The Biochemical Events of Mitosis. I. Synthesis and Properties of Colchicine Labeled with Tritium in Its Acetyl Moiety*

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ABSTRACT: Colchicine, labeled with tritium in the acetate group, has been prepared with a specific activity of 250 mc/mmole. U.S.P. colchicine was purified, then hydrolyzed to trimethylcolchicinic acid. The trimethylcolchicinic acid was next methylated to a mixture of deacetylcolchicine and isodeacetylcolchicine. The deacetylcolchicine was separated from the isodeacetylcolchicine by silica gel column chromatography and acetylated to colchicine with tritiated acetic anhydride. The newly synthesized colchicine (acetyl-^{3}H) was separated from the reaction mixture and purified on a Bio-Sil-A silicic acid column. A method for identification of colchicine and several of its derivatives has been developed, utilizing the decrease in absorbancy that occurs at 350 mμ when these compounds are exposed to ultraviolet light. Rate constants, different for each compound tested, have been determined under standard conditions. A simple method for separation and identification of colchicine, deacetylcolchicine, and isodeacetylcolchicine has been developed by use of silica gel G thin layer chromatography with methanol as the developing solvent. The R_F for colchicine is this system is 0.56, for deacetylcolchicine 0.4, and for isodeacetylcolchicine 0.3.

The understanding of mitosis has been greatly benefited through the widespread use of the plant alkaloid, colchicine, whose powerful and unique action in interrupting cell division was first recognized in 1934. It is widely believed that colchicine exerts its effect by interfering with the normal function and structural integrity of the mitotic spindle (Eigisti and Dustin, 1955; Sauaia and Mazia, 1961; Dustin, 1963; Taylor, 1965). However, some recent work with deacetyl-*N*-methylcolchicine indicates that colchicine may have no effect on the spindle, but causes inhibition of cell division by preventing the normal movement or function of the centrioles (Brinkley, 1965; Stubblefield, 1965). The present study was initiated as a beginning approach in this laboratory to the more biochemical aspects of mitosis and the mechanism of its inhibition by colchicine. It is hoped that by the use of colchicine (acetyl-^{3}H), some of the molecular events of mitosis as yet unrevealed by the usual cytological procedures with light or electron microscopy will be uncovered.

Several groups have utilized radioactive colchicine for studies on the mechanism of the mitotic inhibition as well as in more general tracer experiments. Raffauf *et al.* (1953a) synthesized several derivatives of colchicine-^{14}C with specific activities of about 1 mc/mmole. Walaszek *et al.* (1952, 1960) isolated colchicine-^{14}C from *Colchicum autumnale* previously grown in an atmosphere of CO_2-^{14}C. More recently, Taylor (1965) prepared colchicine of high specific activity (2.5 c/mmole) by methylation of colchicine with diazomethane in tritiated water. This procedure yielded colchicine specifically labeled with tritium in the methyl group of ring C. Taylor has studied the kinetics of inhibition and binding of colchicine-^{3}H in cultures of human cells. This communication describes a practical method (outlined in Scheme I) for labeling the acetyl moiety of colchicine with tritium.

Materials and Methods

Tritiated acetic anhydride (sp act. 500 mc/mmole) was obtained from New England Nuclear Corp. U.S.P. colchicine was a gift from Eli Lilly and Co. BDH chromatographic aluminum oxide was used in the purification of U.S.P. colchicine. Final purification of colchicine (acetyl-^{3}H) was performed with Bio-Sil-A (100–200 mesh) silicic acid obtained from Bio-Rad Laboratories. The methanol and chloroform (ethanol free) used in all procedures was of spectrophotometric grade. Melting points were measured with the Nalge melting point apparatus and are corrected. Spectra were obtained with the Cary Model 14 recording spectrophotometer. Tritium was determined with a Tri-Carb scintillation counter in 1.2% 2,5-diphenyloxazole and 0.5% 2,2′-*p*-phenylenebis(5-phenyloxazole) in dioxane–anisole–dimethoxyethane 6:1:1 (Davidson and Feigelson, 1957).

All thin layer chromatography was carried out as follows: plates of glass (8 in. long by 2 or 3 in. wide) were coated with a layer of Brinkmann silica gel G for thin layer chromatography (250 μ thick) by applying a slurry of 25 g of the silica gel G/50 ml of water.

* Department of Pharmacology, Tufts University School of Medicine, Boston, Massachusetts 02111. *Received March 23, 1966.* This investigation was supported by Public Health Service Graduate Training Grant 5T1-GM-765 from the Institute of General Medical Sciences.

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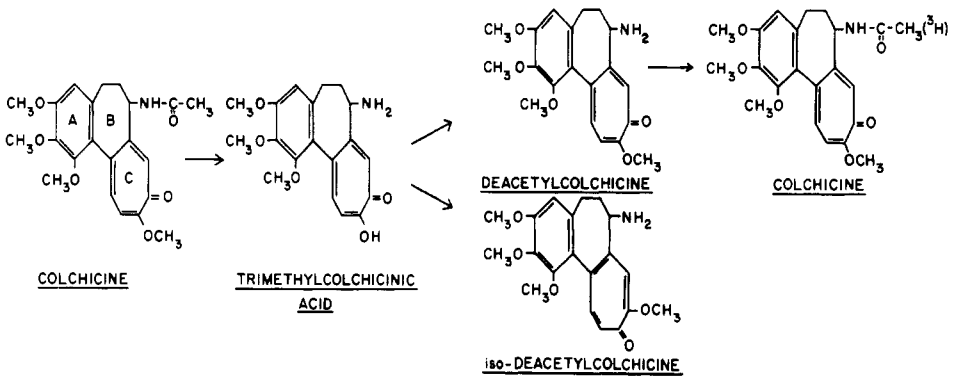

SCHEME I

After drying at 80° for 1.5 hr, the coated plates were ready for use. Usually 100–200-μg quantities of colchicine and its derivatives were applied and the chromatograms were developed with 100% methanol. By this method, the following R_F values were obtained: isodeacetylcolchicine, 0.3; deacetylcolchicine, 0.4; and colchicine, 0.56; seen as yellow spots under ultraviolet light.

Since colchicine and some of its derivatives are very unstable in light and oxygen, special care must be taken in the handling and storage of these compounds. During the purification of colchicine, the product was collected on filters under oxygen-free nitrogen. All columns were wrapped with aluminum foil. All crystalline preparations and solutions of colchicine and its derivatives were stored under nitrogen in the dark at −20°. Material applied to thin layer plates for chromatography was dried with a stream of nitrogen.

Experimental Section

Purification of Colchicine. Colchicine was purified by passage over a column packed with alumina followed by recrystallization from ethyl acetate according to the method of Ashley and Harris (1944). Pure white crystals of colchicine were obtained melting at 150–153°. The ultraviolet spectrum in 95% ethanol showed the expected pattern (two peaks), λ_{max} 350 mμ (ε 16,740) 244 mμ (ε 30,000) (Horowitz and Ullyot, 1952).

Hydrolysis of Colchicine to Trimethylcolchicinic Acid. Colchicine was deacetylated by acid hydrolysis according to a modified method of Fernholz (1953). The methyl group of ring C, which is more labile than the acetate group, is also removed and must therefore be replaced later.

Purified colchicine (5 g) was dissolved in 60 ml of a 1:1 mixture of methanol and concentrated HCl and refluxed for 12 hr. The resulting solution was diluted by the addition of 40 ml of cold distilled water, then slowly neutralized with solid sodium carbonate. The flocculent precipitate of trimethylcolchicinic acid was collected by centrifugation, washed with cold distilled water, and dried *in vacuo*. Crystallization from methanol containing 0.5% acetic acid yielded pale yellow, needle-shaped crystals, mp 152–155°, $[\alpha]_D^{24}$ −226.7° (*c* 1, chloroform) (Raffauf *et al.*, 1953b; Modelli and Vercellone, 1955). The ultraviolet spectrum in 95% ethanol showed a maximum at 355 mμ. Crude trimethylcolchinic acid was obtained in 65–70% yield. Upon recrystallization 60% was recovered with an over-all yield from colchicine of 40%.

Methylation of Trimethylcolchicinic Acid. Trimethylcolchicinic acid was methylated to a mixture of deacetylcolchicine and isodeacetylcolchicine (see Scheme I) according to the method of Raffauf *et al.* (1953b). A diethyl ether solution of diazomethane [15 ml, made from 2.48 g of nitrosomethylurea, 7.2 ml of 50% KOH, and 36 ml of diethyl ether in the usual manner, (Blatt, 1943)], was added to 0.955 g of trimethylcolchicinic acid in 25 ml of methylene chloride and maintained at 0–5° until a green color was no longer obtained upon addition of 1% ferric chloride to an aliquot of the reaction mixture. The mixture of the two isomers thus obtained was evaporated to dryness *in vacuo* and redissolved in methylene chloride. The isomers again were evaporated to dryness *in vacuo*. The evaporation procedure was repeated twice more to assure removal of all excess diazomethane. Essentially all of the trimethylcolchicinic acid was converted to the two methylated derivatives, with a ratio of deacetylcolchicine to isodeacetylcolchicine of 25:75. The dried residue of isomers was dissolved in 100% ethanol for storage.

Separation of Deacetylcolchicine from Isodeacetylcolchicine. Deacetylcolchicine and isodeacetylcolchicine can be conveniently separated by thin layer chromatography (see Methods). On the basis of these findings, an excellent large-scale separation of the isomers was obtained by use of a column packed with Brinkmann silica gel G for thin layer chromatography (Figure 1).

Several problems were encountered in our early attempts to separate deacetylcolchicine from isodeacetylcolchicine. Two methods have been described for the separation of deacetylcolchicine and isodeacetylcolchicine (Raffauf *et al.*, 1953b; Modelli and Vercellone, 1955). One involves precipitation of the *d*-tartrate of deacetylcolchicine from a solution of the two isomers by addition of *d*-tartaric acid. The other involves separation by alumina column chromatography. We could not isolate pure deacetylcolchicine with either

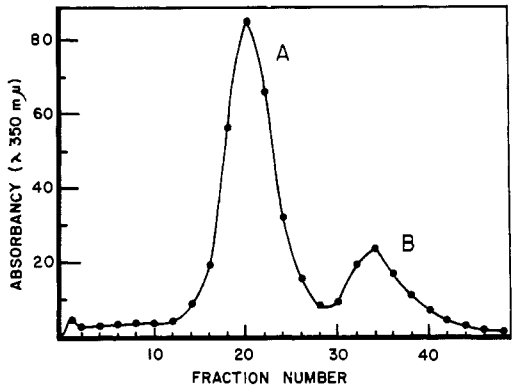

FIGURE 1: Separation of deacetylcolchicine and isodeacetylcolchicine. Silica gel G was scraped from 9 (20 × 20 cm) thin layer plates and with this material a 2.5-cm column was prepared to a height of 33 cm. Suction was used to pull the dry adsorbant down into a well-packed mass. A mixture of deacetylcolchicine and isodeacetylcolchicine (50 mg) was deposited on a 3-cm diameter spot on another plate. The silica gel with the adsorbed isomers was scraped from the plate and onto the top of the silica gel G in the column and then overlayered with 3 cm more of silica gel G. The isomers were eluted (slowly for maximum resolution) with 100% methanol from a 1-l. reservoir 3 ft above the column. Fractions (2.8 ml) were collected at 5-min intervals. Aliquots were taken and diluted with methanol for absorbancy measurements. The material in peak A was deacetylcolchicine showing a characteristic peak in the ultraviolet at 350 mμ (see Figure 2) and an R_F of 0.41 (one spot) by thin layer chromatography (see Methods). The material in peak B was isodeacetylcolchicine (λ_{max} at 344 mμ, as shown in Figure 2) and an R_F of 0.31.

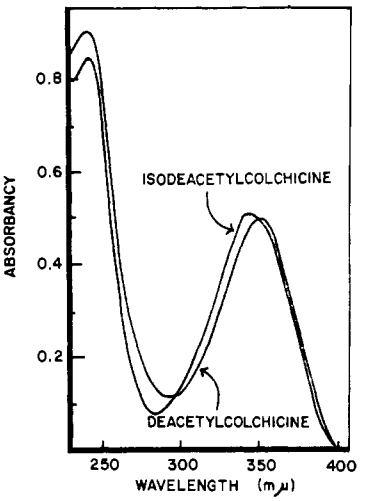

FIGURE 2: Spectra of deacetylcolchicine and isodeacetylcolchicine in 95% ethanol.

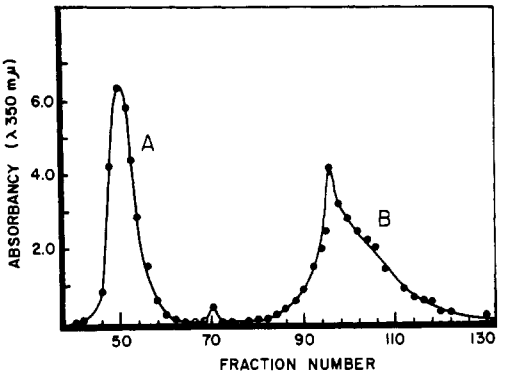

FIGURE 3: Separation of deacetylcolchicine and colchicine. A slurry of silicic acid (Bio-Sil-A), prepared by suspending 25 g in 100 ml of methanol, was poured into a 1-cm diameter column to a height of 30 cm after packing. A mixture of 5 mg of colchicine and 4 mg of purified deacetylcolchicine in methanol was applied to the column and chromatographed with methanol. The flow rate was adjusted to 1 drop/15 sec with a pressure bulb. This slow flow rate gave maximum resolution. Fractions (5 min) were collected and aliquots diluted with 95% ethanol for absorbancy measurements. Based on ultraviolet spectra and thin layer chromatography, the material in peak A was colchicine and in peak B, deacetylcolchicine.

of the above procedures. In each case the deacetylcolchicine was contaminated with varying amounts of isodeacetylcolchicine which, if present, would be acetylated in the next step to isocolchicine. Deacetylcolchicine could only be separated from isodeacetylcolchicine when conditions for thin layer chromatography were duplicated on a column (Figure 1). Several other silica gel preparations were ineffective.

Acetylation of Deacetylcolchicine to Colchicine. Deacetylcolchicine is readily converted to colchicine by acetylation with acetic anhydride (Raffauf *et al.*, 1953b). Since the two compounds can be separated quickly by thin layer chromatography (see Methods), the conversion of deacetylcolchicine to colchicine can be followed easily. For example, a small quantity of deacetylcolchicine was partially acetylated with acetic anhydride in pyridine for 30 min at 98°. The reaction mixture was spotted on a thin layer plate and chromatographed with methanol in the usual manner. The acetylated mixture gave two spots, one with an R_F of 0.39, corresponding to deacetylcolchicine, and a second with an R_F of 0.56, corresponding to a colchicine control of 0.57. On the basis of these findings a method was developed for large-scale separation of deacetylcolchicine and colchicine by column chromatography (Figure 3) and use was made of this simple procedure for the preparation of tritium-labeled colchicine as described in the following section.

Acetylation of Deacetylcolchicine with Acetic Anhydride-^3H. Deacetylcolchicine (65 mg, based on $\epsilon = 16{,}740$

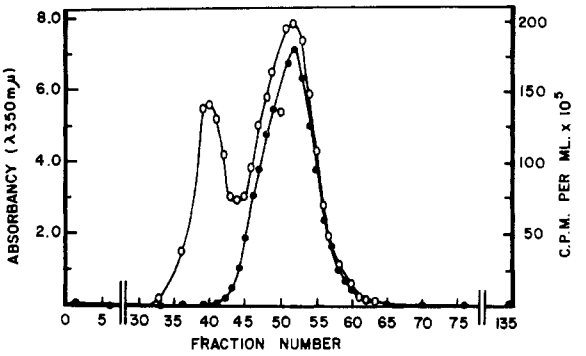

FIGURE 4: Purification of colchicine (acetyl-^{3}H). Impure colchicine (acetyl-^{3}H) in ethanol (1 ml of solution S, equivalent to product from 5.4 mg of deacetylcolchicine) was applied to an 8 × 260 mm column of silicic acid and eluted with methanol as described in Figure 3. Fractions (20 drops) were collected (1 drop = 10 sec). Each fraction was diluted with 95% ethanol for absorbancy measurements. Counting vials contained 10 μl of each fraction, 15 ml of scintillation fluid (see Methods), and 1.95 ml of H_2O. Open circles = radioactivity; closed circles = absorbancy.

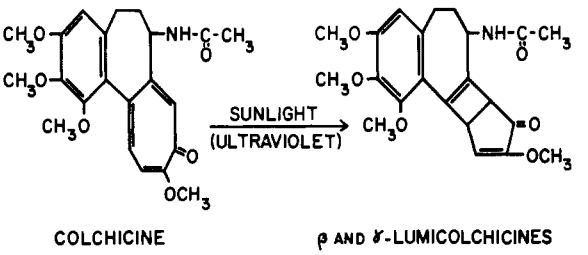

FIGURE 5: Conversion of colchicine to β- and γ-lumicolchicines.

at 350 mμ), contained in a small Pyrex tube, were dissolved by the addition of 1.5 ml of pyridine. After the further addition of 20.4 mg of acetic anhydride-^{3}H (500 mc/mmole) in 0.080 ml of benzene, the reaction mixture was heated for 1 hr at 98°. The contents of the tube were then transferred to a 300-ml round-bottom flask by rinsing with 2 ml of benzene and then evaporated to dryness *in vacuo* for 1 hr. Ethanol (12 ml) was added to dissolve the colchicine and then this solution (labeled S) was divided into 2-ml portions for convenient storage. Thin layer chromatography of a small quantity of the impure colchicine solution gave one spot with an R_F of 0.56, λ_{max} 350 mμ, indicating that complete conversion of deacetylcolchicine to colchicine had occurred.

Purification of Colchicine (Acetyl-^{3}H). Colchicine (acetyl-^{3}H) prepared as above was subjected to chromatography on a Bio-Sil-A column as previously described (Figure 3). The elution pattern thus obtained (Figure 4) indicated that the deacetylcolchicine was completely converted to colchicine as expected with good separation of tritium-containing impurities from the product. Furthermore, the radioactivity of the major peak coincided with the absorbancy, indicating probable purity of the colchicine (acetyl-^{3}H). Further purification of colchicine (acetyl-^{3}H) by rechromatography of combined tubes representing more than 90% of the colchicine yielded an elution profile with complete correspondence between absorbancy at 350 mμ and radioactivity.

Criteria for Purity of Colchicine (Acetyl-^{3}H). 1. CONSTANT SPECIFIC ACTIVITY UPON REPEATED CRYSTALLIZATION OF COLCHICINE (ACETYL-^{3}H). A sample of colchicine (acetyl-^{3}H) was taken from one of the peak fractions obtained by chromatography (Figure 4) and added to 74 mg of purified, twice recrystallized natural colchicine. The specific activity of the resulting mixture was determined, and again determined each time after three successive crystallizations from ethyl acetate–ether. The specific activity of the first mixture was 4.03, followed by values of 4.05, 4.09, and 3.82 (cpm × 10^4/mg). Another sample from another portion of the peak yielded similar results.

2. THIN LAYER CHROMATOGRAPHY. Thin layer plates were spotted with colchicine, colchicine (acetyl-^{3}H) and a mixture of colchicine plus the radioactive product (see Methods). After development with methanol, radioactivity was found only in small areas coincident with colchicine (R_F 0.58 ± 0.02).

3. SPECTRUM. The synthetic colchicine (acetyl-^{3}H) has a spectrum in 95% ethanol identical with that of purified natural colchicine (λ_{max} 350 and 243 mμ).

4. BREAKDOWN OF COLCHICINE AND SOME DERIVATIVES UPON IRRADIATION WITH ULTRAVIOLET LIGHT. Colchicine and its derivatives are sensitive to sunlight in aqueous solution, being converted to a mixture of isomers of colchicine (α-, β-, and γ-lumicolchicines) (Grewe and Wolfe, 1951; Gardner *et al.*, 1957; Forbes, 1955). It appears that the β- and γ-lumicolchicines are stereoisomers formed by a rearrangement of ring C of colchicine as shown in Figure 5. Forbes (1955) found that the ratio of α-, β-, and γ-lumicolchicines was approximately 1:16:1, while Grewe and Wolfe (1951) reported a ratio of 4:3:2.

By use of a strong ultraviolet source, colchicine and also its derivatives can rapidly be converted to the lumicolchicines when dissolved in 95% ethanol. Previous irradiation studies with the compounds dissolved in water required more extended periods of time. Our irradiation system (Figure 6) appeared to yield mainly β- and γ-lumicolchicines as determined by comparison of ultraviolet spectra (Grewe, 1951). Additional proof that β- and γ-lumicolchicines were the main products of irradiation of colchicine was the appearance of two isosbestic points at 305 and 255 mμ. The rearrangement can be followed easily by measuring the decrease of absorbancy at 350 mμ.

The absorbancy of colchicine or its derivatives was found to decrease according to first-order kinetics described by the relationship $Y = Y_0e^{-kx}$ (where Y = absorbancy at 350 mμ, x = time, Y_0 = the initial absorbancy at 350 mμ, and k = the rate constant).

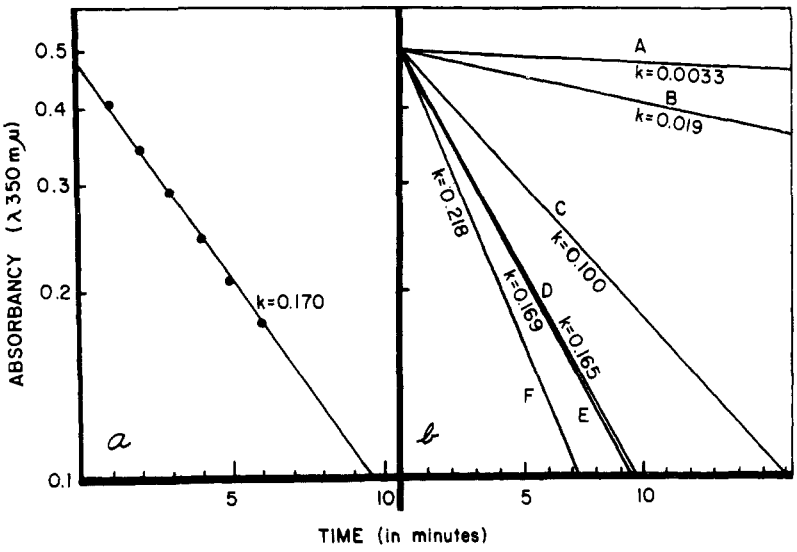

FIGURE 6: Rate constants for ultraviolet-induced decrease of absorbancy at 350 mμ for colchicine and derivatives Each compound was dissolved in 95% ethanol, adjusted to a concentration yielding an absorbancy of 0.5 at 350 mμ and then placed in a 1.4-ml quartz cuvet (1-cm light path) exactly 12 in. from a black ray long-wave ultraviolet lamp, Model B100 A (Ultraviolet Products, Inc., San Gabriel, Calif.). The apparatus was in a dark room to avoid extraneous light. After irradiation, the cuvet was quickly transferred to the spectrophotometer for an absorbancy measurement at 350 mμ, and then returned for further irradiation. Isodeacetylcolchicine (A) and isocolchicine (B) were irradiated for 10-min periods. Trimethylcolchicinic acid (C), colchicine (D), colchicine (acetyl-^{3}H) (E), and deacetylcolchicine (F) were irradiated for 1- to 2-min periods. (a) A typical irradiation experiment with colchicine (acetyl-^{3}H). (b) Decay curves for all compounds tested. All rate constants, as determined for each compound (results of two or three experiments), were averaged and in each curve, values of x were calculated with $Y_0 = 0.5$ (see formula in text). Values obtained in separate experiments were within 5%. Trimethylcolchicinic acid was the only compound tested which did not strictly adhere to the first-order relationship. Its rate of absorbancy loss increased slightly with time.

The rate of rearrangement appears to be dependent upon substitutions in rings B and C, since the rate of absorbancy decrease was found to be different in each derivative tested. Therefore, it is possible to identify several derivatives of colchicine by measuring the rate constant under controlled conditions. Figure 6a shows data obtained in a typical irradiation experiment with colchicine (acetyl-^{3}H). Rate constants from data of several experiments (as in Figure 6a) were averaged for each derivative and theoretical decay curves were collected for comparison on a single graph (Figure 6b).

It can be seen that the iso derivatives have a much slower breakdown rate than normal derivatives and, therefore, the change in position of oxygen and the methoxy groups in ring C from the normal to the iso configuration (see Scheme I) has a marked effect in stabilizing the molecule to ultraviolet light. It is quite evident that the synthetic colchicine (acetyl-^{3}H) has a rate constant of decay almost identical with that of purified natural colchicine. This is considered another important criterion of purity and identity of the radioactive preparation.

The semisynthetic procedure for preparation of purified colchicine (acetyl-^{3}H) yielded a product with a specific activity of 250 mc/mmole. More highly labeled material is dependent on the use of acetic anhydride-^{3}H. Commercial sources now offer acetic anhydride-^{3}H with an activity of 3 c/mmole.

The present procedure involves a direct acetylation which results in the formation of only the desired product, *i.e.*, labeled colchicine, whereas Taylor's procedure (1965) gives a mixture of both labeled colchicine and isocolchicine. The radiochemical impurity reported by Taylor has not been encountered in the present preparation. In metabolic studies, the availability of colchicine with isotopic labels in different positions should prove to be of value. We hope that the availability of this simple method for producing large quantities of highly labeled colchicine will provide a powerful tool for further studies on the many and varied biological effects of this unique substance.

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Reactions of Diacylamines Containing N-Protected Aminoacyl Groups*

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ABSTRACT: Diacylamines of the type $R_1CON(R_2)$-COC_6H_5, where R_1CO is derived from a N-protected α-amino acid or peptide, have been synthesized by the reaction of imidoyl chlorides with appropriate carboxylic acids. Evidence for the diacylamine structure assigned to the products is offered by (a) the formation of the substituted imidazolone-5 after removal of the N-protecting group, and (b) their reactivity with hydroxylamine. Hydroxylaminolysis yields preferentially the hydroxamic acid derived from the carboxylic acid of higher apparent pK_a; the mechanism of this reaction is discussed.

The reactivity of the amide group has received recurrent attention in relation to the chemistry of peptides and proteins. Several communications (McConnan and Titherley, 1906; Wieland *et al.*, 1955; Battersby and Robinson, 1955, 1956; Clayton *et al.*, 1956; Brenner *et al.*, 1957; Schofield, 1964; Shemyakin *et al.*, 1965) describe the base-catalyzed reactions of the amide group with other functional groups, in particular when such reactions are favored by stereochemical factors. The intermediates postulated or shown to be formed belong to the general class of acylated amides (diacylamines).[1] Diacylamines have been proposed as intermediates in the intramolecular participation of the CONH group in the solvolysis of esters (Bernhard *et al.*, 1962; Shafer and Morawetz, 1963; Behme and Cordes, 1964) and have been considered as possible intermediates in the catalytic action of pepsin (Neumann *et al.*, 1959; Fruton *et al.*, 1961; Bender and Kézdy, 1965; Delpierre and Fruton, 1965). Relatively few diacylamines containing N-protected aminoacyl groups have been described (Bergmann *et al.*, 1929; Bergmann and Tietzman, 1944; Wieland and Urbach, 1958; Kopple and Renick, 1958; Schellenberg and Ullrich, 1959; Cramer and Baer, 1960), and the current interest in the reactivity of such compounds led us to prepare several new diacylamines containing amino acid units, and to examine their properties.

Experimental Section[2]

Preparation of Imidoyl Chlorides. Powdered benzani-

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[1] Various terms have been used in the recent English and German literature to designate the class of compounds RCON(R')COR''. Among these is the term "imide," which appears to be intended for cyclic compounds [*Chem. Abstr. 56*, 52N (1962)]. Other terms are "diacylimide," "diacylamide," and "diacylamine." Pending a definitive recommendation by an appropriate body, we propose to use "diacylamine," since it appears to define this general class of compounds without ambiguity. In the special case where R' = H, however, the term "imide" has become well established through usage.

[2] All melting points were uncorrected. Microanalyses were performed by Dr. S. M. Nagy, Massachusetts Institute of Technology. Infrared spectra were recorded with a Beckman IR-5 spectrophotometer, and ultraviolet spectra were determined with a Beckman DB spectrophotometer.