erlapped the absorption bands of the hydrocarbons. The absorption spectra of the protein-bound hydrocarbons exhibited shoulder bands in the 280–300-nm region, indicating the involvement of the tryptophan residue in the interaction. The strong enhancement of the B region fluorescence for the hydrocarbons in binding with the protein represents a transition resulting from an energy transfer process because the binding of these compounds with soluble ribonucleic acid, a macromolecule without an energy donor, does not induce the B region fluorescence.

Excluding the fact of energy transfer, the fluorescence of the hydrocarbons is strongly quenched by interacting with the protein and soluble ribonucleic acid. This result probably indicates a stacking interaction between the hydrocarbon and the aromatic moieties of the macromolecules. The association of fluorescence quenching with stacking interactions was reported previously. For example, the binding of protein to nucleic acid always results in strong quenching of the protein fluorescence, which has been shown to be due to the stacking interaction between the tryptophan residues and the nucleic acid bases (3, 4). In a fluorescence study of compounds in which an indole group is connected to nucleic acid bases by a three- or four-atom bridge, Mutai *et al.* (19) showed that the stacked form, in contrast to the fluorescent unstacked form, is nearly nonfluorescent. Benz[*a*]anthracene and benzo[*a*]pyrene are planar molecules. In binding with nucleic acid, benzo[*a*]pyrene intercalates with the nucleic acid bases (8). In protein interactions, it is possible that the binding allows plane parallelism between the tryptophan residue and the hydrocarbon and, therefore, results in fluorescence quenching of both units.

The fact that only bound benz[*a*]anthracene and benzo[*a*]pyrene can receive energy is indicated by the fluorescence studies of tryptophan with the hydrocarbons. Human serum albumin has one tryptophan residue per molecule. However, the addition of tryptophan, in the same concentration range as that of the protein (Fig. 1), does not induce the B region fluorescence. Only in high tryptophan concentrations do the spectra of the hydrocarbons become similar to those observed in protein solutions, since the two units are then close enough for the efficiency of energy transfer. These results suggest that the binding sites for the hydrocarbons are quite close to the tryptophan residue and that the hydrocarbons are favorably oriented with the tryptophan residue to permit interaction and energy transfer. The small R value for benz[*a*]anthracene indeed suggests that the binding site is in the vicinity of the tryptophan residue.

The energy transfer information is important in understanding the interaction of carcinogenic hydrocarbons with proteins. Benz[*a*]anthracene and benzo[*a*]pyrene interact with human serum albumin to approximately the same extent as indicated by the binding constants. This is expected since these compounds are chemically inert and the binding is of a hydrophobic nature. The difference in structure of the two would probably make little difference in their binding affinities. The difference in ring structure, however, may have a profound effect on their orientations on the protein molecule. Conclusions based on binding strength alone could be misleading. Benzo[*a*]pyrene is strongly carcinogenic, whereas benz[*a*]anthracene is not. While the binding constants of the two suggest similar binding, their protein binding sites are distinguished by energy transfer data.

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Rapid Sensitive Fluorometric Analysis of Cephalosporin Antibiotics

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Abstract □ A rapid and sensitive fluorometric analysis for cephalosporins, which can also be applied to penicillins, is presented. The method involves reaction with 0.1 *N* sodium hydroxide at 100°, producing stable fluorescent products. This method was applied to cephalexin and ampicillin with detection at concentrations as low as 0.01 μg/ml.

Keyphrases □ Cephalosporins, various—fluorometric analysis, commercial samples in aqueous solutions □ Fluorometry—analysis, various cephalosporins, commercial samples in aqueous solutions □ Antibacterials—various cephalosporins, fluorometric analysis, commercial samples in aqueous solutions

Several methods for the quantitative measurement of cephalosporins in aqueous solution have been described. They utilize such techniques as microbiological assay (1), GLC (2), formol titration (3), colorimetry (4–7), liquid chromatography (8, 9), iodometry (4, 10), reaction with hydroxylamine (4), and polarography (11). The nonmi-

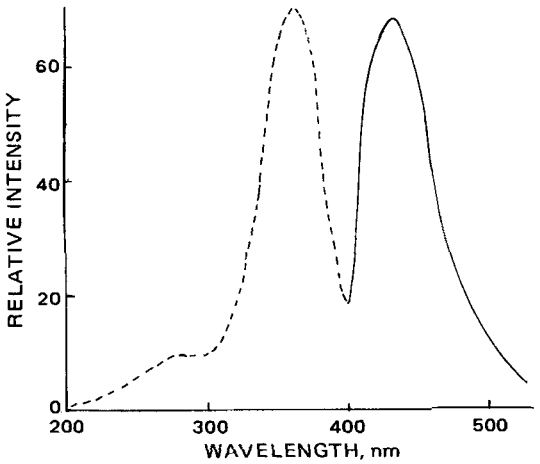

Figure 1—*Fluorescence spectra of caphalothin (1 μg/ml) after reaction at 100° for 75 min in 0.1 N NaOH. Key: - - -, excitation; and —, emission.*

crobiological methods cited are not generally used routinely for analysis of biological samples because of limited sensitivity or, if sensitive enough, because they are less practical than microbiological assays.

This report describes a fluorometric analysis that is simple, rapid, and sensitive enough to be useful for determining cephalosporin levels in biological samples. This same procedure can be applied to ampicillin, amoxicillin, penicillin G, and possibly other penicillins.

EXPERIMENTAL

Samples of cephalothin[1], desacetyl cephalothin[1], cephalexin[1], cephaloglycin[1], cefamandole[1], cefazolin[2], ampicillin[3], amoxicillin[4], and penicillin G[1] were used as received. Stock solutions containing 100 μg/ml of drug in distilled water were freshly prepared prior to use. The stock solution was appropriately diluted with sodium hydroxide solution (various concentrations), and the resulting solutions were sealed in capped[5] culture tubes and heated at various temperatures to produce fluorescent products. After heating in a water bath, the samples were cooled under cold tap water and the fluorescence was measured with a scanning[6] or filter[7] fluorometer.

Separation of the fluorescent products was accomplished with TLC using silica gel precoated plates[8]. The plates were developed for 1.5 hr in chloroform–acetone–acetic acid (50:50:7) and visualized with a UV viewing box.

Standard curves for cephalexin and ampicillin in bovine serum were made using a modification of the extraction procedure described by Jusko (12). This procedure involves adding 0.5 ml of 10% trichloroacetic acid and 0.5 ml of water to 1.0 ml of serum containing drug, mixing for 1 min, and centrifuging at 5000×*g* for 5 min. The supernate (1.3 ml) is mixed with 0.4 ml of 1.0 *N* NaOH and then heated to form fluorescent products as described. After cooling, 0.5 ml of 1 *N* HCl and 8 ml of chloroform–acetone (1:1) are added; the solution is shaken for 15 min and centrifuged at 2000×*g* for 2 min. The organic layer (5 ml) is shaken with 2 ml of 0.1 *N* NaOH for 15 min and centrifuged at 2000×*g* for 2 min. The fluorescence of the aqueous layer is then measured and compared to similarly prepared standards.

RESULTS AND DISCUSSION

Cephalothin—Assay conditions were optimized with cephalothin and then applied with minor variations to other cephalosporins and penicillins. The fluorescence of cephalosporins depends upon their reaction with sodium hydroxide at elevated temperatures. The reaction conditions varied to optimize fluorescence included the sodium hydroxide concentration, the reaction temperature, and the reaction time.

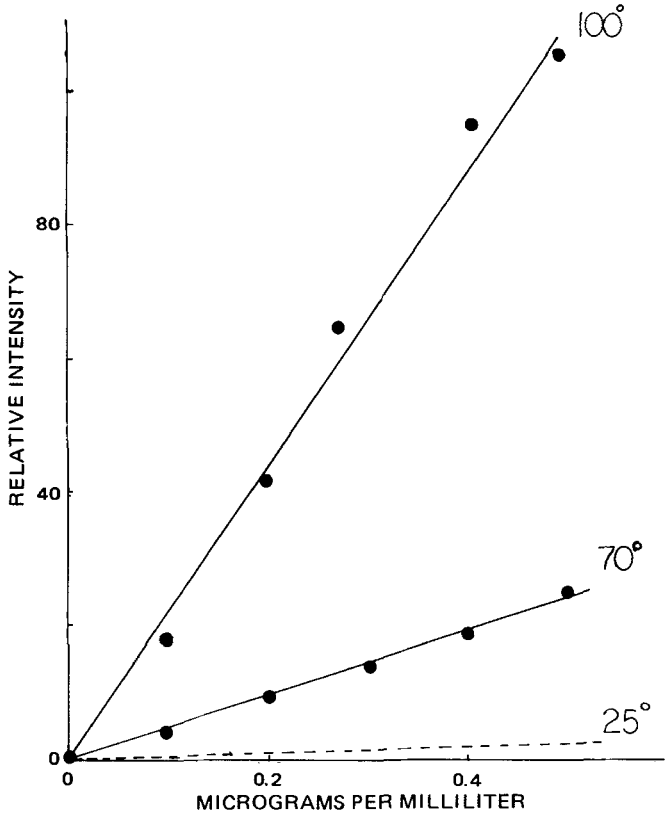

Figure 2—*Effect of temperature on the fluorescence of cephalothin standards reacted in 0.1 N NaOH at 100, 70, and 25° for 75, 240, and 1380 min, respectively. Dashed line is an extrapolation from a linear curve made at concentrations of 1.0–10 μg/ml. Reaction times are optimized for each temperature.*

Figure 1 shows the fluorescence spectrum of a 1-μg/ml solution of cephalothin after reaction at 100° for 75 min in 0.1 *N* NaOH. Figure 2 shows the fluorescence of cephalothin standards reacted in 0.1 *N* NaOH at 100, 70, and 25° for 75, 240, and 1380 min, respectively; 100° was the optimum reaction temperature. At lower reaction temperatures, even at much longer reaction times, the fluorescence intensity did not approach that obtained at 100°. Therefore, all further studies were conducted at 100°.

The effect of sodium hydroxide concentration upon fluorescence is shown in Fig. 3. When 1.0 μg of cephalothin/ml was reacted with 0.1 *N* NaOH, a higher fluorescence was obtained compared to reaction with 0.5 or 1.0 *N* NaOH. At least 75 min was required for maximal fluorescence

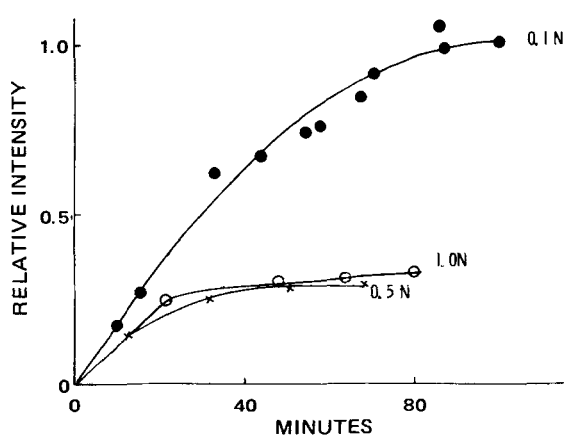

Figure 3—*Effect of sodium hydroxide concentration on the fluorescence of cephalothin (1 μg/ml) reacted at 100°.*

[1] Eli Lilly & Co., Indianapolis, Ind.
[2] Smith Kline and French Laboratories, Philadelphia, Pa.
[3] Bristol Laboratories, Syracuse, N.Y.
[4] Beecham-Massengill, Nashville, Tenn.
[5] Lined with Teflon (du Pont).
[6] Aminco-Bowman SPF spectrophotofluorometer, American Instrument Co., Silver Spring, Md.
[7] Turner model 111 filter fluorometer, Turner Associates, Palo Alto, Calif.
[8] F-254, Brinkmann Instruments, Inc., Westbury, N.Y.

Table I—Relative Fluorescent Intensities and Reaction Conditions for Various Cephalosporins

Compound	Reaction Conditions at 100°	Excitation (Ex), Emission (Em)	Relative Intensity
Cephalothin	0.1 *N* NaOH, 75 min	360 Ex 435 Em	1
Desacetyl cephalothin	0.1 *N* NaOH, 75 min	360 Ex 435 Em	0.33
Cephaloridine	0.1 *N* NaOH, 2.5 hr	360 Ex 435 Em	2.5
Cefazolin	0.05 *N* NaOH, 35 min	330 Ex 415 Em	0.14
Cephaloglycin	0.1 *N* NaOH, 30 min	342 Ex 430 Em	0.45
Cephalexin	0.1 *N* NaOH, 30 min	340 Ex 425 Em	0.55
Cefamandole	0.1 *N* NaOH, 12 hr	310 Ex 390 Em	0.03

to develop in 0.1 *N* NaOH, while only half that time was required for 0.5 or 1.0 *N* NaOH. If assay sensitivity is not as important as rapidity of assay, the higher sodium hydroxide concentrations may be more advantageous.

After maximal fluorescence was attained with 0.1 *N* NaOH, longer reaction times up to 2.5 hr did not affect the fluorescence, indicating fairly stable products. Sodium hydroxide concentrations as low as 0.05 *N* did not significantly alter the fluorescence from that observed with 0.1 *N* NaOH. Cephalothin standards exhibited linear fluorescence from 0.05 up to 5 μg/ml. Linearity may extend beyond concentrations of 5 μg/ml, but such experiments were not performed.

Other Cephalosporins—Table I and Fig. 4 summarize the fluorescence results for various cephalosporins. The fluorescence properties of the alkaline reaction products varied from one cephalosporin to another with respect to excitation and emission maxima, the degree of fluorescence, and optimum reaction time. Minor variations in excitation and emission wavelengths can be ignored if a filter fluorometer[7] is used with specific excitation[9] and emission[10] filters. A filter fluorometer does not alter the detection limits of the assay and may be more convenient.

Since reaction conditions were not completely optimized for each cephalosporin (except reaction time), the fluorescence possibly could be increased by altering the sodium hydroxide concentration or reaction temperature for each cephalosporin. In general, detectable fluorescence was obtained at 0.05 μg/ml (except for cefamandole) and linearity extended to 0.5–1.0 μg/ml or above. Certainly, this assay is applicable in nonlinear regions by comparison with actual standard curves.

Penicillins—Fluorescence was observed with penicillin G, ampicillin, and amoxicillin when subjected to the same reaction conditions producing fluorescence with cephalosporins. Penicillin G was only weakly fluorescent compared to cephalothin, requiring concentrations above 10 μg/ml to obtain detectable fluorescence after reaction times over 3 hr. The fluorescence obtained, though, confirms an earlier observation that a fluorescent product is produced from penicillin G when subjected to alkaline degradation conditions (13).

Ampicillin and amoxicillin were much more fluorescent than cephalothin after reaction in 0.1 *N* NaOH. Ampicillin standards exhibited linear fluorescence down to concentrations as low as 0.005 μg/ml. Comparison with the results of Jusko (12) is difficult, but the limits he suggested (0.01–20 μg/ml) for his fluorescent assay indicate that the sodium hydroxide reaction is of approximately the same sensitivity. Jusko obtained maximal fluorescence in pH 2 reaction solutions at 75° and observed much reduced (~10-fold) fluorescence when alkaline conditions up to pH 9 were employed.

This study indicates that the reaction profile for ampicillin may have two pH maxima (pH 2 and 13) or that entirely different products are obtained under extremely alkaline conditions compared to acidic conditions. This latter possibility is quite likely, since penicillin G and cephalothin were nonfluorescent after reaction under acidic conditions but fluorescent after reaction under high temperature and high pH conditions.

Fluorescent Products—The literature on cephalosporins gives no hint to possible fluorescent products under extremely alkaline conditions.

[9] Corning 7-60.
[10] Combination of Wratten 2A and 47B.

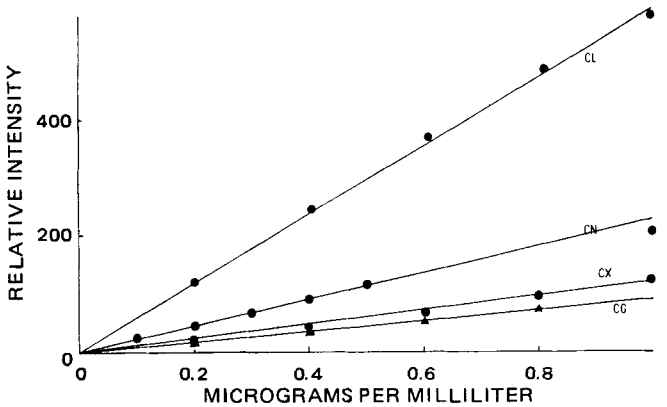

Figure 4—*Fluorescence of cephalosporin standards reacted with sodium hydroxide according to the conditions shown in Table I. Key: CL, cephaloridine; CN, cephalothin; CX, cephalexin; and CG, cephaloglycin.*

Alkaline degradation studies on cephradine, which is structurally similar to cephalexin, indicate the formation of a diketopiperazine product (14) similar to the structure Jusko (12) proposed for the fluorescent product obtained by acidic hydrolysis of ampicillin. Neither of these products would account for the fluorescence of penicillin G or cephalosporins without the α-aminobenzyl side chain.

The thin-layer chromatograms of the final reaction solutions for a number of cephalosporins after heating with 0.1 *N* NaOH for 1 hr are shown in Fig. 5. In each case, except for cefamandole and cefazolin, at least two fluorescent spots were obtained, one with a high R_f value and one with a low R_f value. Cephalexin also gave two fluorescent spots with intermediate R_f values. At this time, the nature of these fluorescent products is speculative. Nevertheless, further investigation of the relationship between cephalosporin and penicillin degradation products is warranted.

It has been observed that penicillenic acid-type compounds and oxazolones, in general, are not obtained from cephalosporins (15). This may be true under the reaction conditions studied to date. However, under the high temperature and extreme alkaline conditions employed in our studies, such degradation pathways possibly may become preferred or at least comparable in rate to other pathways. Oxazolone production could account for the general fluorescence obtained from cephalosporins and penicillins of varying side chains. The various degrees of fluorescence could also be explained on the basis of faster parallel pathways for certain cephalosporins (*i.e.*, cefamandole) leading to a greater proportion of nonfluorescent products than fluorescent products. Studies are in progress to elucidate these fluorescent species.

Fluorescence in Biological Fluids—To assess the utility of this fluorometric method for the analysis of biological samples, cephalexin and ampicillin standards were prepared in serum and treated as described previously, and their fluorescence intensity was measured. Although the fluorescence intensity was reduced compared to aqueous samples, linearity was obtained over the concentration range studied (0.05–10.0 μg/ml).

It is expected that other cephalosporins could be treated similarly or

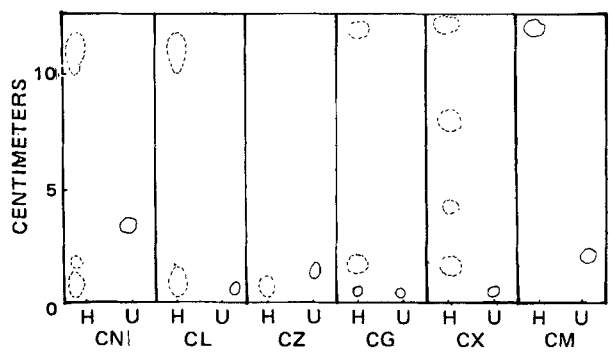

Figure 5—*Chromatogram of hydrolyzed (H) and unhydrolyzed (U) cephalosporins. Dotted lines indicate fluorescence under long UV light. Key: CN, cephalothin; CL, cephalexin; CZ, cefazolin; CG, cephaloglycin; CX, cephalexin; and CM, cefamandole.*

extracted and then reacted with sodium hydroxide to give sufficient fluorescence for detection in serum or tissue samples.

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Vaginal Drug Absorption in Rhesus Monkeys I: Development of Methodology

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Abstract □ Earlier reports from these laboratories described a procedure for determining vaginal drug absorption in the rabbit based upon a perfusion system, and data on the vaginal absorption of the straight-chain aliphatic alcohols and carboxylic acids were given. These studies have been extended to the rhesus monkey. Rib-cage-type cells were designed for intravaginal insertion through the vulval orifice and to fit the specific dimensions of the monkey vagina. The general design of the cell was similar to that used in the rabbit vaginal absorption studies. The perfusion system was checked by using ^{3}H-polyethylene glycol 4000, and no significant leaks from the cell were found. The absorption of the alcohols followed first-order kinetics. The computed apparent permeability coefficients for the alcohols were of comparable magnitude to those previously reported for the rabbit vaginal membrane.

Keyphrases □ Absorption, vaginal—various straight-chain aliphatic alcohols, perfusion system designed, rhesus monkeys □ Vaginal absorption—various straight-chain aliphatic alcohols, perfusion system designed, rhesus monkeys □ Perfusion systems—vaginal drug absorption, various straight-chain aliphatic alcohols, rhesus monkeys □ Alcohols, straight-chain aliphatic, various—vaginal absorption, perfusion system, rhesus monkeys

Research efforts in these laboratories have been based upon the premise that the pivotal event in regional or systemic therapy *via* the vaginal absorption route may be drug transport across the vaginal membrane. A method was described (1) for evaluating drug absorption in the vagina, using the rabbit doe as a prototype animal. A rib-cage-type cell, which provided a closed absorption compartment in the vaginal tract, was designed and surgically implanted. Drug absorption was determined by perfusing the drug solution through this system.

In subsequent reports, the absorption of straight-chain aliphatic alcohols (2) and aliphatic carboxylic acids (3) was described. The data agreed with a physical model involving a diffusion layer in series with a membrane consisting of two parallel pathways: a lipoidal pathway and an aqueous "pore" pathway. The surface pH of the rabbit vaginal membrane under the conditions prevailing during the experiments could be well approximated by the pH of the bulk lumenal solution (4).

The rabbit doe was chosen for these studies because it does not exhibit an estrous cycle and the vaginal tissues exhibit a constancy in their histological, biochemical, and physiological properties not seen with other rodents and most other mammals (5). The lack of a sexual cycle in the rabbit was expected to provide minimum variability in the membrane permeability behavior (1–4).

In the human female, however, the secretion of estrogenic hormones in the ovarian cycle induces cyclic changes in the histology, biochemistry, and physiology of the vaginal tissues; therefore, the vaginal membrane might exhibit a corresponding cyclic barrier behavior. Thus, the rationale for the present investigation was to extend the studies to a sexually cyclical animal, closely approximating the human female with regard to ovarian-induced membrane changes. The Macaques rhesus species of the higher primates was selected. The rhesus has an ovarian cycle of approximately 28 days, as does the human female. It is widely believed by researchers in the fertility area that the rhesus and human reproductive systems are functionally similar in that they have comparable anatomy and phys-