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3′,5′-DIESTERS OF 5-FLUORO-2′-DEOXYURIDINE AND THYMIDINE: HYDROLYSIS BY ESTERASES IN HUMAN, MOUSE, AND INSECT TISSUE*

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Abstract—3′,5′-Aliphatic diesters of 5-fluoro-2′-deoxyuridine (FUDR) and thymidine are hydrolyzed by homogenates and centrifugal fractions of normal and tumor tissues taken from humans and mice, and by homogenates of houseflies and pea aphids. The esterases involved are widely distributed among all tissues and tissue fractions examined; are more active in most of the normal mouse tissues than in the mouse tumor or human tissues studied; generally have optimal activity with FUDR esters containing C_4 to C_8 acids; hydrolyze thymidine esters more rapidly than FUDR esters; and are sensitive to inhibition by organophosphates and eserine. The rate and site of initial attack on the 3′,5′-diesters depend on the esterase source and the ester chain length. Activity of these esterases is inhibited *in vivo* by administration of selective organophosphates.

THE carcinostatic activity and/or toxicity after oral administration of a series of 3′,5′-diesters of 5-fluoro-2′-deoxyuridine (FUDR), made from carboxylic acids containing four to sixteen carbon atoms, is greater than that of FUDR against certain transplanted mouse tumors. The dibutyryl, dihexanoyl, and dioctanoyl were the most effective antitumor agents of the FUDR diesters with aliphatic acids. However, such diesters with nonaliphatic acids as well as certain other esters do not have a distinct advantage for this use over aliphatic diesters.[1] Also, the FUDR esters are less potent than FUDR in inhibiting the reproductive potential of houseflies and aphids ingesting them.[1] It is assumed that these actions result from intial hydrolysis of the esters *in vivo*, followed by the intracellular formation of 5-fluoro-2′-deoxyuridine-5′-monophosphate, an inhibitor of thymidylate synthetase and, consequently, of DNA biosynthesis. After hydrolysis of the aliphatic esters, the FUDR formed is also susceptible to cleavage by nucleoside phosphorylase to yield 5-fluorouracil (FU) which is incorporated into RNA, replacing uracil.[2–5] The biological activity of these esters might therefore be determined by the nature and distribution of the esterases effecting their hydrolysis. Differences in activity or specificity, if any, of the esterases in tumor tissues compared with normal tissues are probably important in contributing to the selective action of these esters.

Esterases hydrolyzing nucleoside aliphatic esters have not been extensively studied. Human, rat, and mouse livers hydrolyze 3′-monoacetyl FUDR and 3′,5′-diacetyl FUDR to the nucleoside, the esteratic activity residing primarily in the particulate

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fraction. The 3′-monoacetyl FUDR cleaves to FUDR much more rapidly than does the 3′,5′-diacetyl FUDR, suggesting that the 5′-acetyl group is the more resistant to hydrolysis. Ehrlich ascites cells degrade acetylated FUDR at an extremely low, although significant, rate. Deacetylation is the rate-limiting reaction for the ultimate formation of FU via the intermediate, FUDR, according to studies *in vitro*.[4, 5]

One type of structural modification that has been utilized in deriving purine and pyrimidine antagonists useful in cancer chemotherapy is esterification of the hydroxyl groupings in their ribonucleosides and deoxyribonucleosides. 3′,5′-Diacetyl FUDR and 2′,3′,5′-triacetyl-6-azauridine have been most extensively studied in this respect, and these acetylated derivatives have been found to have greater stability *in vivo* than FUDR or azauridine (AzUR) respectively. This characteristic of the acetylated nucleosides gives rise to some or all of the following advantages of the esters over the nucleosides as carcinostatic drugs: an effectiveness on oral administration of the esters equal to or greater than intravenous administration of FUDR or AzUR; more rapid or more complete absorption through the gastrointestinal tract; higher blood and urine levels after oral administration to cancer patients, in which case the 5′-acetyl derivatives of FUDR and AzUR are the major persisting intermediates in the release of free FUDR and AzUR; less rapid cleavage to FU or 6-azauracil; and higher and/or more persistent blood levels of FUDR and AzUR, allowing higher levels of their respective 5′-phosphates to be formed intracellularly to effect their carcinostatic activity.[3–11]

It is apparent that additional work was warranted (i) to determine the activity and behavior of the esterases in mammalian and insect tissues that effect the hydrolysis of 3′,5′-diesters of FUDR and related nucleosides; and (ii) to find ways and means of altering or varying the release rate of the nucleosides from their esters. Consequently, experiments were made to determine the rate of hydrolysis of a series of 3′-5′-diesters of FUDR and thymidine by esterases obtained from normal and tumor tissues from experimental animals and humans; and the inhibiting action of selective organophosphorus esterase inhibitors. The rate of hydrolysis was determined by manometric and radioactive tracer techniques.

MATERIALS AND METHODS

Materials and apparatus

The FUDR esters used included the monoacetyl and monobutyryl derivatives and those diesters listed in Table 1; they were from a lot previously described.[1] In addition, the five carbonyl-^{14}C-labeled FUDR esters used were those previously described and included: 3′-acetyl-^{14}C,5′-acetyl-^{14}C FUDR; 3′-acetyl-^{14}C,5′-acetyl FUDR; 3′-acetyl,5′-acetyl-^{14}C FUDR; 3′-acetyl-^{14}C FUDR; 5′-acetyl-^{14}C FUDR.[1] Their specific activity was 36,000 cpm/μmole for those with one acetyl-^{14}C group and 72,000 cpm/μmole for the single compound with two acetyl-^{14}C groupings. Three 3′,5′-diesters of thymidine were prepared by the same procedure used to make the corresponding esters of FUDR[1] as follows. (i) Diacetyl, m.p. 126°–127° (reported m.p., 125°[12] and 126°–127°[13]), prisms, recrystallized from benzene (found: C, 51·55; H, 5·58; N, 8·68. $C_{14}H_{18}N_2O_7$ requires C, 51·53; H, 5·56; N, 8·59%). (ii) Dipropionyl, m.p. 92°, needles, recrystallized from benzene–hexane mixture (found: C, 53·92; H, 6·16; N, 7·92. $C_{16}H_{22}N_2O_7$ requires C, 54·23; H, 6·26; N, 7·91%). (iii) Dibutyryl, m.p. 110°–111°, needles, recrystallized from benzene–hexane mixture (found: C,

56·62; H, 6·85; N, 7·41. $C_{18}H_{26}N_2O_7$ requires C, 56·53; H, 6·85; N, 7·33%). The other ribosides or riboside esters utilized were provided by Dr. S. A. Schepartz and Dr. R. B. Ross of the Cancer Chemotherapy National Service Centre, National Institutes of Health, Bethesda, Md.

The white mice were normal 25-g females (Dan Rolfsmeyer Co., Madison, Wis.). The selected strains of mice with tumors were provided by Professor C. Heidelberger of the McArdle Memorial Laboratory, University of Wisconsin, Madison, Wis.[1] Designations used for these tumors were as follows: ascites, for Ehrlich ascites; AD-755 for adenocarcinoma 755; L-1210 for leukemia 1210; S-180 for sarcoma 180. The tumors ranged from 0·5 to 1·0 g per mouse for S-180 and AD-755 and 1·5 to 2·5 ml per mouse for ascites and L-1210. The mice were in fairly good health at the time of sacrifice and tumor removal; no necrotic tissues were used. The human tissues were either fresh samples provided by Professor Heidelberger from biopsy or autopsy and were held at 5° in isotonic saline solution for a few hours prior to assay; or were samples quickly frozen after removal, which were provided by Dr. T. C. Hall and Dr. D. Roberts, Children's Cancer Research Foundation, Inc., Boston, Mass., and Dr. C. E. Shonk, Research Laboratories, Merck, Sharp and Dohme, Rahway, N.J., through arrangements made by Dr. F. R. White of the Cancer Chemotherapy National Service Center. These tissues were classified as normal, benign tumors, or malignant tumors, based on pathological examination prior to their use in the present study. The pea aphids, *Acrythosiphon pisum* (Harris), and houseflies, *Musca domestica* L., were adults supplied by the Wisconsin Alumni Research Foundation, Madison, Wis.

Radioactive measurements were made with a model 314EX Packard Tri-Carb liquid scintillation spectrometer in glass scintillation vials with 15 ml of 2:1 toluene: methyl Cellosolve containing 0·55% 2,5-diphenyloxazole. Melting points were determined on microscope cover slips on a hot block by observing single crystals with a microscope; they were not corrected. The manometric esterase assays were made in an 18-unit GME model RWB-3 Warburg apparatus (Gilson Medical Electronics, Middleton, Wis.) in 10-ml Warburg flasks with a single side arm. Each microcell esterase assay was made on a $\frac{3}{4}$ in. circular glass cover slip, No. 1 thickness, held in a plastic micromount holder (item C881, Ward's Natural Science Establishment, Inc.. Rochester, N.Y.). A glass homogenizer (Potter-Elvehjem type) was used in preparing the tissue homogenates.

Triton X-100 emulsifier was obtained from Rohm and Haas Co., Philadelphia, Pa. The bicarbonate buffer solution contained $NaHCO_3$ (0·0357 M) and NaCl (0·164 M); a gas mixture of 5% CO_2 and 95% N_2 was bubbled through it just before use. The DEAE-cellulose material for column chromatography was Cellex-D, anion exchange quality rated at 0·52 mEq/g (Calbiochem, Los Angeles, Calif.).

Preparation of tissues and fractions for manometric esterase assay

Tissues from normal mice and tumors from selected strains of mice were removed rapidly after sacrifice. The blood was heparinized and centrifuged to separate plasma and corpuscles. The samples of human tissue, other than plasma and corpuscles, were taken for assay in the frozen state. When used directly (i.e. without fractionation), the insects and tissues, other than blood, were homogenized at 20% (w/v) in the bicarbonate buffer solution described above. With plasma and certain liquid

tumors, a higher concentration was occasionally used; with insects, the whole pea aphid was used, but only the abdomen from the houseflies was utilized.

In preparing the centrifugal fractions, the tissues were homogenized in 0·25 M sucrose solution, and centrifuged at 15,000 *g* and 5° for 30 min to separate the soluble (S) and particulate (P) fractions. However, the mouse liver homogenates were further separated by differential centrifugation into fractions designated as follows: nuclei, precipitated at 1500 *g* for 30 min; mitochondria, precipitated at 15,000 *g* for 30 min; microsomes, precipitated at 105,000 *g* for 60 min; soluble, supernatant from the 105,000 *g* centrifugation. The particulate fractions were reconstituted to the original homogenate volume with 0·25 M sucrose, mixed for washing of the precipitate, resedimented, and prior to assay made up again to the original homogenate volume with fresh 0·25 M sucrose.

The microsome fraction from mouse liver was further fractionated for one study. An acetone powder, prepared from the microsomes, was homogenized in water, and the protein components were fractionated with ammonium sulfate. The most active precipitate fraction was dissolved directly in 0·02 M (pH 7·6) Tris buffer and chromatographed on a DEAE-cellulose column (8 g, 2·2 × 18 cm), packed in Tris buffer, and developed with increasing concentrations of potassium chloride (0, 0·02, 0·06, 0·2, and finally 0·6 M) in Tris buffer. The buffer and potassium chloride did not interfere with the manometric assays (technique described later) on the column effluent fractions with the aliquot size that was used.

Manometric esterase assay

Esterase activity was determined indirectly by assay of the acid products which result in CO_2 evolution from a bicarbonate buffer at a pH of approximately 7·6 in an atmosphere of 5% CO_2 and 95% N_2 at 38°. A 0·40-ml portion of enzyme preparation (see above) was added to the side arm of the flask (in the absence or presence of added inhibitor), and 1·60 ml of substrate solution or emulsion (0·0025 M) was put into the main compartment of the flask (with or without added inhibitor). The flask and unmixed contents were incubated for approximately 20 min at 28° and for 10 min at 38° before tipping the flask twice to mix the enzyme and substrate portions. Manometer readings were made immediately before tipping the flask and at 5-min intervals thereafter for 30 min (or more).

If the CO_2 evolution was not linear for the first 20 min, the test was repeated with 0·40 ml of a more dilute enzyme preparation (diluted with buffer solution) until the desired linearity was achieved. In certain cases, the reaction was allowed to proceed for more than 30 min, until completion of hydrolysis was indicated by no further manometer change or until a stoichiometric volume of CO_2 had been evolved for the acid groups of interest.

Since many of the esters were of low water solubility, emulsions were prepared by adding Triton X-100 in acetone to dissolve the ester, evaporating the solvent, and adding the buffer with thorough mixing to yield a final concentration of 0·5 mg Triton X-100/ml. This level of emulsifier was routinely used with all except the formyl, acetyl, propionyl, and butyryl esters.

For tissue blanks the substrate was replaced with buffer, and for substrate blanks, the tissue was replaced with buffer. Triton X-100 was added to the blanks if it was used in the assay.

Protein levels were determined by the biuret procedure, with human plasma albumin (fraction V) as the standard.[14] Reaction rates were calculated only from the initial rate of CO_2 evolution, and results were expressed either as micromoles per milligram protein per hour or micromoles per 100 milligrams fresh tissue weight (or equivalent) per hour. The substrate level of the flask contents (after mixing) was 0·0020 M in all experiments unless otherwise stated.

Microcell esterase assays with radiolabeled substrates

Enzymatic reactions were made in a volume of 20 μliters on a circular glass cover slip held in a plastic micromount holder. The $\frac{1}{8}$-in. deep well created by placing the circular cover slip in the holder was sealed, to minimize evaporation during the reaction, by placing a second, larger cover slip over the top of the holder and then allowing a small water drop to flow under the edges of the upper cover slip. However, on reaction times of less than 15 min, the upper cover slip was omitted. (The reaction took place on the lower cover slip in a 20-μliter drop which did not touch the mount or upper cover slip. This procedure was based on one described that used acetyl-^{14}C-choline for assay of cholinesterase activity.[15])

Mouse liver was homogenized at 30% (w/v) in 0·25 M sucrose and the mitochondria and microsome fractions separated by differential centrifugation under conditions previously given. Each fraction was resuspended and washed with 0·25 M sucrose and finally reconstituted to the original volume of the homogenate with 0·50 M sucrose. To 10 μliters of the microsome or mitochondria suspension on each cover slip was added 10 μliters of an acetyl-^{14}C FUDR ester at 0·0040 M in 0·05 M (pH 7·3) potassium phosphate. Mixing was achieved by careful tilting of the reaction cells. The reaction was allowed to proceed at 28° for 0 to 40 min prior to the addition of 15 μliters of 0·1 N hydrochloric acid to stop the reaction and convert all free acetate-^{14}C to acetic-^{14}C acid. After evaporation of the acidified reaction mixture at room temperature with a gentle stream of air, the residue was heated for 3 to 5 min at 110° in an oven until the first signs of browning of the sucrose were evident. The cover slips were placed in scintillation vials, the scintillation mixture added, and the whole was held for 36 to 48 hr at 5° in the dark, with occasional swirling to achieve solution of the radioactive material deposited on the cover slip. The residual ^{14}C present after incubation, acidification, and heating was determined by liquid scintillation counting. In the same experiment, controls were made with acetate-1-^{14}C (in place of acetyl-^{14}C FUDR) to correct for incorporation or other effects that would yield the acetate added or released during hydrolysis in a nonvolatile form. (The necessary correction was usually about 3% and never more than 10% of the initial counts.)

Expiration studies with acetyl-^{14}C-labeled 3′,5′-diacetyl FUDR in mice

Acetyl-^{14}C-labeled 3′,5′-diacetyl FUDR was administered intraperitoneally, in 0·10 ml propylene glycol, to normal 25-g female white mice to yield a dose of 53·7 mg/kg. For comparison or as controls, similar mice were treated with equimolar acetate-1-^{14}C. The rate of $^{14}CO_2$ expiration was followed by the method of Jeffay and Alvarez[16] and, in certain cases, the total radiocarbon excreted in the urine of the mice was assayed. One such set of mice was treated 24 hr before the radioactive material with 0·10 ml corn oil, and another set was treated with 0·10 ml corn oil containing sufficient *S,S,S*-tributylphosphorotrithioate (DEF) to yield a dose of 80 mg/kg. Replication of the experiments was as follows: four DEF-treated and four control

mice were used with 3′-acetyl-^{14}C,5′-acetyl-^{14}C FUDR; one DEF-treated mouse was used for each of 3′-acetyl-^{14}C,5′-acetyl FUDR, and 3′-acetyl,5′-acetyl-^{14}C FUDR; two DEF-treated and two control mice were used for acetate-^{14}C.

Recovery and identification of FUDR and thymidine in hydrolysis reaction mixtures

Under the conditions described above (manometric esterase assay), 3′,5′-dibutyryl esters of FUDR and thymidine were hydrolyzed enzymatically to near completion as evidenced by manometric measurement of the CO_2 evolved. Comparable reaction mixtures containing only the deoxyribonucleosides, FUDR and thymidine, were also incubated in the same manner, and these gave no CO_2 evolution. The reaction mixtures were removed from the Warburg flasks and evaporated to dryness. The mixture was treated with methanol to dissolve all soluble material, the insolubles filtered off, and the solvent removed from the filtrate by evaporation. The impurities were removed by washing the residue with hot absolute ether, and subsequently the deoxyribonucleosides were extracted from the residue with hot absolute ethanol. The ethanol was removed by evaporation, and the residual material was recrystallized two or three times from ethyl acetate. The melting point of the resulting crystals was determined with and without admixture with the respective pure deoxyribonucleoside.

Esterase inhibition by DEF in vivo

DEF was administered to 20-g female mice by intraperitoneal injection at a uniform dose of 20 mg/kg. The DEF was injected in corn oil; the control group received corn oil alone. The animals were kept in individual jars at ambient laboratory conditions and were fed standard rations and water *ad libitum*. At intervals of 1, 6, 24, and 72 hr after receiving the injections, three animals were sacrificed, homogenates immediately made of their liver and small intestine, and their blood plasma separated. Each preparation was used, in the manometric esterase assay procedure, to hydrolyze the following substrates: 3′,5′-diacetyl thymidine, 3′-butyryl FUDR, 5′-butyryl FUDR, and 3′,5′-dihexanoyl FUDR. However, at 120 hr after the injections, only two mice were sacrificed and tested. Three animals from the control group were sacrificed and assayed at the beginning of the test. Calculation of the percentage inhibition of the esterase activity was based on the activity found for these control animals.

EXPERIMENTAL

Esters of FUDR, having acid radicals with one to eighteen carbon atoms, were incubated with various esterase sources in accordance with the manometric esterase assay procedure. Plasma or homogenates were used as follows:

Human	*Mouse*		*Insects*	
Normal	*Normal*	*Tumor*	*Fly*	*Aphid*
Liver	Liver	Ascites	Abdomen	Whole
Jejunum	Small intes.	AD-755		
Plasma	Plasma	L-1210		
	Kidney	S-180		
	Spleen			

Protein levels were determined on all the tissues but not on the insects. The esters used and the results obtained are given in Table 1.

TABLE 1. EFFECT OF CHAIN LENGTH ON RATE OF HYDROLYSIS OF ALIPHATIC 3′,5′-DIESTERS OF 5-FLUORO-2′-DEOXYURIDINE (FUDR) BY HOMOGENATES OF NORMAL HUMAN TISSUES, NORMAL AND TUMOR MOUSE TISSUES, HOUSEFLY ABDOMENS, AND WHOLE APHIDS

3′, 5′-Diester of FUDR	Acid products (μmoles/100 mg fresh wt./hr)													
	Human, normal			Mouse, normal					Mouse, tumor				Insects	
	Liver	Jejunum	Plasma	Liver	Sm. int.	Plasma	Kidney	Spleen	Ascites	AD-755	L-1210	S-180	Fly	Aphid
Formyl	1·0	0·4	0·00	28	0·8	1·3	18	0·9	0·3	5·1	0·4	0·0	1·4	1·6
Acetyl	2·4	0·5	0·02	17	4·5	0·1	11	2·1	0·6	1·1	0·4	0·3	0·9	2·8
Propionyl	9·1	2·1	0·45	74	25	1·0	39	2·7	1·4	1·4	0·5	0·5	3·0	8·1
Butyryl	23	13	1·07	201	164	5·0	48	5·1	3·9	2·1	0·6	1·6	5·9	14
Pentanoyl	28	14	0·25	283	200	6·0	61	9·4	6·6	4·0	1·6	2·4	9·3	17
Hexanoyl	48	21	0·15	294	201	7·0	76	9·8	6·8	3·5	1·9	3·4	15	16
Heptanoyl	47	15	0·11	220	128	6·8	69	7·9	5·6	2·0	1·4	1·5	14	5·7
Octanoyl	27	8·1	0·07	111	63	2·5	37	2·8	2·6	1·8	0·5	0·9	6·6	3·2
Nonanoyl	4·9	1·6	0·02	20	28	0·9	5·4	1·6	0·4	0·8	0·4	0·8	1·2	1·2
Decanoyl	0·2	0·4	0·06	9·4	14	0·5	3·6	0·7	0·5	0·8	0·5	0·9	0·2	1·0
Lauroyl	0·0	0·0	0·03	2·1	4·8	0·3	1·0	0·7	0·4	0·5	0·3	0·1	0·2	1·0
Myristoyl	0·0	0·3	0·01	2·6	3·4	0·1	1·4	0·6	0·3	0·4	0·3	0·3	0·3	1·1
Palmitoyl	0·0	0·0	0·07	1·6	2·5	0·1	1·8	0·3	0·3	0·3	0·4	0·4	0·9	1·3
Stearoyl	0·0	0·3	0·07	0·8	0·5	0·0	0·0	0·3	0·3	0·1	0·0	0·0	0·2	0·9
Oleoyl	0·0	0·2	0·04	2·6	1·1	0·1	1·1	0·6	0·3	0·5	0·4	0·5	1·0	1·2
Mg protein (biuret)/ 100 mg fresh wt.	33·2	22·2	6·9	28·5	10·0	6·3	19·5	19·0	12·2	12·9	14·8	19·9		

Hydrolysis of several other ribonucleoside and deoxyribonucleoside esters was also examined on a preliminary basis, with mouse liver homogenate and the manometric assay procedure. The maximum velocity, V, and Michaelis constant, K_m, were determined for the diacetyl and dipropionyl esters of FUDR and thymidine by the classical methods. After complete hydrolysis of the dibutyryl esters of FUDR and thymidine, the free deoxyribosides were separated and characterized.

The 3′,5′-diesters of FUDR listed in Table 1 were hydrolyzed by four centrifugal fractions from mouse liver homogenates, by means of the manometric assay procedure. Protein levels were determined in each fraction. The results are given in Fig. 1 in terms of the carbon atoms in the acid radical. Mono- and diacetyl esters of FUDR were hydrolyzed by mouse liver microsomes and mitochondria in accordance with the microcell esterase assay procedure, with the four radioactive substrates. The esters used and the results obtained are given in Fig. 2.

Homogenates prepared from a larger number of normal and tumor tissues from humans were fractionated by centrifugation at 15,000 *g*, and the resulting soluble and particulate fractions were used to hydrolyze dibutyryl thymidine according to the manometric assay procedure. Protein levels were determined on each esterase source. The tissues used and rates of hydrolysis found are given in Table 2.

Soluble and particulate fractions were prepared from blood of normal mice and homogenates of four normal tissues and four tumors from mice, as follows:

Normal tissue	*Tumors*
Blood	Ascites
Kidney	AD-755
Liver	L-1210
Small intestine	S-180
Spleen	

Each fraction was used to hydrolyze dibutyryl thymidine in accordance with the manometric esterase assay procedure. A series of similar experiments was made in the presence of varied amounts of eserine and di*iso*propyl phosphorofluoridate (DFP), with concentrations suitable for determination of the 50% inhibition level. All concentrations stated were those during both the inhibition reaction and the assay of the esterase. The molar concentration producing 50% inhibition was determined by plotting the percentage inhibition against the logarithm of inhibitor concentration for each combination of esterase source and inhibitor. The results are expressed as percentage inhibition or the negative logarithm of the molar inhibitor concentration resulting in 50% inhibition of esteratic activity (pI_{50}). Protein levels were determined on each esterase source. The results are given in Table 3.

DEF was administered to a number of mice at 20 mg/kg, the animals sacrificed after various intervals, and the degree of esterase inhibition determined (by the *in vivo* procedure given above) for esterases from the plasma, liver, and small intestine (of each animal) against four substrates. Protein levels were determined on the esterase sources from the control animals. The results obtained are given in Table 4.

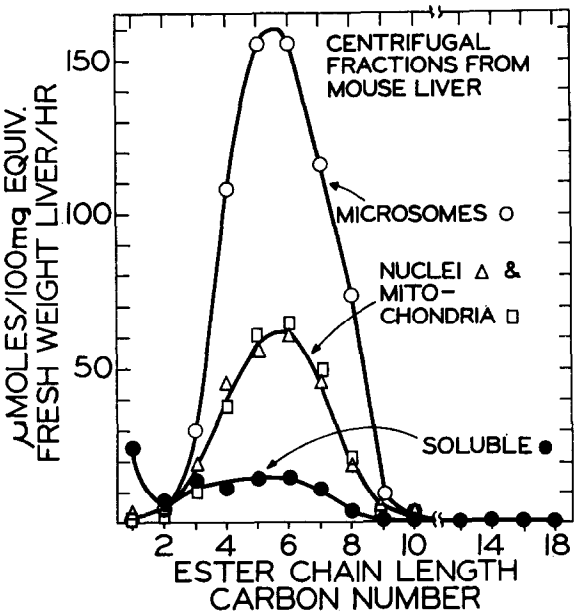

FIG. 1. Chain-length specificity for hydrolysis of 3′, 5′-diesters of FUDR by centrifugal fractions from mouse liver homogenates. Milligrams protein (biuret) per 100 mg fresh weight equivalent of liver: 7·6 for nuclei, 2·4 for mitochondria, 3·5 for microsomes, and 15 for soluble fractions.

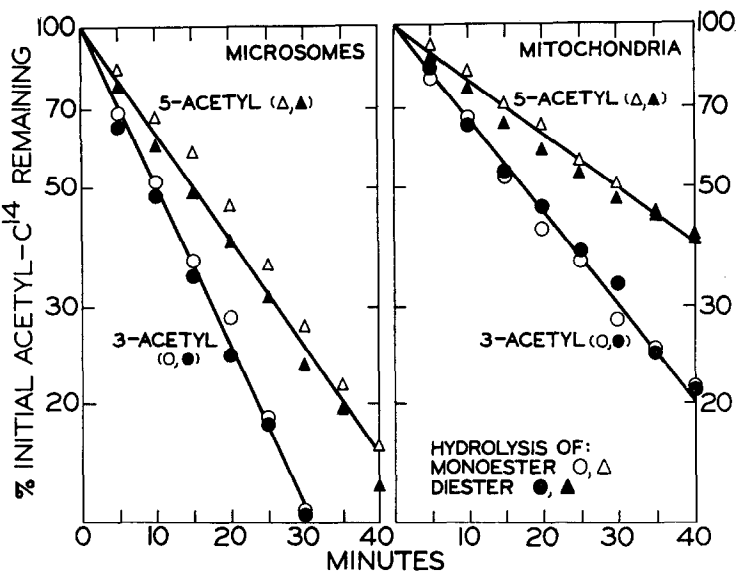

FIG. 2. Rate of hydrolysis of mono- and diacetyl esters of FUDR by mouse liver microsomes and mitochondria as determined with a microcell esterase assay with radiolabeled substrates. Conditions: 20-μliter reaction volume; microsomes or mitochondria from 3·0 mg mouse liver; 0·25 M sucrose and 0·025 M (pH 7·3) potassium phosphate; 0·0020 M radiolabeled substrate. Radiolabeled substrates: 3′-acetyl-^{14}C,5′-acetyl FUDR (●); 3′-acetyl,5′-acetyl-^{14}C FUDR (▲); 3′-acetyl-^{14}C FUDR (○); and 5′-acetyl-^{14}C FUDR (△).

TABLE 2. RATE OF HYDROLYSIS OF 3′,5′-DIBUTYRYL THYMIDINE BY SOLUBLE (S) AND PARTICULATE (P) FRACTIONS FROM NORMAL AND TUMOR TISSUE HOMOGENATES OF HUMANS

Tissue	Condition*	Number†	Acid products (μmoles/mg protein/hr) S	P
Blood	n	4	0·35	‡
Bone marrow	n	1	0·13	0·00
	l	7	0·07	0·03
Brain	n	2	0·20	0·15
	m	2	0·49	0·20
Breast	n	3	0·30	0·04
	b	1	0·23	0·00
	m	3	0·32	0·00
Colon	n	2	0·38	0·13
	b	1	0·00	0·04
	m	4	0·55	0·16
Diaphragm	n	1	0·41	0·01
Duodenum	n	1	0·89	0·00
Fat	n	7	0·09	0·02
Heart	n	4	0·92	0·04
Ileum	n	8	1·86	0·21
Jejunum	n	3	3·04	0·21
Kidney	n	2	0·98	0·27
	m	2	0·14	0·09
Liver	n	4	1·81	0·43
Lung	n	6	0·22	0·03
	m	2	0·27	0·04
Muscle	n	3	0·18	0·06
	m	2	0·38	0·01
Neuroblastoma	m	2	0·25	0·02
Omentum	n	1	0·39	0·07
Ovary	n	3	0·27	0·03
	m	1	0·21	0·18
Parotid	m	1	0·58	0·04
Placenta	n	2	0·23	0·04
Prostate	b	2	0·25	0·04
Skin	n	4	0·46	0·04
Spleen	n	5	0·17	0·05
Stomach	n	4	0·53	0·09
	m	2	0·83	0·06
Thyroid	n	1	0·27	0·09
	b	2	0·15	0·05
	m	1	0·30	0·60
Tonsils and adenoids	n	5	0·47	0·10
Uterus and cervix	n	2	0·46	0·09
	b	4	0·36	0·06
	m	3	0·29	0·10

* Condition of tissue based on pathological examination prior to assay; n = normal, b = benign, l = leukemia, m = malignant.

† number of tissues from different individuals included in the average results reported.

‡ Specific activity of erythrocytes was 0·015 μmole/μliter/hr when reconstituted to the original blood volume. Interfering color did not allow expression of results on protein basis.

RESULTS

Hydrolysis of nucleoside diesters by mouse liver esterases

When various nucleosides or their esters were incubated with mouse liver homogenate in a bicarbonate buffer, no CO_2 was evolved with any of the nucleosides, but CO_2 evolution, approaching theory for complete hydrolysis, was observed with

TABLE 3. RATE OF HYDROLYSIS OF 3′,5′-DIBUTYRYL THYMIDINE BY SOLUBLE (S) AND PARTICULATE (P) FRACTIONS FROM NORMAL AND TUMOR TISSUE HOMOGENATES OF MOUSE, AND SENSITIVITY TO INHIBITION BY ESERINE AND DI*iso*PROPYL PHOSPHOROFLUORIDATE (DFP)

Tissue	Acid products (μmoles/mg protein/hr)		pI_{50}*			
			Eserine		DFP	
	S	P	S	P	S	P
Normal						
Blood	2·1	†	6·4	12%‡	7·6	7·5
Kidney	8·7	8·3	4·1	39%‡	8·5	7·6
Liver	16·2	29·6	4·8	5·5	7·6	7·9
Small intestine	21·0	22·9	31%‡	36%‡	8·1	8·1
Spleen	0·7	0·3	32%‡	4·0	7·2	7·1
Tumors						
Ascites	0·9	0·6	6·0	6·2	8·9	8·7
AD-755	0·9	0·2	5·0	31%‡	7·7	7·0
L-1210	1·2	0·1	5·8	38%‡	8·4	7·1
S-180	0·4	0·1	4·5	45%‡	8·3	5·7

* pI_{50} = negative logarithm of molar concentration producing 50% inhibition.

† Specific activity of erythrocytes was 0·033 μmole/μliter/hr when reconstituted to the original blood volume. Interfering color did not allow expression of results on protein basis.

‡ Values indicated as percentages are level of inhibition at 1×10^{-4} M eserine.

TABLE 4. DEGREE OF INHIBITION OF ESTERASES IN MOUSE LIVER, PLASMA, AND SMALL INTESTINE HYDROLYZING ESTERS OF THYMIDINE AND FUDR AT VARIOUS TIMES AFTER INTRAPERITONEAL ADMINISTRATION OF 20 MG *S*, *S*, *S*,-TRIBUTYLPHOSPHOROTRITHIOATE (DEF) PER kg

Tissue and substrate	Normal activity, acid products (μmoles/100 mg fresh wt./hr)*	Per cent inhibition at indicated hours after DEF				
		1	6	24	72	120
Liver						
3′,5′-Diacetyl thymidine	71	70	93	95	56	12
3′-Butyryl FUDR	66	71	100	100	60	10
5′-Butyryl FUDR	227	87	100	100	80	26
3′,5′-Dihexanoyl FUDR	491	83	97	100	75	28
Plasma						
3′,5′-Diacetyl thymidine	1·3	89	100	100	57	58
3′-Butyryl FUDR	1·7	92	100	100	82	64
5′-Butyryl FUDR	6·9	94	100	100	79	46
3′,5′-Dihexanoyl FUDR	13·7	88	97	98	76	59
Small intestine						
3′,5′-Diacetyl thymidine	10	33	84	71	0	0
3′-Butyryl FUDR	14	42	100	73	13	0
5′-Butyryl FUDR	37	71	90	69	0	0
3′,5′-Dihexanoyl FUDR	359	68	89	71	0	0

* Milligrams protein (biuret) per 100 mg fresh weight: 28·5 for liver, 6·3 for plasma, and 10·0 for small intestine.

many of their esters. When the substrates were soluble or easily formed stable emulsions, the rate of CO_2 evolution was directly proportional to the concentration of liver homogenate. The activity of liver homogenates in hydrolysis of diacetyl and dibutyryl FUDR was heat-labile, such that 30 min at 50° resulted in loss of half the activity for homogenates prepared in isotonic potassium chloride solution.

Esters of FUDR with aliphatic acids of carbon numbers one to nine were hydrolyzed at varying rates, dependent on the nature of the acyl group, by mouse liver homogenates and nuclei, mitochondria, microsomes and soluble fractions prepared from such homogenates. Higher aliphatic esters were hydrolyzed quite slowly, if at all (see Table 1 and Fig. 1). The other 3′,5′-diesters of FUDR reported by Nishizawa *et al.*[1] were also hydrolyzed slowly, if at all, by mouse liver esterases and included the cyclohexylcarbonyl, benzoyl, phenoxyacetyl, phenylacetyl, methylsulfonyl, N-methylcarbamoyl, and 0,0-diethylphosphoryl derivatives. The same was true for the 5′-(*p*-toluenesulfonyl) ester. Mouse liver esterases also hydrolyzed the following: 3′,5′-di*iso*butyryl and 3′,5′-di(2-methyl-DL-butyryl) FUDR; 3′,5′-diacetyl, dipropionyl and dibutyryl thymidine; 2′,3′,5′-tributyryl thioguanosine; 2′,3′,5′-triacetyl and tributyryl 6-mercaptopurine riboside. In comparing the hydrolysis of thymidine and FUDR esters by liver homogenate, the maximum velocity, V, and Michaelis constant, K_m, as micromoles per 100 milligrams fresh weight per hour and as molar substrate level, respectively, were as follows: diacetyl thymidine, 279 and 0·0054; diacetyl FUDR, 128 and 0·0054; dipropionyl thymidine, 893 and 0·0025; dipropionyl FUDR, 744 and 0·0036.

Thymidine and FUDR were recovered as the crystalline deoxyribonucleoside products, and identified by mixed melting points with authentic compounds from reaction mixtures of mouse liver with the following: thymidine; 3′,5′-dibutyryl thymidine; FUDR; 3′,5′-dibutyryl FUDR. The product and mixed melting point range, uncorrected, was 183°–184° for thymidine and 143°–144° for FUDR.

Relative activity of various mouse, human, and insect tissues for hydrolysis of FUDR and thymidine esters

Mouse liver, small intestine, and kidney were the most active of the tissues examined from this species for hydrolysis of FUDR esters and diacetyl and dibutyryl thymidine (Tables 1, 3, and 4). The mouse plasma and spleen were also usually more active than any of the four tumors assayed (Table 1). A major proportion of the activity for hydrolysis of dibutyryl thymidine appeared in the particulate fraction on centrifugation at 15,000 *g* only in the case of the tissues with the highest total activity—the liver, small intestine, and kidney (Table 3). Further differential centrifugation of mouse liver homogenates yielded nuclei, mitochondria, microsomes, and soluble fractions, all of which were very active in hydrolyzing FUDR esters (Figs. 1 and 2). The highest specific activity appeared with mouse liver microsomes, followed by mitochondria and nuclei of mouse liver (Figs. 1 and 2). A small degree of purification was achieved on the mouse liver microsome esterases. Little if any esteratic activity was lost on holding the microsomes frozen for several days or in preparing an acetone powder of the microsomes. This acetone powder (0·46 mg protein/mg total weight) could be increased 3- to 5-fold in esteratic activity by homogenizing in water and utilizing ammonium sulfate for fractionation. The most active fraction remained in the 15,000-*g* supernatant when 3·0 g of ammonium sulfate was added to 10 ml of

enzyme preparation, but was precipitated with an additional 1·5 g of ammonium sulfate and centrifugation.

The human tissues were considerably less active than the mouse tissues, with the greatest activity appearing in the liver, ileum, and jejunum (Tables 1 and 2). No consistent differences in rate of dibutyryl thymidine hydrolysis appeared between the normal human tissues and the benign and malignant tumors with the limited number of such tissues examined (Table 2).

Homogenates of whole pea aphids and housefly abdomens were also quite active in hydrolysis of FUDR esters (Table 1).

Effect of chain length on hydrolysis rates of diesters

In preliminary tests, the thymidine esters were always hydrolyzed at a rate 2·5 to 10 times greater than that of the FUDR esters, and the hydrolysis rate always increased from diacetyl to dipropionyl to dibutyryl. This was the case for mouse lung, heart, and erythrocytes as well as the normal and tumor tissues of mouse listed in Table 1. Mouse brain was almost inactive. Human tissues found to give the same relationship were normal large intestine, liver, lymph nodes, plasma, spleen, and stomach, and tumors from the bowel, breast, colon, esophagus, lung, and stomach.

As shown in Table 1 and Fig. 1 (containing data obtained with a variety of esterase sources), the most rapid rate of hydrolysis of 3′,5′-diesters of FUDR was obtained for those having acid chains containing four to eight carbon atoms, the dihexanoyl ester usually being the most rapidly hydrolyzed. Human plasma was the only major exception to this relationship and, in this case, the dibutyryl ester was the most rapidly and the dipropionyl ester the next most rapidly hydrolyzed. Diformyl FUDR was usually hydrolyzed at a rate below that of diacetyl FUDR. However, more rapid hydrolysis of formyl than acetyl occurred with mouse kidney and housefly homogenates, and particularly with AD-755 tumors, mouse plasma, and the soluble fraction of mouse liver (Table 1 and Fig. 1).

Effect of esterase source on rate of hydrolysis at 3′- or 5′-ester site of FUDR esters

Manometric studies with mono- or di-, acetyl or butyryl, esters of FUDR showed that the rate of acid liberation on enzymatic hydrolysis of the diesters approximated the summation of the rates with the two corresponding monoesters. The hydrolysis rate of 5′-butyryl FUDR was 2·6- to 4·1-fold greater than that for 3′-butyryl FUDR with the liver, plasma, and small intestine of mice (Table 4). Centrifugal fractionation of mouse liver homogenates yielded similar distribution of activity among the fractions on the 3′-butyryl and the 5′-butyryl FUDR, with the 5′-ester always being the more rapidly hydrolyzed. The sensitivity of the esterases to inhibition by 2×10^{-6} M eserine varied among the mouse liver fractions, but for each fraction it was about the same with either 3′-butyryl FUDR or 5′-butyryl FUDR as the substrate. Hydrolysis of 5′-butyryl FUDR proceeded at 2·5 times the rate of 3′-butyryl FUDR in the presence of an acetone powder of mouse liver microsomes which had been further fractionated with ammonium sulfate. All other mouse tissues assayed on the two monobutyryl esters also hydrolyzed the 5′-ester more rapidly than the 3′-ester. These included erythrocytes, kidney, spleen, heart, lung, and the AD-755, L-1210, and S-180 tumors. A human stomach tumor also hydrolyzed 5′-butyryl more rapidly

than 3′-butyryl FUDR, but a sample of human spleen hydrolyzed the two monoesters at about equal rates. Activity on the acetyl esters was considerably less than on the butyryl esters, and 3′-acetyl FUDR was usually hydrolyzed more rapidly than 5′-acetyl FUDR. 3′-Acetyl FUDR was hydrolyzed at about twice the rate of 5′-acetyl FUDR by mouse liver homogenates, or by an acetone powder of mouse liver microsomes which was further purified by ammonium sulfate fractionation. This selective action by esterases on acetyl esters seemed to vary with mouse tissues, because the 3′-acetyl ester was more rapidly hydrolyzed by esterases from the liver and kidney; with erthrocytes, similar hydrolysis rates were observed for the two monoesters, while the 5′-acetyl ester was the one more rapidly hydrolyzed by esterases from the plasma and spleen.

By means of the microcell technique with radioactive substrates, it was found that with microsomes and mitochondria from the mouse liver, the 3′-acetyl group was more readily hydrolyzed than the 5′-acetyl group, this being true for monoesters as well as diesters (Fig. 2). It appeared, however, that the 5′-acetyl group was hydrolyzed slightly more rapidly from the diester than from the monoester. The rate of acetyl-^{14}C loss from 3′-acetyl-^{14}C,5′-acetyl-^{14}C FUDR was intermediate between that of 3′-acetyl-^{14}C,5′-acetyl FUDR and 3′-acetyl,5′-acetyl-^{14}C FUDR. The curve for 3′-acetyl-^{14}C,5′-acetyl-^{14}C FUDR hydrolysis, not shown in the figure, gave the slope change anticipated for more rapid initial hydrolysis of the 3′-acetyl-^{14}C group, followed by somewhat slower subsequent hydrolysis of the 5′-acetyl-^{14}C group; however, the the data were not adequate to show that none of the initial hydrolysis occurred at the 5′ position.

Nature and inhibition of mouse esterases hydrolyzing deoxyribonucleoside and ribonucleoside esters

Hydrolysis of a deoxyribonucleoside ester, dibutyryl thymidine, by normal and tumor tissues of mouse was inhibited by low levels of DFP but was less sensitive to inhibition by eserine (Table 3). The molar level of DFP for 50% inhibition of these esterases was generally between 1×10^{-9} and 1×10^{-7} M, while the amount of eserine required was usually 1×10^{-6} to greater than 1×10^{-4} M. Preliminary studies with various normal and tumor tissues from mouse and human indicated that the sensitivity to inhibition by eserine and DFP was the same with diacetyl, dipropionyl, and dibutyryl FUDR and with the corresponding esters of thymidine in any given system.

Sodium fluoride at 1×10^{-3} M yielded 66–77% inhibition of hydrolysis of dibutyryl FUDR by mouse liver, plasma, and small intestine. *p*-Chloromercuribenzoate and *o*-iodosobenzoic acid at 1×10^{-4} M gave little if any inhibition of these enzymes. Hydrolysis of a ribonucleoside ester, 2′,3′,5′-triacetyl 6-mercaptopurine riboside, was apparently effected by the same mouse enzymes hydrolyzing 3′,5′-diacetyl FUDR or by similar ones. The activity distribution among the four centrifugal fractions of mouse liver and sensitivity of these fractions to DFP and eserine inhibition were similar for the two substrates. The relative activity of the normal mouse tissues for hydrolysis of this mercaptopurine riboside triester was the same as that found for the FUDR diesters indicated in Table 1.

Cholinesterase was not the effective enzyme in mouse liver for hydrolysis of FUDR esters. Prostigmine at 1×10^{-5} M gave little inhibition of the hydrolysis of the

diacetyl, dipropionyl, or dibutyryl esters of FUDR but, even at 1×10^{-7} M, prostigmine resulted in nearly complete inhibition of the hydrolysis of acetyl, propionyl, and butyryl esters of choline. Choline at 10 mg/ml yielded much higher inhibition of choline ester than FUDR ester hydrolysis. The hydrolysis rate for mixtures of choline esters and FUDR esters by mouse liver approximated that expected for summation of the rates on the individual substrates.

One of the enzymes in mouse liver that hydrolyzes dibutyryl FUDR may also hydrolyze tributyrin. DFP added to mouse liver homogenates at 1×10^{-7} M almost completely inhibited the hydrolysis of tributyrin and dibutyryl FUDR but had little effect on the hydrolysis of methyl butyrate and phenyl butyrate. When lower DFP concentrations were used, it was found that the hydrolysis of tributyrin and dibutyryl FUDR was of similar sensitivity to inhibition. The activity in hydrolysis of both these substrates was undiminished on preparing an acetone powder of the microsomes and, on further fractionation of this esterase source with ammonium sulfate, the activity in hydrolyzing both substrates appeared in the same fraction (procedure as referred to earlier). After this purification, the hydrolysis of tributyrin and dibutyryl FUDR was of similar sensitivity to inhibition by DFP. Further, on passing the most active fraction from ammonium sulfate fractionation through a DEAE-cellulose column (procedure as referred to earlier), two peaks active in hydrolyzing tributyrin and dibutyryl or dihexanoyl FUDR were obtained; Tris buffer alone (0·02 M, pH 7·6) eluted the first peak; Tris buffer containing 0·6 M potassium chloride eluted the second peak, which contained about one third of the total activity present in the first peak.

The major activity of certain mouse tumors for hydrolysis of dibutyryl FUDR and tributyrin probably results from different enzymes. The 15,000 *g* soluble fraction from Ehrlich ascites homogenates was DFP-sensitive at 1×10^{-7} M when assayed with dibutyryl FUDR but was resistant to this level of DFP when assayed with tributyrin as the substrate. The enzyme active on dibutyryl FUDR in the soluble fraction from ascites could be precipitated with either acetone at 25% (v/v) or ammonium sulfate at 25% (w/v). With the soluble fraction from L-1210 homogenates, eserine at 1×10^{-5} M resulted in much greater inhibition of the dibutyryl thymidine than of the tributyrin hydrolysis.

Inhibition of esterases hydrolyzing FUDR and thymidine esters after treatment of mice with an organophosphate ester

In the tissues obtained from mice treated intraperitoneally with DEF, the plasma esterases were the most, and the small intestine esterases the least, sensitive to prolonged inhibition when tested with diacetyl thymidine and three FUDR esters (Table 4). The pattern of esterase inhibition and recovery after DEF administration was similar with all four substrates, indicating that the same esterases in these tissues may be involved in the hydrolysis of the thymidine and FUDR esters, at both the 3′- and 5′-positions, and with both the shorter (diacetyl) and longer (dihexanoyl) chain lengths.

Expiration of $^{14}CO_2$ by mice receiving acetyl-^{14}C-labeled 3′,5′-diacetyl FUDR

In the experiment in which 3′-acetyl-^{14}C,5′-acetyl-^{14}C FUDR and acetate-1-^{14}C were administered to mice, with and without pretreatment with DEF at a dose of

80 mg/kg 24 hr before administration of the labeled compounds, the percentage of the administered radioactivity from 3′-acetyl-^{14}C,5′-acetyl-^{14}C FUDR given off as $^{14}CO_2$ in a 24-hr period dropped from 68 (64–71 range) to 59 (54–61) as the result of the DEF treatment. Also, the number of hours until half the administered radioactivity was recovered as $^{14}CO_2$ increased, owing to the DEF treatment, from 1·6 (1·2–1·9) without the DEF to 3·4 (2·5–5·0) with the DEF. If the DEF effect involved inhibiting the oxidation rate of the liberated acetate-1-^{14}C rather than the hydrolysis of the acetyl-^{14}C groupings from labeled diacetyl FUDR, the rate and extent of $^{14}CO_2$ liberation on direct administration of acetate-1-^{14}C should also be inhibited; this was not the case because within 24 hr after treatment with acetate-1-^{14}C, 72% of the administered dose was recovered as $^{14}CO_2$ without DEF, and 73% was recovered with DEF pretreatment; and the number of hours until half the dose was lost as $^{14}CO_2$ was 1·5 without and 1·6 with DEF pretreatment. The radioactivity in the urine from the 3′-acetyl-^{14}C,5′-acetyl-^{14}C FUDR within 24 hr was about 10% of the administered dose without DEF pretreatment and 20–30% with DEF pretreatment.

When mice which had been pretreated with DEF at a dose of 80 mg/kg 24 hr earlier were given (per kg) 53·7 mg of either 3′-acetyl-^{14}C,5′-acetyl FUDR, or 3′-acetyl, 5′-acetyl-^{14}C FUDR, it appeared that the 3′-acetyl group was hydrolyzed more rapidly. Half the administered radioactivity appeared as $^{14}CO_2$ in 2·5 hr with 3′-acetyl-^{14}C,5′-acetyl FUDR, and 5·0 hr were required when 3′-acetyl,5′-acetyl-^{14}C FUDR was administered.

DISCUSSION

Thymidine and FUDR were selected for this investigation because of their closely related chemical structures, interaction in certain biological and biochemical systems, and the finding that certain of their aliphatic esters appeared to be susceptible to enzymatic hydrolysis. Esterases effective in catalyzing this hydrolysis were found in many insect, mouse, rat, rabbit, and human tissues. Because of availability, the studies were concentrated on normal and tumor tissues from mouse and human.

Comparable esterase activity was obtained from fresh and frozen tissue samples when both were available for comparison. All results tabulated for human tissues, other than for plasma and corpuscles, were obtained from frozen samples; the results with mouse tissues were based on fresh samples. Manometric assay of 0·4-ml aliquots from homogenates of mouse liver prepared in water, bicarbonate buffer, or 0·25 M sucrose yielded similar results for esteratic activity. The presence of Triton X-100 apparently caused no interference in the assays because, with soluble esters of lower chain length, the enzymatic hydrolysis rate by mouse liver and small intestine was not altered by the addition of Triton. Further, the emulsifier level used in certain of the assay systems did not appear to alter the reaction of the inhibitors with the enzymes. However, solubility limitations and the necessity for testing higher esters as emulsions made it uncertain whether the substrate optima observed (see Table 1 and Fig. 1) reflect only the specificity of the enzyme or whether they are greatly influenced by the physical properties of the system.

The esterase activity of homogenates of adenocarcinoma-755 tumors is of particular interest since the toxicity and/or carcinostatic activity of FUDR esters, after oral administration to mice with these tumors, is known.[1] Such homogenates showed

optimal activity on the FUDR esters with C_1, and C_4 to C_8 acids, and the latter esters were more potent carcinostatic agents than the shorter-chain, less readily hydrolyzed diacetyl and dipropionyl esters. (See Table 2 of Nishizawa *et al.*,[1] and Table 1 of this report).

Since the site of initial esteratic attack on the 3′- or 5′-position varied with the ester and esterase involved, mixed esters might be used to effect release of both the 3′- and 5′-hydroxyl groupings at the desired rate. The mouse tumors were generally lower in esteratic activity than many of the normal tissues. This difference was not as clear in the case of the human tumors. Esters of naturally occurring nucleosides that are more readily hydrolyzed by normal than by tumor tissues might be used in preventing toxicity without preventing the therapeutic effect of the purine and pyrimidine antagonists administered in the nonesterified riboside or deoxyriboside form, where these differences in esterase activity (between normal and tumor tissues) are of significant magnitude.

The possibility of further modifying the release rate of the nucleosides from their aliphatic esters by treatment of the animals with esterase inhibitors, particularly organophosphates, has been shown in these experiments. Esterase inhibition resulting from DEF increased toxicity and possibly also the carcinostatic activity of dibutyryl FUDR in mice with adenocarcinoma-755 tumors. (See Table 4 of Nishizawa *et al.*,[1] and Table 4 of this report). The testing of combinations of nucleoside esters with organophosphates is severely restricted by other pharmacological actions of these organophosphates, although it appears that the rate of ester hydrolysis *in vivo* can be inhibited at critical levels of selective organophosphates which do not give gross indications of poisoning. The selectivity of these organophosphates for inhibition of esterases in normal versus tumor tissues has not been examined *in vivo*. Inhibition studies *in vitro*, made with DFP, did not clearly differentiate the potential selectivity *in vivo* for esterase inhibition.

Esterases active in hydrolyzing aliphatic 3′,5′-diesters of thymidine and FUDR to their deoxyribonucleosides are present in many tissues. Human serum has been noted to hydrolyze 2′,3′,5′-triacetyl AzUR,[9] and mouse liver homogenates hydrolyze 2′,3′,5′-triacetyl and tributyryl 6-mercaptopurine riboside and 2′,3′,5′-tributyryl thioguanosine. The esterases hydrolyzing FUDR and thymidine esters could be easily differentiated by inhibitor studies from those hydrolyzing choline esters, phenyl butyrate, and methyl butyrate. They could not be differentiated by limited inhibitor and purification studies from certain mouse liver esterases hydrolyzing tributyrin. This is important, because the hydrolysis of the aliphatic esters of ribonucleosides and deoxyribonucleosides may be due in part to such "aliesterases".

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