recovery from whole blood was performed by running replication studies on five samples. In each run the amount of coumarin added to whole blood was 0.4 μg/ml of blood. Each sample was analyzed by the proposed method. The relative standard deviation for the five replicate samples was 6.21% at this concentration level (Table III).

Umbelliferone—As mentioned previously, the major metabolites of coumarin are 3-hydroxycoumarin and 7-hydroxycoumarin (umbel*liferone*); these metabolities are conjugated mainly as sulfates and glucuronides. These conjugates are not isolated by the proposed extraction procedure, and they do not fluoresce (10). Umbelliferone does show the phenomenon of excited-state ionization, so its fluorescence behavior is completely different than that of the 3-isomer. The 3-isomer shows negligible fluorescence between pH 9 and 12 (9). Under the proposed experimental conditions, only free umbelliferone is measured. As shown in Fig. 1, umbelliferone showed an emission maximum of 450 nm when activated at 370 nm in pH 10.0 glycine buffer. The fluorescence was stable for at least 90 min (Table I).

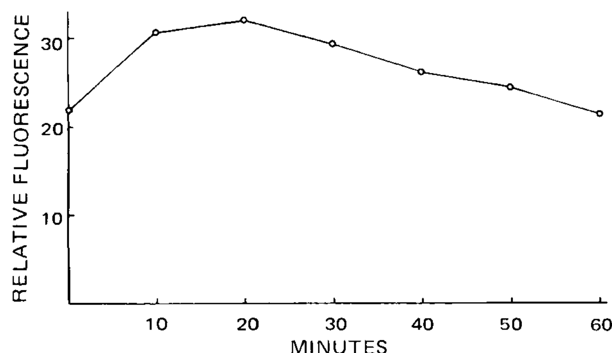

Figure 2—*Effect of time of light exposure on fluorescence development of coumarin.*

The concentration–fluorescence plot shows that the fluorescence response is linear with concentration in the investigated range of 1–10 ng/ml. Therefore, the proposed procedure is approximately 10 times more sensitive than the one previously reported by Mead *et al.* (10). The linear regression line is $F_{370/450} = 10.25C + 0.33$ with a correlation coefficient of 0.9998, where F is expressed in relative fluorescence units and C in nanograms per milliliter[9]. The standard error of the estimate of F on C is 0.382.

The recovery data of umbelliferone from whole blood are presented in Table II. The overall percent recovery for two samples was 88.2%. This value is in agreement with recovery data from liver homogenates reported by Creaven *et al.* (9).

Coumarin–Umbelliferone Mixtures—Results shown in Table IV indicate that the recovery of umbelliferone in the presence of coumarin was in agreement with its recovery when it alone was present in blood. However, the percent recoveries of coumarin were lower than expected. Presumably, a fraction of the coumarin undergoes hydrolysis during the extraction process with pH 10.0 glycine buffer to form the *o*-coumarinate anion which is insoluble in ether. Since the glycine fraction was not subjected to UV radiation, this solution showed no fluoresence at the coumarin activation and emission wavelengths. Thus, measurement of umbelliferone in this glycine buffer solution is not interfered with by coumarin. To keep the hydrolysis of coumarin to a minimum, *cold* glycine buffer must be used for the extraction.

SUMMARY

The developed fluorometric procedure was sensitive and specific for coumarin and umbelliferone and was adapted to drug level determination in whole blood. Among the advantages of the present procedure are its reproducibility, its sensitivity, and the relative lack of interference by constituents in blood. It also allows for measurement of free umbelliferone. In addition, both the umbelliferone conjugates and the other hydroxycoumarins do not interfere. Precipitation of proteins in blood with agents such as trichloroacetic acid is not necessary, as shown by the recovery studies.

This method is currently being applied to biopharmaceutical and pharmacokinetic studies of coumarin.

REFERENCES

(1) C. A. Scholten, *VASA*, **2,** 96(1973).

(2) M. Kaighen and R. T. Williams, *J. Med. Pharm. Chem.*, **3,** 25(1961).

(3) J. Duncan and R. Dustman, *Ind. Eng. Chem., Anal. Ed.*, **9,** 471(1937).

(4) M. Szymanska, *Acta Pol. Pharm.*, **29,** 511(1972); through *Chem. Abstr.*, **78,** 81717z(1973).

(5) J. R. Clopton, *J. Agr. Food Chem.*, **6,** 457(1958).

(6) V. Anger and H. E. Richter, *Mikrochim. Ichnoanal. Acta*, **1965,** 1025; through *Chem. Abstr.*, **64,** 14777b(1966).

(7) F. Feigle, H. E. Feigle, and D. Goldstein, *J. Amer. Chem. Soc.*, **77,** 4162(1955).

(8) K. Blyaim, *Biokhimiya*, **21,** 689(1956).

(9) P. J. Creaven, D. V. Parke, and R. T. Williams, *Biochem. J.*, **96,** 390(1965).

(10) J. A. R. Mead, J. N. Smith, and R. T. Williams, *ibid.*, **61,** 569(1955).

(11) "Scientific Tables," 7th ed., K. Diem and C. Lentner, Eds., Ciba-Geigy, Basle, Switzerland, 1971, p. 280.

[9] Olivetti Programma 101 minicomputer.

ACKNOWLEDGMENTS AND ADDRESSES

Received February 26, 1975, from the *College of Pharmacy, University of Cincinnati, Cincinnati, OH 45221*

Accepted for publication April 3, 1975.

Presented at the 22nd Canadian Conference on Pharmaceutical Research, Montreal, Canada, May 1975.

* Recipient of Research Fellowship from Schaper und Brümmer KG, Saltzgitter-Ringeheim, Germany.

ˣ To whom inquiries should be directed.

Binding of Salicylate and Sulfathiazole by Whole Blood Constituents

CHARLES A. CRUZE * and MARVIN C. MEYER ˣ

Abstract □ The binding of salicylic acid and sulfathiazole to bovine whole blood, plasma proteins, and purified albumin fraction was investigated using a dynamic dialysis system. The binding profiles for salicylic acid were quite similar in bovine plasma and 4% bovine serum albumin. In contrast, the binding of sulfathiazole was significantly greater in the plasma than in solutions of fraction V bovine serum albumin. Data from dynamic dialysis binding studies of the compounds, conducted in whole blood and suspended erythrocyte systems, did not lend themselves to analysis by classical methods. Hemolysis and alteration in the nature of the protein binding sites during the binding studies were shown to be factors that could explain the unusual binding observed in the whole blood system.

Keyphrases □ Salicylate—binding to bovine whole blood, plasma proteins, and purified albumin fractions compared □ Sulfathiazole—binding to bovine whole blood, plasma proteins, and purified albumin fractions compared □ Binding—salicylate and sulfathiazole to bovine whole blood, plasma proteins, and purified albumin fractions compared □ Plasma proteins—compared to bovine whole blood and purified albumin fraction binding of salicylate and sulfathiazole □ Albumin—purified fractions, compared to bovine whole blood and plasma protein binding of salicylate and sulfathiazole

Numerous drugs and endogenous substances are reported to be bound by serum proteins (1). Plasma albumin is generally thought to be the primary serum protein responsible for the binding of most drugs. Thus, drug binding studies aimed at determining the affinity of a drug for plasma proteins generally have employed albumin as the macromolecule.

In vitro studies with various protein fractions have shown that the summation of the binding to the individual fractions of the plasma may exceed the binding observed in the whole plasma (2). Therefore, binding studies employing purified protein fractions may not accurately reflect the binding that may occur *in vivo*. Several reports have dealt with the binding of drugs to plasma constituents (3–7). However, relatively little consideration has been given to the binding of drugs by erythrocytes (8, 9) or the binding in whole blood systems (10, 11).

The use of classical methods to study the binding of drugs to plasma and whole blood is difficult and subject to error. In equilibrium dialysis, the time necessary to establish equilibrium may be sufficient to permit significant denaturation of the blood and plasma components. The use of ultrafiltration methods for whole blood binding studies may result in separation of the blood components and blockage of the pores of conventional dialysis membranes. In view of these problems, the dynamic dialysis technique of Meyer and Guttman (12) seemed to hold promise for the quantitation of drug binding in whole blood. The time required for the determination of a complete binding profile is relatively short, temperature can be conveniently controlled, and no centrifugation, vacuum, or pressure is required.

Salicylic acid and sulfathiazole were selected as model compounds to test the applicability of the dynamic dialysis technique to the study of drug binding to bovine whole blood, erythrocytes, plasma, and purified serum albumin.

EXPERIMENTAL

Materials—Fraction V bovine serum albumin[1], sodium salicylate[2], and sulfathiazole sodium[3] were obtained from commercial sources. Regenerated cellulose dialysis tubing[4] was conditioned prior to use by rapidly running distilled water through the tubing for several hours, with the tubing immersed in distilled water. The tubing was then stored in distilled water at 4° until used.

Bovine blood was obtained from the jugular vein of freshly slaughtered cattle and was collected into large vessels containing either heparin[5], 50 units/ml of blood, or anticoagulant citrate dextrose solution A USP, 0.15 ml/ml of blood. The collected blood was divided into two portions. One portion was employed in the whole blood studies, and the other portion was centrifuged to separate the plasma and formed elements. The buffy coat was removed from the packed cells, and the erythrocytes were washed several times with pH 7.38, 0.128 *M*, isotonic phosphate buffer solution. The erythrocytes were then resuspended with the phosphate buffer to produce the same volume present before the plasma was removed.

Dynamic Dialysis Studies—The dynamic dialysis system of Meyer and Guttman (13) was employed with a few modifications. The dialysis behavior of the drug was first characterized with a control solution of the drug in pH 7.38, 0.128 *M* phosphate buffer. A 5-ml sample of the control drug solution was placed inside a 6.5-cm long dialysis sac, secured at one end with a knot and suspended in a jacketed beaker containing 175 ml of the phosphate

[1] Calbiochem, San Diego, Calif.
[2] J. T. Baker, Phillipsburg, N.J.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Union Carbide dialysis membrane.
[5] The Upjohn Co., Kalamazoo, Mich.