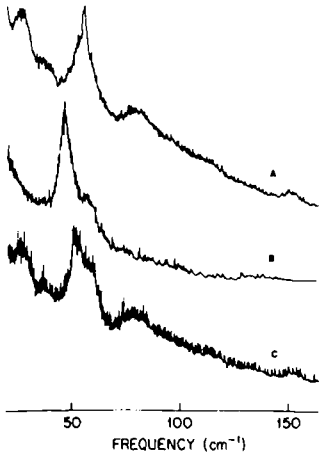

Figure 4—*Phonon spectra of the conjugates having sulfathiazole–povidone weight ratios of 3:1 (A) and 1:2 (B) are compared with that of sulfathiazole grown from propanol (C).*

and intramolecular vibrations have shown that at low povidone weight fractions (10:1, 5:1, and 1:1 conjugates), sulfathiazole was not present as the ethanol solvate but as the unsolvated sulfathiazole. In the sulfathiazole–povidone conjugate region 3:1–1:1.5, a second (higher dissolution rates) plateau was observed. Simonelli *et al.* postulated that the initial rate of dissolution exhibited at the higher plateau could rise from the presence of a new higher energy form of sulfathiazole. However, this higher energy form of sulfathiazole exhibited much faster dissolution than the previously known polymorphic forms of sulfathiazole. In the sulfathiazole–povidone region 3:1–1:1, we observed two different kinds of phonon and intramolecular spectra, one belonging to the sulfathiazole A form and the other being the polymorphic form obtained from propanol. The method of preparation (*i.e.*, temperature and/or concentration of solution) probably determines which of the two forms of sulfathiazole is present in the conjugates with drug–polymer ratios of 3:1–1:1.5. No other crystalline forms of sulfathiazole were observed in the drug–polymer conjugates. We therefore attribute the higher dissolution rate observed to arise from the particle size reduction of the drug in the presence of the polymer, whereby the increased effective surface area leads to an enhanced dissolution rate.

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Electrostatic Effects in Acylation of Hemoglobin by Aspirins

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Abstract □ Carboxylate substituents added to the salicylate ring increase the effectiveness of a variety of aspirins and diaspirins in acylating hemoglobin. Even more effective are a series of monoesters of dicarboxylate derivatives. Bis(5-carbomethoxysalicyl)fumarate and -succinate at 5 mM concentrations modify ~100% of the hemoglobin in solution and should alter the aggregation behavior of sickle hemoglobin.

Keyphrases □ Diaspirin—hemoglobin, sickle cell anemia, carboxylate substituents □ Hemoglobin—carboxylate-substituted diaspirins, acylation, sickle cell anemia

Previous studies have shown that aspirin and its analogues are capable of acylating hemoglobin (1). Acetylsalicylate, the prototype monoester, tends to transfer its acetyl group largely to the βLys[1] 59 and βLys 144 residues. These are in areas of the protein with a high local concentration of cationic side chains; *e.g.*, βLys 59 is part of a surface constellation constituted of βLys residues 59, 61, 65, and 66. The double-headed bissalicylates, in turn, preferentially go to the β-cleft of hemoglobin. In this canyon, both β chains contribute their βVal 1, βHis 2, βLys 82, and βHis 143 residues to provide a highly cationic environment.

In view of these observations, it seemed likely that even greater acylation of hemoglobin might be achieved with salicylate esters carrying additional negative charges. Furthermore, different structural dispositions of anionic charges on the reagents might lead to alternative specificities in binding to and acylation of Lys residues in hemoglobin. These might be directed toward placing substituents near βVal 6 or near βPhe 85 or βLeu 88 of hemoglobin S, residues evidently at a contact interface in sickled hemoglobin (2, 3). Therefore, several aspirin analogues with added substituents, particularly anionic ones, have been prepared. These substances are members of different classes of compounds: substituted monosalicylates [substituted carboxyphenyl acetates (I–V)]; substituted bissalicylates of succinic acid [bis(substituted carboxyphenyl)succinates (VI, VII, X, and XI)], of fumaric acid [bis(substituted carboxyphenyl)fumarates (VIII and IX)], or of anhydromethylene citric acid [bis(substituted

[1] Key: (Lys) lysine; (Val) valine; (His) histidine; (Phe) phenylalanine; (Leu) leucine.

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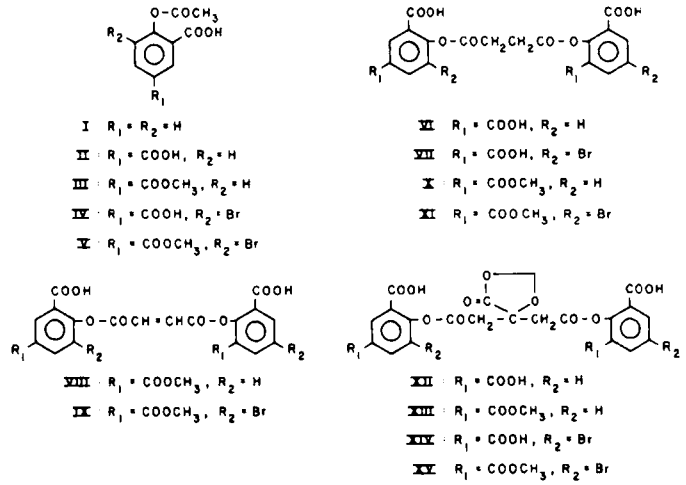
carboxyphenyl)4-oxo-1,3-dioxolane-5,5-diacetate (XII–XV)]. The extent of modification of hemoglobin by these compounds has been explored.



RESULTS AND DISCUSSION

Chemistry—Some of the compounds (Table I) proved to be very elusive. One of the key intermediates, 4-hydroxy-5-bromo-isophthalic acid monomethyl ester could not be obtained from the diester by conventional methods (4), which in this case produced the diacid. However, the simple procedure of stirring the diester in an appropriate alkaline solution yielded the desired monoester, from which V and IX could be prepared. The bisfumarates of the diacids 4-hydroxy-isophthalic and its 5-bromo analogue were so sensitive to traces of water in the environment that they could not be isolated even though their presence was established (TLC) in the dry solvent in which their synthesis was carried out. In contrast, the corresponding monoesters were comparatively stable.

As mentioned previously (5), the methylene citrate derivatives had to be protected under petroleum ether in the cold, but they were sufficiently stable to carry out modification experiments if they were added quickly to the hemoglobin solution.

Activity—A comparison (Table I) of aspirin (I) with its doubly anionic analogue 4-(acetyloxy)-1,3-benzenedicarboxylic acid (II) shows that the extent of modification of hemoglobin is significantly greater for the latter. This increase was expected for the doubly charged reagent. To our surprise, however, the monomethyl ester, 4-(acetyloxy)-1,3-benzenedicarboxylic acid 1-methyl ester (III), is much more effective than either analogue I or II. We conclude, therefore, that the presence of the added carbomethoxy moiety is much more influential than the additional charge. Evidently this second electron-withdrawing group in the *para* position to the acetoxy substituent activates the latter substantially so that it is much more effective in transferring its acetyl radical to the hemoglobin. The dipole of the $-COOCH_3$ moiety may also provide some electrostatic interaction with a cationic locus on the protein.

A bromo substituent on the aromatic ring, producing IV, also increases effectiveness (compared with II) of the aspirin analogue in modifying hemoglobin. This effect of the polarizable bromine atom has been seen previously (1). A particularly marked increase in modification is manifested with the monoanionic monoester V, the bromo analogue of III. Again we see that the *para* carbomethoxy substituent has a marked activating effect on the acetoxy group.

Turning to the bissalicylates, we find similar substituent effects, even more marked in these diaspirins. Bissalicyl succinate, the single-COOH reference analogue of VI, at 5 mM concentration modifies ~15% of hemoglobin (1, 6). In contrast, the additional —COOH provided by VI increases the degree of modification to almost 80%. An additional bromo substituent increases the reactivity even further, as is evident with VII (Table I).

It has been shown previously (5, 6) that the fumarate diaspirin, bis(2-carboxyphenyl)fumarate, is more effective than the corresponding salicylate derivative. This superiority is maintained in the present series of compounds. Thus, VIII is more reactive than X, and IX is better than XI. With all of these compounds the extent of modification of hemoglobin is very high.

In the methylene citric acid series (XII–XV), the beneficial effects of the $-COOCH_3$ substituent continue to be expressed. Thus, XIII is superior to XII, and XV is better than XIV.

The shifts in p_{50} values (oxygen pressure at half-saturation of hemoglobin) provide some insight into the location of the acyl group affixed by the aspirin

Table I—Modification of Hemoglobin by Salicylates

Compound	Conc., mM	Percent Modification of Oxyhemoglobin A, 1 mM	p_{50} for Oxygen Binding, mm Hg: Without Inositol Hexaphosphate	p_{50} for Oxygen Binding, mm Hg: With 2 mM Inositol Hexaphosphate
None	—	—	5	40
Aspirin; 2-(acetyloxy)-benzoic acid (I)	20	26	5	34
	10	15	4	35
	5	8	4	36
4-(Acetyloxy)-1,3-benzenedicarboxylic acid (II)[a]	20	39	5	21
	10	23	4	30
	5	10	4	36
4-(Acetyloxy)-1,3-benzenedicarboxylic acid 1-methyl ester (III)	10	70	5	22
	5	43	5	32
4-(Acetyloxy)-5-bromo-1,3-benzenedicarboxylic acid (IV)	10	68	7	28
	5	48	7	36
4-(Acetyloxy)-5-bromo-1,3-benzenedicarboxylic acid 1-methyl ester (V)	10	83	7	20
	5	60	7	28
Bis(2,4-dicarboxyphenyl)-succinate (VI)[b,f]	5	78	6	14
	1	31	6	38
Bis(6-bromo-2,4-dicarboxyphenyl)succinate (VII)[b,c,f]	5	86	5	11
		86	6	12
	1	46	6	40
		39	7	35
Bis(4-carbomethoxy-2-carboxyphenyl)-fumarate (VIII)[b,f]	5	97	6	7
	1	45	7	36
Bis(6-bromo-4-carbomethoxy-2-carboxyphenyl)-fumarate (IX)[b,d,f]	5	98	4	7
		100		
		100		
	1	61	5	26
		49		
		58		
Bis(4-carbomethoxy-2-carboxyphenyl)-succinate (X)[b,f]	5	77	7	12
		81		
	1	33	6	39
		34		
Bis(6-bromo-4-carbomethoxy-2-carboxyphenyl)-succinate (XI)[b,e,f]	5	63	6	24
		74		
	1	17	6	37
		24		
Bis(2,4-dicarboxyphenyl)4-oxo-1,3-dioxolane-5,5-diacetate (XII)	5	41	6	33
	1	13	7	39
Bis(4-carbomethoxy-2-carboxyphenyl)4-oxo-1,3-dioxolane-5,5-diacetate (XIII)	5	76	7	23
	1	34	7	38
Bis(6-bromo-2,4-dicarboxyphenyl)4-oxo-1,3-dioxolane-5,5-diacetate (XIV)	5	40	6	34
	1	15	7	38
Bis(6-bromo-4-carbomethoxy-2-carboxyphenyl)-4-oxo-1,3-dioxolane-5,5-diacetate (XV)	5	94	6	23
	1	42	7	38

[a] Some qualitative experiments with this compound have been described by Kokkini *et al.* (11). [b] Satisfactory analytical data (±0.4% for C, H) were reported for each of these compounds. [c] Calc. for Br, 26.44; found, 25.82. [d] Calc. for Br, 25.36; found 24.91. [e] Calc. for Br, 25.28; found 24.83. [f] Melting points (°C): VI, 228–230; VII, 212–213; VIII, 200–202; IX, 233–235; X, 179–181; XI, 178–180.

reagent. If p_{50} is shifted back to near 40 mm (Table I) when inositol hexaphosphate is added to the hemoglobin solution, then this effector is clearly able to enter the β-cleft of the protein to shift the conformation to the low-affinity form. As has been shown previously (1), the diaspirins are capable of denying inositol phosphate access to the β-cleft by forming a covalent blocking bridge across the portal. It is apparent that these features of the double-headed aspirins are maintained in the current series of compounds (VI–XI), all of which, at least at a concentration of 5 mM, keep p_{50} from returning to the normal value in the presence of inositol hexaphosphate. The

two most striking agents in this regard are the monomethyl esters VIII and IX, which at 5 mM concentration keep p_{50} in the presence of inositol hexaphosphate at essentially the same value as in its absence. Thus, these two compounds are essentially 100% effective in blocking access to the β-cleft. The bis(carbomethoxysalicyl)fumarate (VIII), devoid of the bromo substituent, should be a particularly attractive compound from a pharmacological viewpoint for testing as an antisickling agent.

EXPERIMENTAL SECTION[2]

3-Bromo-5-carbomethoxy Salicylic Acid—Partial hydrolysis of 5-bromo-4-hydroxy-isophthalic acid dimethyl ester was affected by stirring the mixture of diester (2.0 g) with 25 mL of 33% KOH in methanol and 25 mL of water for several hours at room temperature. The unchanged diester in suspension was removed by filtration. The filtrate was cooled and acidified with 1 M HCl. The resulting precipitate was removed by filtration and then recrystallized from dilute ethanol to give the monoester, mp 237–238°C. IR: 1720 cm^{-1}; ^{1}H-NMR (Me_2SO-d_6): δ 3.85 (3, s, —$COOCH_3$) and 8.0–8.4 ppm (2, t, ArH).

Anal.—Calc. for $C_9H_7BrO_5$: C, 39.30; H, 2.57; Br, 29.05. Found: C, 39.34; H, 2.58; Br, 29.18.

Preparation of Substituted Isophthalic Acids and Methyl Esters—*4-(Acetoxy)-isophthalic Acid (II) and 4-Acetoxy-isophthalic Acid 1-Methyl Ester (III)*—These compounds were prepared as described in the literature (4).

4-(Acetoxy)-5-bromo-isophthalic Acid (IV)—Compound IV was prepared in a manner similar to that in the literature (4) for II and was recrystallized from chloroform–petroleum ether, mp 197–199°C. ^{1}H-NMR (Me_2SO-d_6): δ 2.45 (3, s, —$OCOCH_3$), 8.4 (2, t, ArH), and 11.61 ppm (br s, 2, 2-COOH).

Anal.—Calc. for $C_{10}H_7BrO_6$: C, 39.63; H, 2.33. Found: C, 39.74; H, 2.32.

4-Acetoxy-5-bromo-isophthalic Acid Monomethyl Ester (V)—Ester V was also prepared by a procedure (4) analogous to that used for III and was recrystallized from chloroform–petroleum ether, mp 133–135°C. IR: 1725 cm^{-1}; ^{1}H-NMR ($CDCl_3$-Me_2SO-d_6): δ 2.37 (3, s, —$OCOCH_3$), 3.92 (3, s, $COOCH_3$), and 8.34–8.53 ppm (2, d, ArH).

Anal.—Calc. for $C_{11}H_9BrO_6$: C, 41.67; H, 2.86; Br, 25.20. Found: C, 41.40; H, 2.80; Br, 25.75.

General Method for the Synthesis of Bissuccinates (VI and VII)—Sodium hydride (0.06 mol) was added to a solution of substituted salicylic acid (0.02 mol) in 30 mL of dry tetrahydrofuran, and the mixture was stirred overnight at room temperature. Succinyl chloride (0.01 mol) was dissolved in a minimum quantity of tetrahydrofuran (~5 mL) and added in a dropwise manner, over a period of 0.5 h, to the stirred suspension of sodium salt of the substituted salicylate. The resulting mixture was further stirred for an additional day. After removal of solvent under reduced pressure, the gummy residue was treated with ethyl acetate and acidified with aqueous 1 M HCl while being cooled in an ice bath. The organic layer was removed, washed with cold saturated NaCl solution, and dried over $MgSO_4$. After removal of solvent, the residue was recrystallized from a mixture of ethyl acetate–petroleum ether. Properties of the compounds obtained (VI and VII) are summarized in Table I.

General Method for the Synthesis of Bisfumarates and Bissuccinates (VIII–XI)—To a stirred mixture of substituted salicylic acid (2.0 mmol) and *N,N*-dimethylaniline (6.2 mmol) in 80 mL of dry benzene was added in a dropwise manner fumaryl or succinyl chloride (1.02 mmol) dissolved in 20 mL of benzene. The resultant mixture was stirred overnight at room temperature. After removal of the solvent under reduced pressure, the residue was suspended in 50 mL of ethyl acetate and extracted several times with ice-cold 1 M HCl (50 mL) and once with water (50 mL). The organic layer was dried ($MgSO_4$), filtered, and evaporated under reduced pressure. The residue was further recrystallized from either ethanol or a mixture of ethyl acetate–petroleum ether. The compounds obtained (VIII–XI) are listed in Table I. ^{1}H-NMR spectra were consistent with the structures.

General Method for the Synthesis of Biscitrates (XII–XV)—The preparations of XII–XV were described in a previous publication (5).

Hemoglobin Experiments—Whole blood (with anticoagulant) was obtained from local hospitals. Hemolysate was prepared by the method of Drabkin (7) except that chloroform was used to facilitate separation of the membranes. Hemolysate from normal adults was used without further purification. After freezing in liquid nitrogen, the hemolysate and hemoglobin samples were stored at −80°C. Hemoglobin concentrations in solution were measured after conversion to the cyanomet derivative (8).

Chemical modifications with the acylating compounds were performed with 1–5-fold molar ratio of reagent to hemoglobin for the bissalicylate esters and with a 5–20-fold molar excess for the monosalicylates. The reactions were carried out in 0.1 M Tris-HCl buffer, pH 7.3, which the hemoglobin had been dialyzed against. All reactions were performed with oxyhemoglobin. Modification reactions were initiated by suspending the compound in buffer, partially dissolving it, and adding hemoglobin within 30 s. Stirring was continued for 2 h at 37°C. The reaction mixture was then dialyzed overnight against cold 0.13 M glycine (pH 6.0).

The extent of modification of the hemoglobin was assessed by isoelectric focusing (9, 10) in a flat gel[3] using the procedures described by the manufacturer, with the following alterations. A 1-mm gel (instead of 3 mm) was prepared between a glass plate (bottom) and a piece of plexiglass (top) with a polytef gasket between plates. The gel composition for 100 mL of solution was 4 g (0.56 M) of acrylamide[4], 0.16 g (0.01 M) of *N,N'*-methylenebisacrylamide[5], 5 g (0.54 M) of glycerol, 5.4 mL of ampholytes, pH 6.5–9[6], 1.8 mL of ampholytes, pH 6–8[7], 0.05 g (4.3 mM) of *N,N,N',N'*-tetramethylethylenediamine[4], and 0.05 g (2.2 mM) of ammonium persulfate[8]. All hemoglobin samples were saturated with gaseous CO and made 0.01 M in NaCN; 2.5 μL was then applied to the gel surface on small pieces of filter paper. The anolyte was 1 M H_3PO_4; 2 M NaOH served as catholyte. Isoelectric focusing was carried out at 10°C for ~90 min with a power level of 30 W. Immediately after the power was turned off, the gel was fixed in an aqueous solution of 10 g/100 mL (0.6 M) of trichloroacetic acid[9] and 6 g/100 mL (0.2 M) of 5-sulfosalicylic acid[10] for 20–30 min, and then placed for 30–60 min in a preservative solution of water–ethanol–acetic acid–glycerol (35:5:1:1). After the gel had dried overnight, the modification pattern in each reaction channel was analyzed by recording a densitometric scan with a soft laser scanning densitometer with an integrator[11]. Oxygen affinities of chemically modified and unmodified hemoglobins were determined in the presence and in the absence of 2 mM inositol hexaphosphate[12] using an oxygenator[13].

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[2] Elemental analyses were performed by Micro-Tech Laboratories, Skokie, Ill. IR spectra were obtained with a Perkin-Elmer 283 spectrometer. ^{1}H-NMR spectra were recorded using Varian EM-360 (60 MHz) and JEOL-FX-270 spectrometers, and chemical shifts are listed as δ values in ppm relative to $(CH_3)_4Si$ as internal standard. Melting points were determined with a Buchi capillary apparatus and are uncorrected. The 4-hydroxy-isophthalic acid (or 5-carboxysalicylic acid) used herein as a starting material was purchased from the Chemolog Co., N.Y.

[3] LKB 2117 Multiphor instrument with a Savant power source.
[4] Eastman.
[5] Aldrich Chemical Co.
[6] Pharmacia.
[7] LKB.
[8] Bio-Rad.
[9] Mallinckrodt.
[10] Baker.
[11] Zeineh; BioMed Instruments.
[12] Sigma Chemical Co.
[13] Hemoscan; American Instruments.