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THE ABILITY OF NONSTEROID ANTI-INFLAMMATORY COMPOUNDS TO ACCELERATE A DISULFIDE INTERCHANGE REACTION OF SERUM SULFHYDRYL GROUPS AND 5,5′-DITHIOBIS(2-NITROBENZOIC ACID)*

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Abstract—Anti-inflammatory compounds were studied for the unusual ability to accelerate a reaction between serum protein sulfhydryl groups and 5,5′-dithiobis(2-nitrobenzoic acid). Human serum diluted with an equal volume of 0·1 M phosphate buffer (pH 7·4) was allowed to react with 65 μM dithiobisnitrobenzoic acid for 10 min at 30°. The increase in absorbance at 440 mμ induced by the drug (0·67 mM, 1·33 mM, and 2·00 mM) was taken as a measure of the ability of the drug to accelerate disulfide interchange of the serum sulfhydryl groups and dithiobisnitrobenzoic acid. A measurable increase was associated with the presence of thirteen compounds. In decreasing order of reactivity with serum, these were: indomethacin, oxyphenbutazone, phenylbutazone, flufenamic acid, ibufenac, salicylic acid, dichlorotolylanthranilic acid, hydroxydione, lauryl sulfuric acid, gentisic acid, mefenamic acid, aminophylline, and acetylsalicylic acid. Except for hydroxydione, all these compounds are known to be anti-inflammatory. No measurable effect was noted with 125 other compounds consisting of commonly used drugs and commonly occurring biologicals. These results indicate a highly significant correlation between the anti-inflammatory effects of nonsteroid drugs and the ability of these drugs to accelerate disulfide interchange of serum sulfhydryl groups and an aromatic disulfide *in vitro*.

THE MECHANISM of action of anti-inflammatory compounds is unknown, although several hypotheses have been proposed.[1] Salicylate and several other anti-inflammatory compounds uncouple phosphorylation and oxidation,[2] suppress enzymes such as transaminases[3] and glucosamine 6-phosphate synthetase,[4] and inhibit denaturation of serum albumin by heat.[5] The present report demonstrates an enhancing effect of nonsteroid anti-inflammatory compounds on the rate of interaction of serum protein sulfhydryl groups and 5,5′-dithiobis(2-nitrobenzoic acid) at pH 7·4, and suggests that this effect is almost exclusively limited to these nonsteroid anti-inflammatory compounds.

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METHODS

5,5′-Dithiobis(2-nitrobenzoic acid)[6] (dithiobisnitrobenzoic acid) undergoes a sulfhydryl–disulfide interchange reaction[7] with sulfhydryl groups of serum proteins to release the deeply pigmented, related sulfhydryl compound, 5-thio-2-nitrobenzoic acid ($\varepsilon = 11{\cdot}3 \times 10^3\ M^{-1}\ cm^{-1}$; 440 mμ; pH 7·4). This sulfhydryl–disulfide interchange reaction is represented by the reaction RSSR + R′SH → RSH + R′SSR. Here RSSR represents dithiobisnitrobenzoic acid, R′SH represents a serum protein sulfhydryl group, and RSH represents 5-thio-2-nitrobenzoic acid. In order to study the effect of anti-inflammatory compounds on this reaction, pooled fresh human serum was diluted with an equal volume of 0·1 M phosphate buffer (pH 7·4). Chemical compounds under study were added to the diluted serum in three different final concentrations: 0·67, 1·33, and 2·00 mM. Dithiobisnitrobenzoic acid* (0·1 ml; 2 mM) was added to a final concentration of 65 μM. Absorbance measurements were obtained at 440 mμ prior to, and 10 min after the addition of dithiobisnitrobenzoic acid. The reaction was carried out at 30°. Measurements were made in a Bausch and Lomb Spectronic 20 spectrophotometer in one-half inch test tubes with a 10-mm light path. After suitable corrections for dilution, the per cent increase in the reaction between dithiobisnitrobenzoic acid and serum, induced by the test compound, was calculated. The expression $[(A/B) - 1] \times 100\%$ was used. In this expression, A represents the increase in absorbance in the presence of the study compound and B represents the increase in absorbance in the absence of the study compound. All determinations were performed in triplicate. Compounds were selected for study to include drugs commonly used in the care of patients and compounds found in biological material. A compound was not systematically included if its molecular weight exceeded the arbitrary value of 580, if the recommended dose to patients was less than the arbitrary value of 20 mg/day, or if the compound contained sulfur which could react directly with dithiobisnitrobenzoic acid.

The administration of acetylsalicylic acid to patients was studied for the effect of the drug on the reactivity of the patient's serum with dithiobisnitrobenzoic acid. Measurements were made in the same manner as described above except that the drug was administered to the patient by mouth instead of being added to his serum. The result for a given serum was expressed as the increase in absorbance at 440 mμ due to the reaction between dithiobisnitrobenzoic acid and serum for 10 min at 30°.

Purified serum protein fractions were studied for reactivity with 65 μM dithiobisnitrobenzoic acid; 2% (w/v) bovine serum albumin† and 1% (w/v) human gamma-globulin‡ (pH 7·4, 0·1 M phosphate buffer) were used. These solutions were treated in the same manner as diluted serum except that measurements were made at 412 mμ instead of 440 mμ. The 412 mμ wavelength was chosen because, unlike serum, solutions of bovine serum albumin and human gamma-globulin do not absorb sufficiently at 412 mμ to interfere with measurements of absorbance at this wavelength. The extinction coefficient for 5-thio-2-nitrobenzoic acid at 412 mμ is $13{\cdot}6 \times 10^3\ M^{-1}\ cm^{-1}$ at pH 7·4.

Absorption spectra were obtained with a Beckman DB spectrophotometer, with a

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programmed narrow slit and a 10-mm light path. Recordings were made with a Beckman potentiometric log recorder.

RESULTS AND DISCUSSION

A. Reaction between serum and dithiobisnitrobenzoic acid in the presence of 138 *compounds*

Serum was diluted with an equal volume of 0·1 M, pH 7·4, phosphate buffer. The reaction was carried out in the presence of 65 μM dithiobisnitrobenzoic acid at 30° for 10 min. Thirteen compounds produced a significant increase in the rate of the

TABLE 1. COMPOUNDS CAPABLE OF ACCELERATING THE REACTION BETWEEN SERUM AND 65 μM DITHIOBISNITROBENZOIC ACID

Compound	0·67 mM (% increase)	1·33 mM (% increase)	2·00 mM (% increase)	Anti-inflammatory
Indomethacin	135	149	283	Yes[8]
Oxyphenbutazone	150	196	206	Yes[1, 10]
Phenylbutazone	136	180	189	Yes[1, 10]
Flufenamic acid	58	135	172	Yes[11]
Ibufenac	61	137	165	Yes[12]
Salicylic acid	62	114	139	Yes[1, 9, 10]
Dichlorotolyanthranilic acid	67	124	121	Yes[13]
Hydroxydione	52	94	163	No
Lauryl sulfuric acid	49	98	149	Yes[5]
Gentisic acid	48	69	84	Yes[14, 15]
Mefenamic acid	43	62	75	Yes[10]
Aminophylline	29	63	87	Yes[16]
Acetylsalicylic acid	22	36	46	Yes[1, 9]

Serum was diluted 1:1 with 0·1 M phosphate buffer, pH 7·4. Measurements were taken after 10 min at 30°. Results are expressed as $[(A/B) - 1] \times 100\%$ where A = increase in absorbance in presence of the test compound and B = increase in absorbance in the absence of the test compound.

reaction. These compounds are listed in Table 1 in order of decreasing reactivity. All the compounds except hydroxydione are known to be anti-inflammatory (see Table 1 for references). When the per cent increase in absorbance obtained per 0·67 mM drug (Table 1) was divided by the molecular weight of the drug to give a measure of the reactivity of each drug per unit weight, the following order of decreasing activity was obtained: oxyphenbutazone (0·46), salicylic acid (0·45), phenylbutazone (0·44), indomethacin (0·38), ibufenac (0·32), gentisic acid (0·31), N-(2,6-dichloro-*m*-tolyl)-anthranilic acid (0·23), flufenamic acid (0·21), mefenamic acid (0·18), sodium lauryl sulfate (0·17), hydroxydione (0·16), acetylsalicylic acid (0·07), and aminophylline (0·06). All drugs in Table 1 were active in the serum–dithiobisnitrobenzoic acid system when tested at a concentration of 0·67 mM. This concentration corresponds to a concentration of salicylate of 9·2 mg/100 ml which can readily be obtained in the serum of patients receiving therapeutic doses of aspirin.[1] Serum concentrations of phenylbutazone may reach 0·3 mM in patients receiving therapeutic doses of this drug.[17]

In contrast to the thirteen compounds in Table 1, 125 other compounds (Table 2) selected at random were without measurable effect (viz. 15 per cent per 0·67 mM compound) on the reaction between serum and dithiobisnitrobenzoic acid. Among the list of inactive compounds were antipyrine and phenacetin, two compounds which are

TABLE 2. COMPOUNDS ASSOCIATED WITH LESS THAN A 15 PER CENT INCREASE IN THE RATE OF THE REACTION BETWEEN SERUM AND 65 μM DITHIOBISNITROBENZOIC ACID

Acetamide	Hydantoin-5-acetic acid
4-Acetylaminoantipyrine	Hydralazine
N-Acetyl-D-glucosamine	Hydrochloric acid
Adenosine	*p*-Hydroxybenzoic acid
Alanine	*p*-Hydroxyphenyl-lactic acid
4-Aminoantipyrine	Indole-3-acetic acid
m-Aminobenzoic acid	Isoleucine
α-Aminobutyric acid	Lactic acid
4-Amino-5-imidazolecarboxamide	Leucine
Aminopyrine	Levallorphan
Ammonium chloride	Levopropoxyphene
Amytal	Lysine
Antipyrine	Mandelic acid
Arabinose	Mephenytoin
Arginine	Methergine maleate
Ascorbic acid	Methionine
Asparagine	Methoxyphenamine
Aspartic acid	5-Methoxytryptamine
Benzoic acid	Methylphenidate
Bishydroxycoumarin	Methscopolamine bromide
Cadaverine	Methylethyl ketone
Caffeine	1-Methyl-2-mercaptoimidazole
Caramiphen	N'-Methylnicotinamide
Carbarsone	N-1-Methylnicotinamide iodide
Carnosine	Methyl alcohol
Chloromycetin succinate	Methyprylon
Chloroquine	Methysergide maleate
Chlorthalidone	Niacin
Citraconic acid	Nicotinyl alcohol
Citrate	*n*-Octyl alcohol
Copper sulfate	Orcinol
Cortisone	Ornithine
Creatinine	Orotic acid
Cyclopentamine	Phenacetin
Cycloserine	Phenaglycodol
11-Dehydrocorticosterone	Phenindamine
Dehydroepiandrosterone	Phenmetrazine
11-Desoxycorticosterone	Phenobarbital
Dextromethorphan bromide	Phenol
Diethylstilbestrol	Phenylalanine
Dihydroergotamine	Phenylmercuric acetate
Di-iodo-L-tyrosine	Potassium bromide
2,4-Dinitrophenol	Prednisolone
Dromostanolone	Procainamide
Erythromycin	Progesterone
Estradiol (17-β)	Proline
Estrone	Pronestyl
Ethylenediamine tetraacetic acid	Putrescine
Fluoroacetamide	Pyridostigmine bromide
Fluprednisolone	Serine
Flurandrenolone	Sodium carbonate
Formimino-L-glutamic acid Ba salt	Sodium chloride
Fucose	Sodium hydroxide
Galactosamine	Taurine
Glutamic acid	Thenylpyramine
Glutamine	Threonine
Glutaric acid	Tolazoline
Glycine	Trimethobenzamide
Gramine	Tryptophan
Heptabarbital	Tyrosine
Histamine	Urea
Histidine	Valine
Histidine methyl ester	

Serum was diluted 1:1 with 0·1 M phosphate buffer, pH 7·4. Concentration of compound = 0·67 mM. Measurements were taken after 10 min at 30°.

analgesic and antipyretic[1] but not anti-inflammatory.[1,9,10] 2,4-Dinitrophenol, which like salicylate uncouples phophorylation and oxidation but which is not anti-inflammatory,[3] was inactive (Table 2). EDTA was inactive, suggesting that effects on the serum–dithiobisnitrobenzoic acid interaction are not due to chelation of metals.

B. Study of sera from patients given aspirin by mouth

The sera of 105 patients were studied to determine the effect of the oral administration of acetylsalicylic acid on the reactivity of serum sulfhydryl groups with dithiobisnitrobenzoic acid (10 min, 30°, pH 7·4). Forty-one sera were from patients receiving an average of 2·6 g (S.D. = 1·8) of acetylsalicylic acid per day. Sixty-four sera were from patients receiving no acetylsalicylic acid at the time of the study. There was a highly significant correlation ($r = 0{\cdot}52$, $P < 10^{-4}$) between the increase in absorbance (A) and the acetylsalicylic acid dose in g per day (D). This correlation could be expressed by the regression: $A = 0{\cdot}023 \times D + 0{\cdot}20$. An oral dose of 5 g aspirin per day produced the same effect as 17 mg salicylate added to 100 ml serum, *in vitro*. This correlation is in agreement with what is known about the relationship between aspirin dosage and serum salicylate concentration.[1]

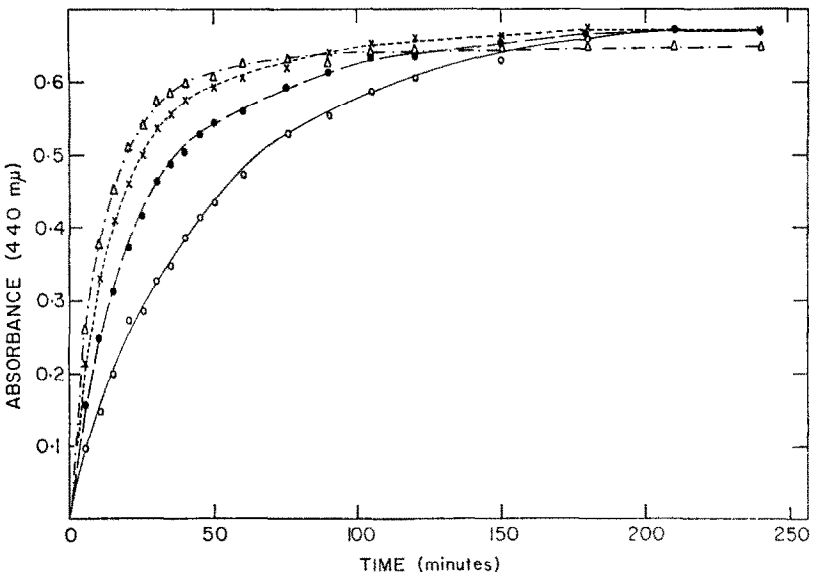

FIG. 1. Effect of time and salicylate on the reaction between serum sulfhydryl groups and dithiobisnitrobenzoic acid (65 μM) at 30°. Serum was diluted with an equal volume of 0·1 M phosphate buffer (pH 7·4). Sodium salicylate concentration: 0 mM (○), 0·67 mM (●), 1·33 mM (×), 2·00 mM (△).

C. Effect of varying concentration of reagents and duration of reaction

A study of the interaction of human serum diluted with phosphate buffer (pH 7·4) and dithiobisnitrobenzoic acid revealed that the extent of this reaction was dependent on the duration of the interaction (Fig. 1) and reached a maximum after 2·5 hr. While salicylic acid accelerated the rate of the reaction, it did not increase the maximal extent of the reaction. In fact, the number of sulfhydryl groups that could

be detected with dithiobisnitrobenzoic acid was not affected by allowing human serum to stand in the presence of 1·33 mM salicylic acid at 4° for 24 hr. Increasing the concentration of dithiobisnitrobenzoic acid increased the extent of the serum–dithiobisnitrobenzoic acid interaction (Fig. 2), but again the maximal extent of the reaction

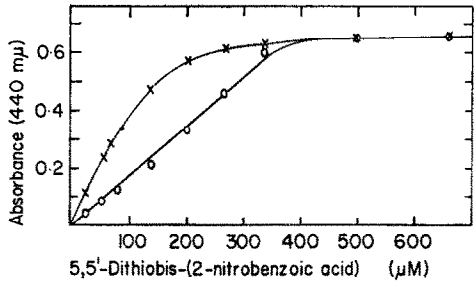

FIG. 2. Effect of the concentration of dithiobisnitrobenzoic acid on the interaction of serum sulfhydryl groups and dithiobisnitrobenzoic acid. Serum was diluted 3:7 with 0·1 M phosphate buffer (pH 7·4). Salicylate concentration: 0·0 mM (○), 2 mM (×).

was independent of the concentration of salicylic acid. These observations indicate that the presence of salicylate is not associated with fission of disulfide bonds and does not render sulfhydryl groups reactive that otherwise would not be reactive in due time. Figure 3 demonstrates the relationship between the increase in absorbance associated with the presence of salicylate and the concentration of salicylate used.

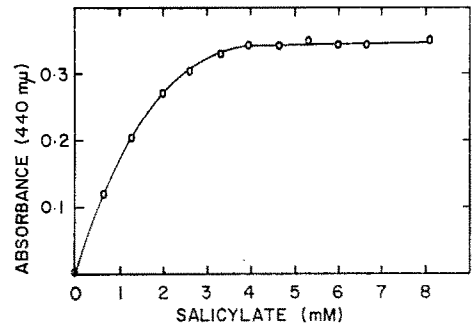

FIG. 3. Effect of the concentration of salicylate on the interaction of serum sulfhydryl groups and dithiobisnitrobenzoic acid (65 μM). Serum was diluted with an equal volume of 0·1 M phosphate buffer (pH 7·4). Reaction carried out at 30° for 10 min.

D. Absorption spectra

Figure 4 depicts the absorption spectra of the products obtained by the interaction of 65 μM dithiobisnitrobenzoic acid and diluted serum in the presence of varying amounts of salicylate (0 mM, 0·67 mM, 1·33 mM, 2·00 mM). All spectra have similar shapes and similar maxima (412 mμ). The reported maximum for 5-thio-2-nitrobenzoic acid is at 412 mμ.[6]

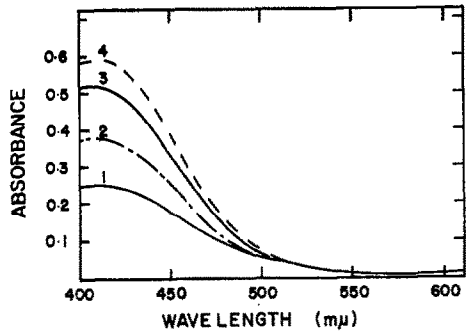

FIG. 4. Absorption spectrum of products formed by interaction of serum sulfhydryl groups and dithiobisnitrobenzoic acid (65 μM). Serum was diluted with an equal volume of 0·1 M phosphate buffer (pH 7·4). Equal volume of buffer was substituted for dithiobisnitrobenzoic acid in reference cuvette. Concentration of salicylic acid: (1) = 0 mM, (2) = 0·67 mM, (3) = 1·33 mM, (4) = 2·00 mM.

E. Reaction between dithiobisnitrobenzoic acid and either bovine serum albumin or human gamma-globulin

Table 3 demonstrates that the reactive compounds in Table 1 were also reactive when 2% (w/v) bovine serum albumin was substituted for diluted human serum.

TABLE 3. COMPOUNDS CAPABLE OF ACCELERATING THE REACTION BETWEEN 65 μM DITHIOBISNITROBENZOIC ACID AND 2% (W/V) BOVINE SERUM ALBUMIN IN PHOSPHATE BUFFER

Compound	0·67 mM	1·33 mM	2·00 mM
	(increase in absorbance)		
Flufenamic acid	0·099	0·459	0·777
Mefenamic acid	0·090	0·460	0·769
Phenylbutazone	0·240	0·354	0·455
Indomethacin	0·053	0·258	0·526
Lauryl sulfuric acid	0·015	0·125	0·550
Salicylic acid	0·100	0·249	0·330
Acetylsalicylic acid	0·066	0·141	0·208
Gentisic acid	0·102	0·117	0·184
Ibufenac	0·008	0·054	0·281
Hydroxydione	0·014	0·061	0·133
Oxyphenbutazone	0·021	0·057	0·084
Aminophylline	0·020	0·036	0·060
Dichlorotolylanthranilic acid		Cloudy	

Buffer 0·1 M, pH 7·4. Measurements were taken after 10 min at 30°. Results are expressed as increase in absorbance associated with the presence of the test compound.

Figure 5 demonstrates the direct linear correlation between the concentration of salicylic acid and the increase in absorbance produced by salicylic acid in the reaction between 2% (w/v) bovine serum albumin and dithiobisnitrobenzoic acid. Figure 5 also demonstrates that the effect of salicylic acid is obliterated by a 2·0 M increase in the concentration of sodium chloride in the reaction mixture.

When 1% (w/v) human serum gamma-globulin was substituted for diluted human serum, only phenylbutazone was reactive. For phenylbutazone, the increases in

absorbance were: 0·037 for 0·67 mM drug, 0·056 (1·33 mM), and 0·070 (2·00 mM). The effect of dichlorotolylanthranilic acid and lauryl sulfuric acid on human serum gamma-globulin could not be studied because gamma-globulin solutions were cloudy in the presence of these two compounds.

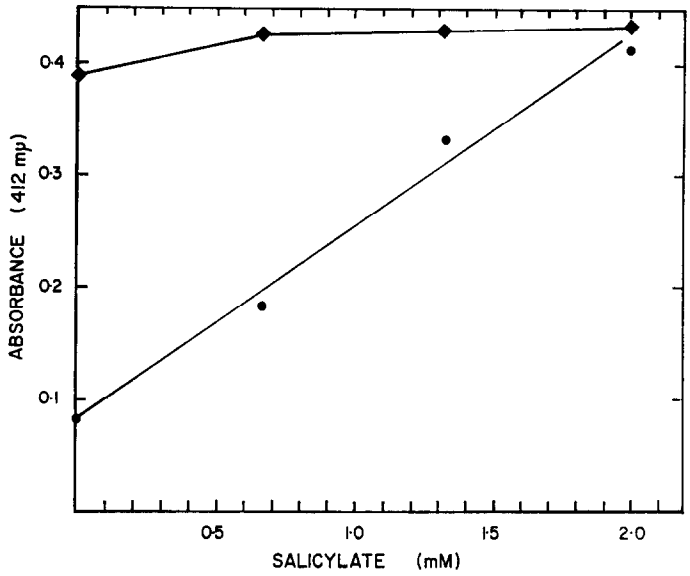

FIG. 5. Effect of salicylate on the interaction of 2% bovine serum albumin (w/v) and 65 μM dithiobisnitrobenzoic acid at pH 7·4; ● = reaction carried out in 0·1 M phosphate buffer; ◆ = reaction carried out in 0·1 M phosphate buffer to which NaCl had been added to a final concentration of 2·0 M.

F. Effect of N-*ethylmaleimide and iodoacetamide on the interaction of dithiobisnitrobenzoic acid and protein solutions*

When either human serum or bovine serum albumin was allowed to react with 1·33 mM N-ethylmaleimide or 1·33 mM iodoacetamide for 15 hr, at 4° no measurable effect on the reaction with dithiobisnitrobenzoic acid could be obtained with any of the compounds in Table 1. No measurable effect was defined as an increase in absorbance of less than 15 per cent of that obtained when the protein solution was not treated with either of these two sulfhydryl-inhibiting reagents.[18, 19] Pretreatment of human gamma-globulin with 1·33 mM iodoacetamide prior to the addition of dithiobisnitrobenzoic acid rendered phenylbutazone inactive. N-ethylmaleimide-treated gamma-globulin was cloudy and so could not be studied.

These results as well as the absorption spectra obtained in (*D*) are in accord with the thesis that the reaction that has been studied is a sulfhydryl–disulfide interchange reaction of serum protein suflhydryl groups and dithiobisnitrobenzoic acid that yields 5-thio-2-nitrobenzoic acid, and it is this reaction that is accelerated by nonsteroid anti-inflammatory compounds.

The effect of nonsteroid anti-inflammatory compounds on a sulfhydryl–disulfide interchange reaction involving protein sulfhydryl groups and an aromatic disulfide

was discovered by chance. The meaning of this observation must await a better understanding of the biochemistry of inflammation. It should not be inferred that non-steroid anti-inflammatory compounds exert their action *in vivo* by an effect on a sulfhydryl–disulfide interchange reaction, although it seems reasonable that an effect on some protein interaction is involved.

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