antimelanoma studies (25, 26), possesses activity; and (*f*) no carboxyl-containing catechol is active.

The data from the testing of the trihydroxyphenethylamines (XV and XVI) indicate that it may be important to restrict potential quinone formation possibilities to only an *o*-quinoid structure. The P-388-inactive neurotoxin, 6-hydroxydopamine (XV), when oxidized, can form either an *o*- or a *p*-quinone. When given this choice, quinones in general and XV in particular (13) form hydroxy-*p*-quinones, which weakly react with sulfhydryl groups (17). The other trihydroxy compound studied, 5-hydroxydopamine (XVI), can form only an *o*-quinone. It has good P-388 activity. Therefore, one would expect that 2-hydroxydopamine, the perdemethyl analog of XVII, should have P-388 activity. Studies are in progress to determine further structure–activity relationships among polyhydroxy derivatives of benzene and pyridine.

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Adsorption of Antineoplastic Drugs to Polyalkylcyanoacrylate Nanoparticles and Their Release in Calf Serum

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Abstract □ Conditions are described for attaching anticancer drugs to polyalkylcyanoacrylate nanoparticles, a new drug delivery system for cells that exhibit endocytic uptake. They are ultrafine, metabolizable, and able to associate with various drugs in a nonspecific manner. Data are given for the *in vitro* degradation and for drug release from this new drug carrier system.

Keyphrases □ Polyalkylcyanoacrylate nanoparticles—adsorption and release of dactinomycin and methotrexate, liberation kinetics, controlled release □ Dactinomycin—controlled-release dosage forms, polyalkylcyanoacrylate nanoparticles, adsorption and release kinetics □ Methotrexate—controlled-release dosage forms, polyalkylcyanoacrylate nanoparticles, adsorption and release kinetics □ Dosage forms, controlled release—polyalkylcyanoacrylate nanoparticles, adsorption and release of dactinomycin and methotrexate

Entrapment of cytotoxic drugs inside endocytizable carriers such as liposomes improves drug specificity and reduces toxicity toward nondiseased cells (1, 2). Work in this field has resulted in the development of polyacrylamide nanocapsules (3, 4). Polyacrylamide nanocapsules also may be useful in promoting cellular uptake *via* endocytosis for compounds that do not gain access to lysosomes (5).

Due to their polymeric nature, these small capsules (diameter of ~200 nm) may be more stable than liposomes in biological fluids and during storage (6–9). Furthermore, they can entrap various molecules in a stable and reproducible way (3, 5). However, this new lysosomotropic carrier is unlikely to be digested by lysosomal enzymes, which may restrict its clinical use. With these considerations, biodegradable nanoparticles made by polymerization of various alkylcyanoacrylate monomers were developed recently (10, 11). Similar polymers are used in surgery as sutures and adhesive agents (12, 13).

This paper describes techniques for attaching two cytostatic drugs to these polyalkylcyanoacrylate particles. Data concerning the degradability of these nanoparticles and drug liberation from this new intracellular formulation (14) are presented also.

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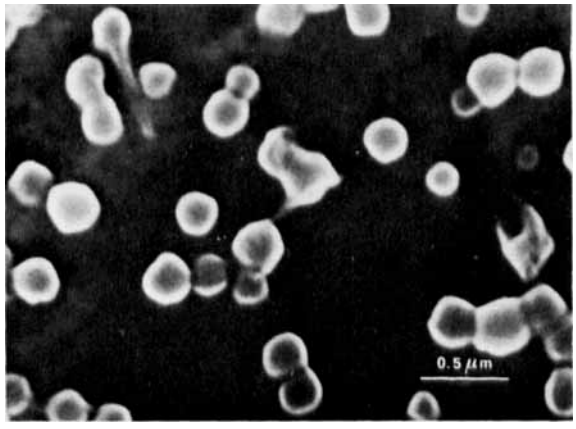

Figure 1—*Scanning electron micrograph of nanoparticles.*

EXPERIMENTAL

Polyalkylcyanoacrylate Nanoparticle Preparation—The nanoparticles were prepared by literature methods (10,11). After dissolution of the cytotoxic drug (methotrexate or ^{3}H-dactinomycin[1]) in 50 ml of water containing 0.25 g of polysorbate 20[2] and 5 ml of 0.1 *M* HCl, 0.83 ml of monomer (methylcyanoacrylate[3] or ethylcyanoacrylate[3]) or of various monomer mixtures was added with stirring. After 30 min, the nanoparticles appeared as a milky suspension displaying the Tyndall effect. They were buffered to pH 7 with 10 ml of a phosphate buffer (15). Scanning electron microscopy showed mostly spherical particles with a diameter of ~0.2 μm (Fig. 1).

Measurement of Dactinomycin Linked to Polymethyl- and Polyethylcyanoacrylate Nanoparticles—Dactinomycin, 1 mg, and ^{3}H-dactinomycin solution, 20 μl, were dissolved in 50 ml of the acid solution containing polysorbate 20. The nanoparticles were prepared as already described. The radioactive concentration of the tritiated dactinomycin solution was 0.5 mCi/ml with a specific activity of 16.5 Ci/mmole. The dactinomycin concentration of the radioactive solution was 90 μg/ml.

The nanoparticle suspensions (10 ml) were centrifuged at 20,000 rpm to separate dactinomycin bound to the particles from free dactinomycin. The sediment was dissolved in 10 ml of dimethylformamide. This solution was diluted to 20 ml with a mixture of calf serum and 0.45% polysorbate 20 in 0.009 *N* HCl (3:1). The supernate was diluted to 20 ml with dimethylformamide. The ^{3}H-dactinomycin in sediment and supernatant layers was measured by scintillation counting.

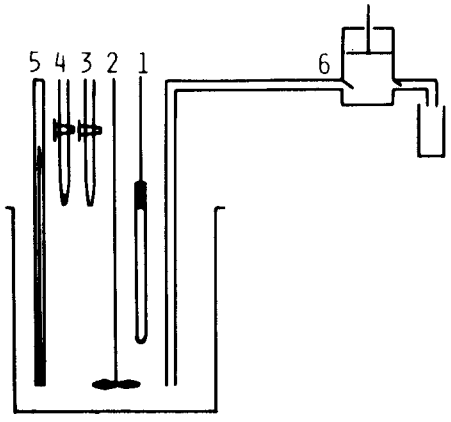

Figure 2—*System for nanoparticle incubation (17). Key: 1, pH measurement electrode; 2, stirrer; 3, automatic buret, hydrochloric acid solution; 4, automatic buret, sodium hydroxide solution; 5, thermometer; and 6, automatic sampler.*

[1] M.B.L.E., The Radiochemical Centre, Amersham, England.
[2] Tween 20, I.C.I., Atlas International Division, Everberg, Belgium.
[3] Loctite, Dublin, Ireland.

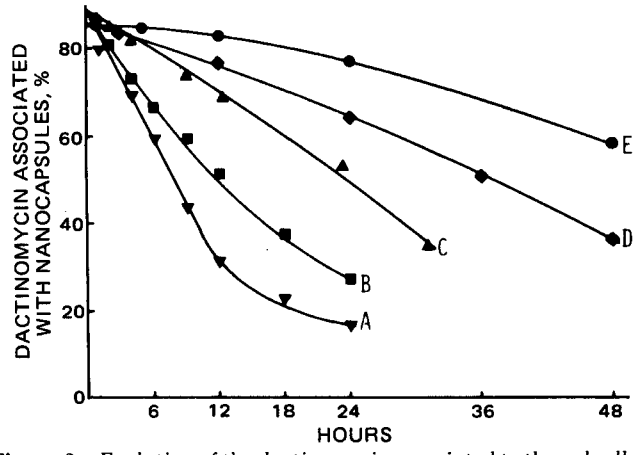

Figure 3—*Evolution of the dactinomycin associated to the polyalkylcyanoacrylate nanospherules as a function of the incubation time. Key: A, polymethylcyanoacrylate; B, mixture of polymethyl- and polyethylcyanoacrylate (2:1); C, mixture of polymethyl- and polyethylcyanoacrylate (1:1); D, mixture of polymethyl- and polyethylcyanoacrylate (1:2); and E, polyethylcyanoacrylate.*

Sample Preparation for Scintillation Counting—One-milliliter aliquots of the sediment and supernate were pretreated with 0.75 ml of 0.5 *N* quaternary ammonium hydroxide in toluene[4]. Isopropanol, 0.75 ml, and 0.5 ml of 30% H_2O_2 were then added to decolorize the samples. After 60 min, 15 ml of xylol[5]–0.5 *M* HCl (9:1) was added, and the samples were counted in a liquid scintillation counter[6]. Quench correction was obtained using a channels ratio method. The results were expressed as the percent of dactinomycin bound to the nanoparticles.

Test for Degradation of Polyalkylcyanoacrylate Nanoparticles—One hundred-milliliter portions of the various buffered polyalkylcyanoacrylate nanoparticle suspensions were incubated at 37° in 200-ml portions of calf serum–modified Eagle-Earle Dulbecco medium (1:9) (16).

The incubation took place in a thermostated cell. The cell was connected to a pH regulator by a glass electrode. The pH regulator was composed of two automatic burets and a pH meter. The burets maintained pH 7 ± 0.05 by delivery of 1.0 *N* HCl or NaOH. An automatic system permitted the uptake of nanocapsule samples at various times (Fig. 2).

Measurement of Methotrexate Linked to Both Polymethyl- and Polyethylcyanoacrylate Nanoparticles—Methotrexate nanoparticles were prepared with various amounts of drug (10–200 μg/ml) and monomers (1–16.7 μl/ml) to determine the influence of these two factors on the adsorptive capacity of polymethyl- and polyethylcyanoacrylate nanoparticles. The determination of the nanoparticle methotrexate content was made according to a fluorometric method (18).

After centrifugation of a 10-ml suspension of methotrexate nanoparticles at 20,000 rpm for 1 hr, the sediment was dissolved in 10 ml of acetone. This solution (40 μl) was added to distilled water (9 ml) and pH 5, 5 *M* acetate buffer[7] (200 μl). Distilled water was then added to exactly 10 ml. After addition of 4% potassium permanganate (100 μl) and a 5-min wait, the solution was decolorized with hydrogen peroxide (200 μl).

The supernate (40 μl) was treated in the same manner. The samples corresponding to the sediment and to the supernate were measured fluorometrically according to the method described previously.

RESULTS AND DISCUSSION

Degradation of Polymethyl- and Polyethylcyanoacrylate Nanoparticles Associated with ^{3}H-Dactinomycin—After dilution of the nanoparticle suspension (100 ml) in the calf serum (200 ml) and before incubation at 37°, 90.2% of the ^{3}H-dactinomycin was linked to the polymethylcyanoacrylate nanoparticles. With polyethylcyanoacrylate, 85.9% of the ^{3}H-dactinomycin was associated with the nanoparticles. The

[4] Soluène 350, Packard Instruments S.A., Brussels, Belgium.
[5] Instagel, Packard Instrument S.A., Brussels, Belgium.
[6] Philips PW 4510 computer, MBLE S.A., Brussels, Belgium.
[7] Prepared by adding 700 ml of 5.0 *M* sodium acetate to 300 ml of 5.0 *M* acetic acid.

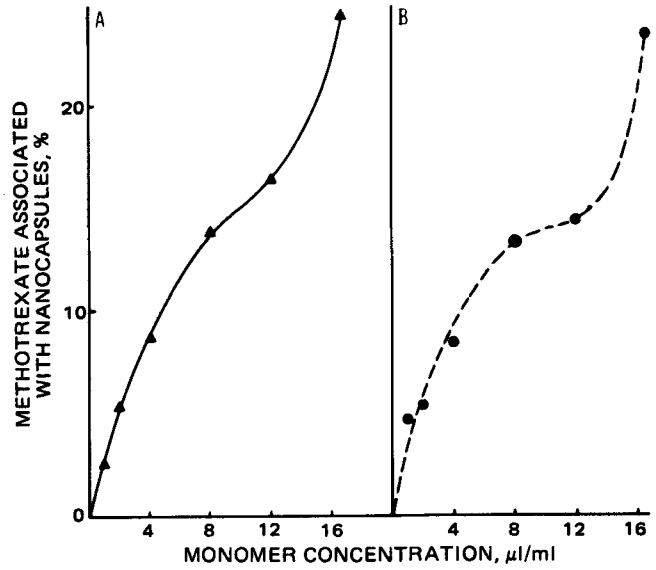

Figure 4—*Isothermic association of methotrexate with the nanoparticles at room temperature (20°). Methotrexate concentration = 100 µg/ml. Key: A, polymethylcyanoacrylate nanoparticles; and B, polyethylcyanoacrylate nanoparticles.*

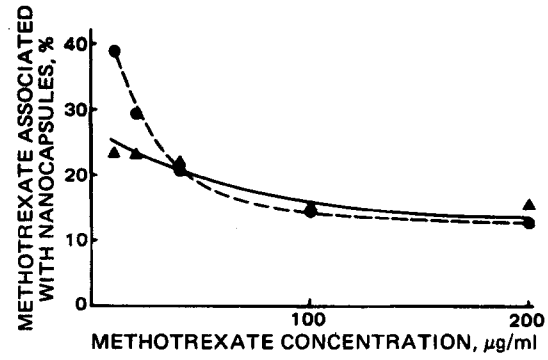

Figure 5—*Decrease of the methotrexate bound to the nanoparticles (percent) as a function of the drug amount in the initial solution. Monomer concentration = 12 µl/ml. Key: ▲—, polymethylcyanoacrylate; and ●—, polyethylcyanoacrylate.*

dactinomycin concentration was 20 µg/ml, and the monomer concentration was 16.6 µl/ml.

The measurement of dactinomycin disappearance from the sediment and its appearance in the supernate provided a good estimation of the nanoparticle degradation in the calf serum. The degradation velocity was related directly to the drug liberation. When the particles were degraded completely, no sediment was observed, and the whole dactinomycin activity was found in the supernate. When the nanoparticles were still intact, the sediment contained most of the dactinomycin activity, and only 10–15% of the drug was in the supernate.

The nanoparticle degradation kinetics, as indicated by dactinomycin release, seem to strongly depend on the alkyl chain length of the cyanoacrylate polymer product.

The polymethylcyanoacrylate nanoparticles (Fig. 3, curve A) were degraded almost entirely after 18 hr, whereas the polyethylcyanoacrylate nanoparticles (Fig. 3, curve E) retained practically 50% of the dactinomycin after 2 days of incubation at 37°. Nanoparticle preparation with various mixtures of methyl- and ethylcyanoacrylates (2:1, 1:1, and 1:2) provided intermediate degradation velocity values (Fig. 3, curves B, C, and D, respectively). These results show that polyalkylcyanoacrylate nanoparticles might represent a new drug delivery system which could degrade at various rates.

Methotrexate Adsorption on Polymethyl- and Polyethylcyanoacrylate Nanoparticles—Methotrexate was bound less extensively to the nanoparticles than was dactinomycin. In the better cases, an adsorption rate of ~25% was observed (Fig. 4). At the same monomer concentration (16 µl/ml), nanoparticles adsorbed about 90% of dactinomycin. Nanoparticles were prepared with various quantities of monomers and methotrexate to determine their influence on the associative tendency of the drug to optimize a nanoparticle formulation containing methotrexate.

The extent of methotrexate bound to the nanoparticles for polymethyl- (Fig. 4, curve A) and for polyethylcyanoacrylate (Fig. 4, curve B) increased sinusoidally with the monomer content in the initial solution. Monomer concentrations above 16.6 µl/ml were practically unusable due to the very high suspension viscosity. The percentage of methotrexate associated with the nanoparticles decreased with increasing drug in the initial solution (Fig. 5). This finding confirmed the poor methotrexate adsorption in comparison with dactinomycin. Indeed, only 38.5% methotrexate was adsorbed for the most dilute solution (10 µl/ml) with the ethyl product.

CONCLUSIONS

Many tumors cells exhibit a high endocytic capacity (19, 20). This knowledge was exploited by several investigators (21, 22) to entrap anticancer agents within phospholipid vesicles (liposomes) and albumin microspheres (23). Likewise, a DNA–daunorubicin complex was developed and was found to be endocytizable (24, 25). The therapeutic potential of nanocapsules-entrapped substances has stimulated numerous studies of their behavior *in vivo* (26, 27) and of their interactions with cells *in vitro* (5). The accumulation of polyacrylamide nanocapsules in cell lysosomes by endocytosis was demonstrated (5). However, since that polymer is unlikely to be degraded in the lysosomes, its use might be restricted because prolonged overloading of lysosomes could ensue.

This paper describes the design of digestible polyalkylcyanoacrylate nanocapsules, which can degrade at various speeds based on the monomer chosen. The drug liberation kinetics may be controlled also. The binding capacity of these nanoparticles to both dactinomycin (90%) and methotrexate (15–40%) exceeds that from the entrapment of these drugs in liposomes. Indeed, percentages of drug entrapment in liposomes are 4–18% for methotrexate (28, 19) and 2.3–11.6% for dactinomycin (30).

Polyalkylcyanoacrylate nanoparticles could be a new drug delivery system for cells that exhibit endocytic uptake since they are ultrafine, degradable, and able to associate with various drugs in a nonspecific manner. Furthermore, the morphology and drug adsorption of these particles are reproducible. Such nanoparticles are possible biodegradable lysosomotropic drug carriers. Future investigation with polyalkylcyanoacrylate nanoparticles will be carried out *in vivo*.

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High-Performance Liquid Chromatographic Studies of Reaction of Hydralazine with Biogenic Aldehydes and Ketones

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Abstract □ To understand hydrazone formation in hydralazine metabolism, the reaction of hydralazine with various biogenic aldehydes and ketones (acetone, pyruvic acid, acetoacetic acid, formaldehyde, and acetaldehyde) in pH 7.4 buffer was studied for potential alterations in hydralazine pharmacokinetics secondary to alcoholism and diabetes. The corresponding hydrazones were isolated, and their structures were characterized. High-performance liquid chromatography was used to monitor the reactions. An aqueous solvent reversed-phase liquid chromatographic system was used to separate hydralazine and its derivatives. Reaction of hydralazine with formaldehyde or acetaldehyde produced the corresponding hydrazones. Formation of an *s*-triazolo ring system yielded the known *s*-triazolo[3,4-α]phthalazine and 3-methyl-*s*-triazolo[3,4-α]phthalazine metabolites, which also were isolated and characterized and suggested nonenzymatic metabolism.

Keyphrases □ Hydralazine—reaction with biogenic aldehydes and ketones, hydrazone formation, high-performance liquid chromatographic analysis □ Antihypertensive agents—hydralazine, reaction with biogenic aldehydes and ketones, hydrazone formation, high-performance liquid chromatographic analysis □ High-performance liquid chromatography—analysis, hydralazine reaction with biogenic aldehydes and ketones, hydrazone formation

The mechanism of hydralazine condensation with biogenic aldehydes and ketones is significant. The reaction, involving hydrazone formation, may be enhanced in alcoholism, diabetes, and fasting states and during prolonged hydralazine administration. Hydrazone formation is important for investigating hydralazine toxicity and alterations in hydralazine metabolism and pharmacokinetics. While hydrazone formation has been evaluated *in vivo* (1–5), no supporting chemical studies have been reported.

A recently developed high-performance liquid chromatographic (HPLC) method (6) is sufficiently sensitive for clinical assay of derivatized hydralazine. The reaction of hydralazine with biogenic aldehydes and ketones apparently is nonenzymatic. The chemistry in an *in vitro* homogeneous solution at physiological conditions should be analogous to the *in vivo* process. Thorough study of this chemistry is complicated by the numerous biogenic ketones and aldehydes available to undergo this condensation *in vivo*.

This report describes the use of HPLC to determine the overall reaction of hydralazine with biogenic aldehydes and ketones to form hydrazones and the subsequent reaction of specific hydrazones to form *s*-triazolo[3,4-α]phthalazine and 3-methyl-*s*-triazolo[3,4-α]phthalazine. HPLC is well suited to this objective since the reversible reaction is usually sufficiently slow to permit discrete analysis of the reaction mixtures. This investigation was undertaken as a part of a long-term study of *in vivo* hydrazone formation from hydralazine in drug toxicity.

EXPERIMENTAL

Materials—Hydralazine[1], acetaldehyde[2], pyruvic acid[2], formaldehyde[2], 8-chlorotheophylline[3], acetone[4], and acetoacetic acid[4] were used as obtained. High purity samples of *s*-triazolo[3,4-α]phthalazine (III*a*) and 3-methyl-*s*-triazolo[3,4-α]phthalazine (III*b*) were prepared (5, 7) and used as standards.

Apparatus—A high-pressure liquid chromatograph[5] was equipped with a septumless injector port[6] and variable-wavelength UV absorption[7] and fluorescence detectors[8]. The column eluate was monitored by UV absorption at 240 nm and by fluorometry with excitation at 240 nm and

[1] Courtesy of Dr. M. Wilhem, Ciba-Geigy, Summit, N.J.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Fisher Scientific Co., Pittsburgh, Pa.
[5] Model 6000A, Waters Associates, Milford, Mass.
[6] Model U6K, Waters Associates, Milford, Mass.
[7] Model SF-770, Schoeffel Instruments Corp., Westwood, N.J.
[8] Model FS-970, Schoeffel Instruments Corp., Westwood, N.J.

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