phenol–I obtained by procedures A and B were dissolved in 0.1 *M* Na phosphate buffer at pH 7.4. Uv spectra were recorded and found to be nearly identical with spectra of solns of the 2 components having molar ratios of I to phenol of 1:2 and 1:6, respectively, in agreement with the compn indicated by elemental anal. of the isolated complexes.

Complex from *N*-Methyl-4-(1-naphthylvinyl)pyridinium Iodide (III) and Phenol.—A soln of 37 mg of III in 100 ml of H_2O (1 m*M*) was poured slowly, with rapid stirring, into 500 ml of 0.67 *M* aq phenol adjusted to pH ~8 with NH_4OH. Glass-distd H_2O and pink light illumination were used in the operations. An amorphous ppt formed immediately, and the pH was raised to ~10 with NH_4OH. The ppt was collected by centrifugation at 3°, and dried to constant wt *in vacuo* over P_2O_5 and mineral oil (3 days) to yield 14 mg of an amorphous, yellow product. The limited aq soly of III in the cold required use of dil solns, and the physical nature of the product contributed to losses during isolation.

The complex did not contain iodide. Uv spectrophotometric anal. of the product in 0.1 *M* Na phosphate buffer, pH 7.4, showed it to contain 3 mols of phenol for each III cation. The compn was calcd by detg the concn of III cation from its ϵ_{max} at 377 nm, where phenol does not absorb, and the ratio of absorbances at 377 nm and 270 nm; both III cation and phenol absorb at 270 nm. The 3:1 ratio of phenol to monocation is equiv to the 6:2 ratio found with dicationic I (preparation B).

Interactions of Quaternary Salts with Polypeptides. Poly(L-tyrosine) (mol wt ~4950), poly(L-histidine) (mol wt ~8750), and poly(L-phenylalanine) (mol wt ~7000) were purchased from Miles-Yeda, Ltd. In a typical expt, 1 mg of the polymer was added to 5 ml of a 50 μM buffered aq soln of a quaternary compd, either at pH 7.4 (0.1 *M* Na phosphate) or pH 10 (0.1 *M* Na_2CO_3). These were covered and stirred magnetically for 3 days at 5°. The resulting polymeric suspensions then were centrifuged at 5000*g* and 3° (International refrigerated centrifuge). Absorption spectra of supernatants were obtained using a Cary Model 15 recording spectrophotometer. These were compared with spectra of the original soln of the ammonium compd, which had been treated in a similar manner in the absence of polymer. With poly(Tyr), 85–95% of the test compd was removed from soln at pH 7.4 or 10, whereas poly(Phe) removed only 5–12%. Poly(His) showed a greater affinity for III then for I, combining with about 26% of the former and only 5% of the latter. The π-electron-deficient pyridinium system in III and electron-donor propensity of unprotonated histidine imidazole moieties may contribute to bonding by charge-transfer interaction.[5,6]

Nonspecific Inhibition of Enzymes by Organic Contrast Media

JOSEPH H. LANG* AND ELLIOTT C. LASSER

Department of Radiology, University of California, San Diego, California 92037

Received May 23, 1970

The activities of a variety of enzymes were measured in the presence of several organic roentgenographic contrast media. The compounds were found to inhibit all of the enzymes tested, and for each compound, the concentrations producing 50% inhibition fell in a relatively narrow range, resulting in a nearly constant order of effectiveness. The order of effectiveness as inhibitors correlates well with the order of binding to serum albumin and appears to parallel the hydrophobic natures of the compounds. The results are interpreted as implying a very general property of proteins: the ability to bind small molecules in a nonspecific way.

This laboratory has been interested for some time in the interactions of organic roentgenographic contrast media with enzymes and other proteins in an effort to understand the mechanisms by which these compounds exert their physiologic effects.[1] Similarity of some symptoms of contrast media toxicity with those produced by ACh led to a study of the inhibition of AChE by contrast media.[2] Other enzymes which might be effected *in vivo* by contrast media were investigated and when a pattern of inhibitory effectiveness began to emerge, the study was expanded to include a variety of different types of enzymes encompassing a wide range of molecular weights and catalytic specificities, without regard to their physiologic significance. In this communication, we report on the inhibition of the enzymes lysozyme, β-glucuronidase (two types), alcohol dehydrogenase, and glucose 6-phosphate dehydrogenase, and we discuss an apparently general relationship between inhibitory strength and structure.

Almost uniquely in medicine, contrast media as clinically employed are present locally in blood vessels and in some tissues in very high concentrations. Concentrations of injectable media are of the order of 0.3 to 1 *M*. In angiography as much as 0.2 mole might be injected. Very high concentrations, therefore, occur at the site of injection into a vessel and until the bolus has passed the first capillary bed. Excretion occurs *via* the kidney or the bile-forming system of the liver, and these organs are presented with high concentrations of the compounds. Oral cholecystographic agents do not appear in high concentrations in the blood, but they are concentrated in bile by way of the liver.

Contrast media are, of necessity, relatively nontoxic, but they are employed in such large doses that mild toxic effects are a common clinical experience, and severe reactions sometimes occur. Consequently, although for some of the compounds relatively high concentrations are required to effect enzymic activity, the findings are relevant to the physiologic situation.

Material and Methods

The contrast media tested were iopanoate (**1**), iodipamide (**2**), diatrizoate (**5**), and iothalamate (**6**), which were obtained as the commercially available pharmaceuticals, and acetrizoate **4** and **3**, which are experimental compounds. The structures are shown in Chart I.

Alcohol dehydrogenase and bacterial β-glucuronidase were products of Sigma Chemical Co., hen's egg lysozyme was obtained from Worthington Biochemical Corp., and bovine liver β-glucuronidase was obtained from Nutritional Biochemicals Corp. Glucose 6-phosphate dehydrogenase was prepared as follows. Erythrocytes of freshly collected heparinized human blood were washed twice with 0.15 *M* NaCl and then lysed with 9 vol of a soln contg 0.01 mg of triphosphopyridine nucleotide and 0.5 μl of 2-mercaptoethanol/ml. The

(1) E. C. Lasser, *Radiology*, **91**, 63 (1968).
(2) E. C. Lasser and J. H. Lang, *Invest. Radiol.*, **1**, 237 (1968).

CHART I
STRUCTURES OF THE CONTRAST MEDIA

I, I, I, $CH_2CHCOOH$, C_2H_5, NH_2
1

COO^-, I, I, I, $NHCO(CH_2)_4CONH$, COO^-, I, I, I
2

COO^-, I, I, I, CH_3CONH, $NHCO(CH_2)_8CONH$, COO^-, I, I, I, $CONHCH_3$
3

COO^-, I, I, I, $NHCOCH_3$
4

COO^-, I, I, I, CH_3CONH, $NHCOCH_3$
5

COO^-, I, I, I, CH_3CONH, $CONHCH_3$
6

supernatant of the centrifuged hemolysate was used without further purification.

Phenolphthalein glucuronide, *Micrococcus lysodeikticus* dried cells, and diphosphopyridine nucleotide were obtained from Sigma Chemical Co. Glucose 6-phosphate and triphosphopyridine nucleotide were products of Calbiochem.

Lysozyme Assay.—The procedure was based on that devised by Shugar[3] in which the activity is measured by the decrease in optical absorbance of a suspension of dried cells of *M. lysodeikticus* in the presence of the enzyme. The rate was not independent of the phosphate buffer concn but there was a broad optimum in the range 0.01–0.05 *M*. The rate in the optimum range was insensitive to ionic strength from 0.02 to 0.08 *M*. Experimental condns were chosen accordingly. The total vol of 4.1 ml consisted of 2 ml of a suspension of dried *M. lysodeikticus* cells (0.6 mg/ml) in pH 7.0 phosphate buffer (0.03 *M*), 2 ml of a soln of the test substance, and 0.1 ml of lysozyme ($\sim$0.03 mg/ml). Absorbance was measured for about 3 min. Although there was sometimes a small decrease in rate as the reaction progressed, no attempt was made to estimate a zero time rate. The amt of enzyme was adjusted so that, in the absence of test substance, the absorbance change was in the range 0.025–0.050 *A*/min.

β-Glucuronidase Assay.—A modification of the method of Fishman, *et al.*,[4] was employed in which the rate of hydrolysis of phenolphthalein glucuronide is used as the measure of enzyme activity. The total vol of 2.1 ml consisted of 1 ml of a soln of the enzyme ($\sim$0.5 mg/ml) in buffer, 1 ml of a soln of the test substance, and 0.1 ml of phenolphthalein glucuronide (0.01 *M*). Reaction time was ordinarily 1 hr. The reaction was stopped and the color developed by addn of 1 ml of 10% aq Na_2CO_3. The enzyme concn was chosen so that the optical density was 0.8–1.0 in 1 hr in the absence of test substrate.

For the bovine liver β-glucuronidase, the enzyme was added in 0.2 *M* acetate buffer, pH 4.5. The bacterial enzyme was added in 0.042 *M* Tris buffer, pH 7.8. In the assay procedure supplied by the Sigma Chemical Co. for the bacterial enzyme, a dilute, pH 6.8, phosphate buffer is used. We found that the rate was only slightly lower at pH 7.8 than at pH 6.8, and it was essentially the same in Tris as in phosphate. We chose to perform the assay at pH 7.8, since this permitted us to test **1**, the solubility of which is too low at pH 6.8 to permit adequate testing.

Alcohol Dehydrogenase Assay.—The activity of this enzyme was measured by the rate of increase in absorbance at 340 nm due to the reduction of the coenzyme diphosphopyridine nucleotide. The procedure was that supplied by the Sigma Chemical Co. The total vol of 3 ml consisted of 1 ml of 0.03 *M* pyrophosphate buffer, pH 8.8, 0.2 ml of 0.012 *M* diphosphyridine nucleotide, 0.2 ml of EtOH, 1.5 ml of test soln, and 0.1 ml of enzyme.

Glucose 6-Phosphate Dehydrogenase Assay.—The procedure was essentially that of Kirkman.[5] The total vol of the assay mixture was 1 ml and consisted of 0.7 ml of test substance, 0.2 ml of "G6PD stock" (Tris, 0.625 *M*, pH 8.0; $MgCl_2$, 0.0625 *M*; TPN, 1.25 mg/ml), 0.05 ml of 0.012 *M* glucose 6-phosphate, and 0.05 ml of enzyme soln. The rate was determined by recording the absorbance change at 360 nm. The curves were generally linear after the first 2 min.

Results

For all the studies, the results are expressed in terms of the relative activity, α, the ratio of the reaction rates in the presence and absence of inhibitor.

β-Glucuronidase from Beef Liver.—The inhibition curves appear in Figure 1A. In addition to the information shown, it was found that NaCl to a concentration of 0.5 *M* had no effect on this enzyme. The effectiveness of the compounds as inhibitors was in the order **2** > **3** > **4** > **5**. Iopanoate was not tested because of its insolubility at the pH of the assay.

β-Glucuronidase from Bacteria.—For this enzyme, the inhibition curves (Figure 1B) show that the compounds inhibited in the order **1** > **2**,**4**,**3** > **5**,**6** > KCl, methylglucamine·HCl (MGA·HCl).

Lysozyme.—Figure 1C illustrates the inhibition of hen's egg lysozyme by the compounds under study. In this case, the order of effectiveness was **1** > **2** > **3** > **4** > **5** > NaCl, NaOAc, MGA·HCl.

Alcohol Dehydrogenase.—The inhibition curves of Figure 1D show that the order of inhibition was **1** > **2** > **3**,**4** > **5**,**6** > KCl, MGA·HCl.

Glucose 6-Phosphate Dehydrogenase.—The results are shown in Figure 1E. The order of effectiveness as inhibitors was **1** > **2** > **3**,**4** > **5**.

Discussion

In this work, all rate measurements have been made under enzyme assay conditions; that is, under condi-

(3) D. Shugar, *Biochim. Biophys. Acta*, **8**, 302 (1952).
(4) W. H. Fishman, B. Springer, and R. Bunetti, *J. Biol. Chem.*, **173**, 449 (1948).
(5) H. N. Kirkman, *ibid.*, **237**, 2364 (1962).

TABLE I

CONCENTRATIONS PRODUCING 50% INHIBITION OF ENZYME ACTIVITY[a]

	β-GUase (liver)	β-GUase (bact)	Lysozyme	ADH	G6PD	AChE[b]
Iopanoate		0.0017	0.0026	0.0007	0.0013	0.0006
Iodipamide	0.0011	0.021	0.015	0.010	0.011	0.010
3	0.016	0.026	0.024	0.045	0.046	
Acetrizoate	0.042	0.022	0.052	0.042	0.050	0.12
Diatrizoate	0.16	0.092	0.084	0.123	0.14	
Iothalamate		0.085		0.128		
KCl	>0.5	0.16		1.2		
NaCl			0.14			
Na acetate			0.17			
MGA·HCl		0.19	0.17	1.2		

[a] Abbreviations: β-GUase, β-glucuronidase; ADH, alcohol dehydrogenase; G6PH, glucose 6-phosphate dehydrogenase; AChE, acetylcholinesterase; MGA·HCl, methylglucamine·HCl. [b] Data from ref 2.

tions for which the rate is effectively independent of substrate concentrations. No attempt has been made to study in detail the kinetics of the inhibition of the enzymes by the contrast media, and no detailed mechanism can be postulated on the basis of the present results. However, certain patterns are visible in the data, and these suggest a general explanation.

The generality of the inhibition is striking. All the compounds were capable of inhibiting all of the enzymes studied, although the enzymes cover a wide range in the types of reactions catalyzed and the types of proteins represented. Lysozyme is among the smallest of enzymes, with mol wt 14,500.[6] Yeast alcohol dehydrogenase is a Zn metalloenzyme of mol wt 150,000 and consisting of 4 subunits.[7] Bovine β-glucuronidase is a glycoprotein of mol wt 280,000.[8] Lysozyme and β-glucuronidase both catalyze hydrolysis of glycosidic linkages, but have vastly different substrate specifities. Alcohol dehydrogenase and glucose 6-phosphate dehydrogenase catalyze H transfer and require coenzymes.

Quantitatively, it may be seen from the positions of the curves in Figure 1 and the data summarized in Table I, that, for each compound, the concentrations required to produce 50% inhibition of all the enzymes lie within a relatively narrow range. The only glaring exception is for **2** with liver β-glucuronidase. As a consequence of this consistency in the concentrations required for 50% inhibition, the order of effectiveness of the compounds as inhibitors is nearly the same for all the enzymes: **1** > **2** > **3**,**4** > **5**,**6**.

In an earlier paper,[9] we reported on the binding of some contrast media to serum albumin as studied by the method of equilibrium dialysis. Iopanoate (**1**) binds at three strong sites and several weak ones; **2** binds at 1–2 strong sites and several weak ones; **4** binds at 1 strong site; **3** is weakly bound at a few sites; binding of **5** is not detectable experimentally. Although in general an unequivocal order of affinity cannot be given, since different numbers of sites are involved, as well as different association constants, nevertheless, for the compounds considered here, the only ambiguity would be between **3** and **4**. Otherwise, the order of affinity is clearly in the order given.

It is now generally agreed that the principle driving force for binding to protein is hydrophobic interaction.[10,11] Indeed, scrutiny of the structures of the compounds considered here with reference to their hydrophobicities would lead, at least roughly, to a correct prediction of the order of affinity to serum albumin. The hydrophilic parts of the molecules are the carboxylate and amide groups. Diatrizoate (**5**) with its 3 symmetrically placed hydrophilic groups on the benzene ring has no extensive hydrophobic region. Compound **3** has 2 rings like that of **5** but has a hydrocarbon bridge con-

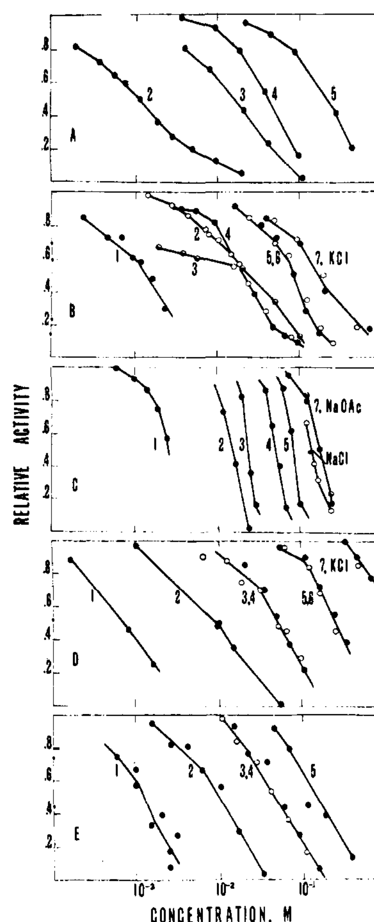

Figure 1.—Plots of relative activity *vs.* concentration for the inhibition of enzymes by contrast media: (A) β-glucuronidase of bovine liver; (B) β-glycuronidase of bacteria; (C) lysozyme; (D) alcohol dehydrogenase; (E) glucose 6-phosphate dehydrogenase; (1) iopanoate; (2) iodipamide; (3) **3**; (4) acetrizoate; (5) diatrizoate; (6) iothalamate; (7) methylglucamine·HCl.

(6) C. C. F. Blake, D. F. Koenig, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Nature (London)*, **206**, 757 (1965).

(7) J. H. R. Kagi and B. L. Vallee, *J. Biol. Chem.*, **235**, 3188 (1960).

(8) B. V. Plapp and R. D. Cole, *Arch. Biochem. Biophys.*, **116**, 193 (1966).

(9) J. H. Lang and E. C. Lasser, *Invest. Radiol.*, **2**, 396 (1967).

(10) W. Kauzmann, *Advan. Protein Chem.*, **14**, 1 (1959).

(11) F. Helmer, K. Kiehs, and C. Hansch, *Biochemistry*, **7**, 2858 (1968).

necting the rings. Acetrizoate (**4**) has a portion of the ring available for hydrophobic interactions, while **2** has two such rings and a hydrocarbon bridge connecting them. In neutral or basic solution **1** has the carboxylate as the only hydrophilic group on a fairly large molecule, and it would be expected to be the most hydrophobic of the compounds studied.

Comparison of the order of binding affinity to serum albumin with the order of inhibition of the enzymes reveals a strong parallel, even though somewhat different information is revealed by the two different types of measurements. Inhibition reveals only those interactions with the enzyme which effect the catalytic activity. It is quite possible that binding can occur without such effects. On the other hand, a direct technique such as equilibrium dialysis permits estimates of affinity constants and numbers of binding sites but fails for very low affinities, where the pertinent quantity being measured becomes comparable to the probable error in the measurement. For example, although **5** binding to serum albumin was undetected by equilibrium dialysis, binding of the low order implied by the inhibition measurements may well occur. While the order of affinity to serum albumin is approximately the same as to the enzymes, the strength of binding to serum albumin is, at least in some instances, much greater. For example, **1** and **4** require, respectively, about 10^{-3} *M* and 10^{-2} *M* concentrations for 50% inhibition of enzymes. If no interactions occur which do not effect enzyme activity, then affinity constants no greater than 10^3 and 10^2 M^{-1} are implied. However, these compounds bind to serum albumin with constants of 7×10^5 M^{-1} (**1**, three sites) and 1×10^6 M^{-1} (**4**, one site).[9] Of course, serum albumin is well known to be exceptional in its ability to bind small molecules, and it may be presumed that whatever structural features are responsible for the general reactivity of proteins with small molecules are highly specialized in serum albumin. Doubtless, more specific types of interactions are sometimes involved just as they are in the binding of substrates to enzymes.

The generality of the inhibition of enzymes by the compounds tested, and the fact that each compound has a characteristic, relatively narrow concentration range for 50% inhibition, implies a quite general ability of proteins to bind small molecules in a nonspecific way. Based on a correlation of the structures of the compounds with their order of effectiveness, it appears that the determining force for the interactions is hydrophobic bonding, and it is implied that the proteins have operationally similar hydrophobic regions. Since the compounds are unrelated structurally to the substrates of the enzymes, they probably act by producing sufficient conformational change to reduce or eliminate activity, rather than by binding to the active site directly.

Hansch and coworkers[11,12] have discovered quantitative relationships between hydrophobicity, as reflected by octanol–H_2O partition coefficients, and binding to serum albumin and a variety of biologic activities. Their findings have led them to conclude that "any sufficiently lipophilic compound will be bound by a variety of macromolecules in a nonspecific way."[11] Our results are in agreement with this conclusion. It is likely that any of the compounds studied (and, of course, others) will affect the function of any protein with which it comes into contact near or above its characteristically effective concentration. This provides a quite general explanation for the toxicity of the contrast media, although the possibility of more specific effects at low concentrations is not excluded.

Acknowledgment.—This work was supported by Grant GM 16593 from the National Institutes of Health.

(12) C. Hansch, *Farmaco Ed. Sci.*, **23**, 293 (1968).

Synthetic Nonenyl Acetates as Attractants for Female Melon Flies

MARTIN JACOBSON,*[1a] IRVING KEISER,[1b] DERRELL L. CHAMBERS,[1b]
DORIS H. MIYASHITA,[1b] AND CHARLES HARDING[1a]

Entomology Research Division, Agricultural Research Service,
United States Department of Agriculture, Beltsville, Maryland 20705, and Honolulu, Hawaii 96804

Received September 25, 1970

The eight possible straight-chain nonenyl acetates with trans configuration were prepared by multistep sequences for evaluation as attractants for female melon flies, *Dacus cucurbitae.* All target compounds showed some attraction in the laboratory, but *trans*-6-nonen-1-ol acetate (**31**) possessed the greatest activity. Although this compound attracted only small numbers of females in field trials, it is the most promising lead in an intensive search for a female attractant.

The first effective synthetic attractants found for the adult melon fly (*Dacus cucurbitae* Coquillet) were benzylacetone and anisylacetone.[2] On the basis of this discovery, related compounds were prepared and tested; the most effective material found thus far is the *p*-acetoxy derivative of benzylacetone,[3] known as Cue-lure. Field traps baited with Cue-lure will attract large numbers of melon flies and thus indicate the location and size of an infestation, but all the attracted flies are males. An effective attractant for the female melon fly is thus badly needed, but until recently, the screening of several thousand chemicals with laboratory olfactometers had failed to uncover any leads to such a substance.

In 1968, we synthesized a small sample of *trans*-7-decen-1-ol (**1**) for testing as a tumor inhibitor. However, many naturally occurring insect sex attractants

(1) (a) Beltsville, Md. (b) Honolulu, Hawaii.

(2) W. F. Barthel, N. Green, I. Keiser, and L. F. Steiner, *Science*, **126**, 654 (1957).

(3) (a) M. Beroza, B. H. Alexander, L. F. Steiner, W. C. Mitchell, and D. H. Miyashita, *ibid*, **131**, 1044 (1960); (b) B. H. Alexander, M. Beroza, T. A. Oda, L. F. Steiner, D. H. Miyashita, and W. C. Mitchell, *J. Agr. Food Chem.*, **10**, 270 (1962).