Biochemical Pharmacology, Vol. 38, No. 22, pp. 3987–3994, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00
Pergamon Press plc

CATALYTIC EFFECT OF SERUM ALBUMIN ON THE *o*-REARRANGEMENT OF *N*-SULFOOXY-2-ACETYLAMINOFLUORENE, A POTENTIAL HEPATOCARCINOGEN IN THE RAT, TO NONMUTAGENIC SULFURIC ACID ESTERS OF *o*-AMIDOFLUORENOLS

BEVERLY A. SMITH, HELMUT R. GUTMANN* and JOHN R. SPRINGFIELD
Research Service, VA Medical Center, Minneapolis, MN 55417, U.S.A.

(*Received* 13 *November* 1988; *accepted* 23 *March* 1989)

Abstract—Bovine serum albumin (BSA) catalyzes the *o*-rearrangement of the reactive electrophile, *N*-sulfooxy-2-acetylaminofluorene (NSF), a potential ultimate hepatocarcinogen in the rat, to the nonmutagenic sulfuric acid esters of 1- and 3-hydroxy-2-acetylaminofluorene. Conversion of NSF was proportional to BSA concentrations ranging from 0.25 to ~4 mg BSA/ml incubation mixture. At concentrations ≥5 mg BSA/ml, ~90% of NSF was converted to the sulfuric acid esters of the *o*-amidofluorenols. Human serum albumin (HSA) likewise catalyzed the *o*-rearrangement of NSF. However, the catalytic activity of HSA was only ~50% of the activity of BSA. The catalytic effect of BSA was abolished by heat denaturation. However, it was not changed by dialysis or by anion exchange chromatography. These observations indicated that the catalytic effect requires intactness of the tertiary structure of BSA and is not due to a contaminant(s) of low or high molecular weight. There were no differences in the catalytic activity of three separate fractions of chromatographed BSA, suggesting that the catalytic activity is associated with the entire BSA molecule. In contrast to serum albumin, γ-globulin (bovine or human) did not catalyze the *o*-rearrangement of NSF. The solvolytic degradation of NSF to 4-hydroxy-2-acetylaminofluorene, a major reaction in the absence of BSA, occurred only to a minor extent in the presence of BSA. These data indicated that the BSA-catalyzed *o*-rearrangement determines the rates of concurrent reactions involved in the degradation of NSF. BSA and HSA did not catalyze the *o*-rearrangement of *N*-acetoxy-2-acetylaminofluorene (*N*-OAC-2-AAF), the acetate ester of *N*-hydroxy-2-acetylaminofluorene (*N*-OH-2-AAF), to the acetic acid esters of the *o*-amidofluorenols. These findings suggest that the albumin-catalyzed *o*-rearrangement occurs preferentially with esters of fluorenylhydroxamic acids that readily ionize in aqueous media.

It is generally accepted that the reactive forms of chemical carcinogens ("ultimate carcinogens") are in many instances metabolically generated electrophiles that interact with DNA and other cellular macromolecules. This interaction is thought to be the initiating step of transformation [1, 2]. In the case of the carcinogenic arylamide, 2-acetylaminofluorene (2-AAF), *N*-sulfooxy-2-acetylaminofluorene (NSF) has been considered a potential ultimate carcinogen of the rat [1, 2]. NSF is the product of the enzymatic sulfonation of the intermediate ("proximate") carcinogen, *N*-hydroxy-2-acetylaminofluorene (*N*-OH-2-AAF), which arises from the microsomal oxidation of 2-AAF [3]. Recently, we reported that synthetic NSF, in the presence of bovine serum albumin (BSA), rearranges quantitatively to 1- and 3-sulfooxy-2-acetylaminofluorene (1-SF and 3-SF), the nonmutagenic sulfuric acid esters of 1- and 3-hydroxy-2-acetylaminofluorene (1- and 3-OH-2-AAF) [4]. The present study, in addition to confirming the initial results, extends the previous investigation to the effects of human and rat serum albumin (HSA and RSA) and of serum γ-globulins on the *o*-rearrangement of NSF. Another question, considered in this study, was whether the rate of *o*-rearrangement is a function of the concentration of BSA. To rule out that the albumin-catalyzed *o*-rearrangement of NSF was due to a contaminant(s) of low or high molecular weight, the reaction was investigated in the presence of BSA that had been dialyzed or subjected to anion exchange chromatography and with BSA essentially devoid of fatty acid (<0.005%). Another aim of this study was to assess the effect of the albumin-catalyzed rearrangement on reactions coincident with the *o*-rearrangement [4]. For this purpose, the formation of 4-hydroxy-2-acetylaminofluorene (4-OH-2-AAF), a major product of the solvolysis of NSF [4], and the extent of the *o*-rearrangement of NSF were determined simultaneously in the presence and absence of serum albumin. Finally, serum albumin was tested for substrate specificity by investigating whether BSA and HSA catalyze the *o*-rearrangement of *N*-acetoxy-2-acetylaminofluorene (*N*-OAc-2-AAF), the acetic acid ester of *N*-OH-2-AAF and a model ultimate carcinogen [2, 5, 6], to 1-acetoxy- and 3-acetoxy-2-acetylaminofluorene (1-OAc-2-AAF and 3-OAc-2-AAF).

MATERIALS AND METHODS

Crystallized and lyophilized bovine serum albumin

* Author to whom correspondence should be sent.

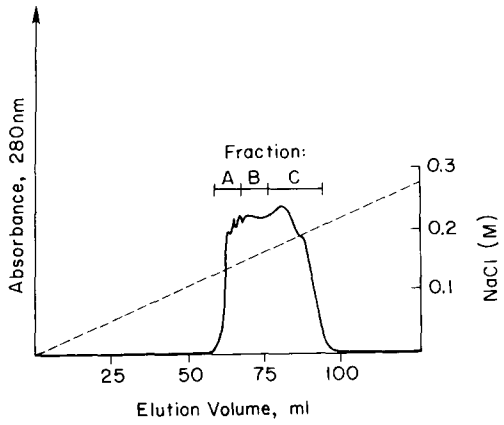

Fig. 1. Elution profile of bovine serum albumin subjected to anion exchange chromatography. BSA (300 mg), dissolved in 0.05 M Tris–HCl buffer (2.0 ml, pH 7.0), was applied to an anion exchange column (DEAE-Trisacryl M, column volume = 37 ml) and eluted with a linear gradient of NaCl (0 to 0.84 M/24 hr); flow rate = 15.6 ml/hr. Prior to the assay of the catalytic effect of the eluted BSA on the *o*-rearrangement of NSF, each BSA fraction was diluted with 0.05 M Tris–HCl buffer to give a protein concentration of 5.0 mg/ml. The assay of the *o*-rearrangement of [^{3}H]NSF was carried out as described in Materials and Methods and in the legend to Fig. 3.

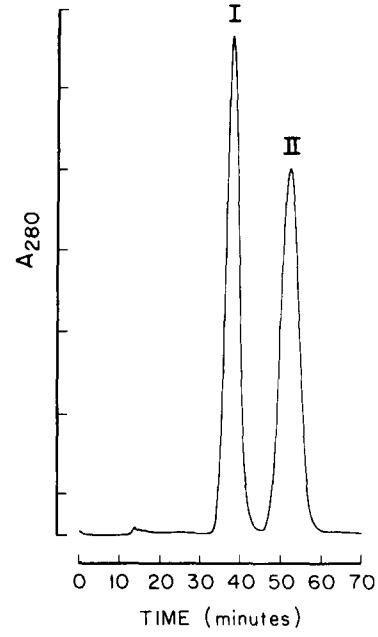

Fig. 2. Separation of 1-OH-2-AAF (I) and 3-OH-2-AAF (II) on Carbowax 400/Porasil C. Eluent: 30% ethyl acetate: 70% *n*-hexane. Pressure: 110 lb/in^2.

(No. A4378), essentially fatty acid-free BSA (No. 7511), human serum albumin (No. A1887), rat serum albumin (No. A4530), and γ-globulins (bovine, No. G5009; human No. G4386) were obtained from the Sigma Chemical Co. (St Louis, MO), Methyl-*tert*-butyl ether (MBE), HPLC grade, was purchased from the Aldrich Chemical Co., (Milwaukee, WI).

Purification of BSA

Method 1. BSA (100 mg) in distilled water (2.0 ml) was dialyzed for 24 hr against 4 l of distilled water. Tris–HCl buffer, pH 7.0, was added to the dialyzed solution to give a final concentration of 0.05 M. Prior to the assay, the BSA concentration was adjusted to 5.0 mg protein/ml with 0.05 M Tris–HCl buffer, pH 7.0, as determined by the method of Lowry *et al.* [7].

Method 2. BSA (300 mg) was subjected to anion exchange chromatography (DEAE-Trisacryl M; LKB Instruments, Inc., Gaithersburg, MD). BSA was applied to the column (column volume = 37 ml) in 0.05 M Tris buffer (2.0 ml, pH 7.0) and eluted with a linear gradient of NaCl (0–0.84 M/24 hr) at a flow rate of 15.6 ml/hr. The recovery of applied BSA from the column was >99%. The eluted protein was collected in three fractions (Fig. 1). Prior to assay of the catalytic activity of the BSA in the three fractions, each fraction was diluted with 0.05 M Tris buffer, pH 7.0, to give a protein concentration of 5.0 mg/ml, as determined by the method of Lowry *et al.* [7].

Preparation of compounds. *N*-Sulfooxy-2-acetylaminofluorene (NSF), labeled with ^{3}H in the fluorene ring ([^{3}H]NSF), and *N*-acetoxy-2-acetylaminofluorene, labeled with ^{3}H (*N*-OAc-2-[^{3}H]AAF), were obtained as previously described [4, 8, 9]. 1-Sulfooxy-2-acetylaminofluorene (1-SF) [4], 3-sulfooxy-2-acetylaminofluorene (3-SF) [4], 1-acetoxy-2-acetylaminofluorene (1-OAc-2-AAF) [10], 3-acetoxy-2-acetylaminofluorene (3-OAc-2-AAF) [10], 1-hydroxy-2-acetylaminofluorene (1-OH-2-AAF) [11], 3-hydroxy-2-acetylaminofluorene (3-OH-2-AAF) [11] and 4-hydroxy-2-acetlamino-fluorene (4-OH-2-AAF) [4], were prepared by the published methods. Melting points and spectral properties (UV, IR) were identical with those reported. The purities of 1-SF, 3-SF, 1-OAc-2-AAF, 3-OAc-2-AAF, 1-OH-2-AAF, 3-OH-2-AAF, and 4-OH-2-AAF were confirmed by high pressure liquid chromatography.

Determination of the effect of serum proteins on the o-rearrangement of NSF to 1- and 3-SF

[^{3}H]NSF (50 nmol, sp. act. 1.9–2.2 × 10^9 dpm/mmol in 0.02 ml dimethyl sulfoxide) was added to the solutions containing the serum proteins under investigation (5.0 mg protein/1.0 ml Tris–HCl buffer, pH 7.0). After incubation for 30 min at 37°, carrier compounds (1- and 3-SF) were added. The incubation mixtures were extracted with MBE (3 × 1.0 ml) to remove 1- and 3-OH-2-AAF. The aqueous phase containing 1- and 3-[^{3}H]SF was acidified with 0.25 M H_2SO_4 (1.0 ml) (and kept at 90° for 60 min. Under these conditions, 1- and 3-SF are hydrolyzed to 1- and 3-OH-2-AAF respectively [4]. The incubation mixtures were extracted with MBE. The MBE was evaporated with a stream of nitrogen. The *o*-amidofluorenols in the residue were separated by reverse phase HPLC [4] or by HPLC on Carbowax 400/Porasil C (Waters Assoc., Inc., Milford, MA) (eluent: 30% ethyl acetate: 70% *n*-hexane; pressure: 110 lb/in^2) (Fig. 2). The amounts of 1- and 3-SF formed were calculated from the quantities of 1- and 3-OH-2-AAF by the method of isotope dilution. Previously, determination of the *o*-rearrangement of NSF had included isolation of 1- and 3-SF, as a single

Table 1. Catalytic effect of serum albumin on the *o*-rearrangement of *N*-sulfooxy-2-acetylaminofluorene to 1-sulfooxy-2-acetylaminofluorene and 3-sulfooxy-2-acetylaminofluorene

Expt.*	Proteins	Formation of rearranged compounds† (nmol) 1-SF	3-SF	*o*-SF	% of NSF rearranged
1	None	1.7 ± 0.1	3.3 ± 0.2	5.0 ± 0.3	10.0
	Serum albumin, bovine	13.6 ± 0.3	34.8 ± 1.4	48.4 ± 1.7	96.8
	Serum albumin, bovine‡	1.5 ± 0.1	2.8 ± 0.3	4.3 ± 0.4	8.6
	Serum albumin, human	6.4 ± 0.1	17.0 ± 0.6	23.4 ± 0.7	46.8
	Serum albumin, human‡	1.4 ± 0.1	2.8 ± 0.2	4.2 ± 0.3	8.4
	Serum globulin, bovine	2.0 ± 0.1	4.2 ± 0.2	6.2 ± 0.3	12.4
	Serum globulin, human	1.9 ± 0.0	3.7 ± 0.3	5.6 ± 0.3	11.2
2	None	0.5 ± 0.0	1.7 ± 0.2	2.2 ± 0.2	4.4
	Serum albumin, rat	4.3 ± 0.1	4.4 ± 0.4	8.7 ± 0.5	17.4
	Serum albumin, rat‡	0.6 ± 0.1	1.7 ± 0.1	2.3 ± 0.2	4.6

Abbreviations: NSF, *N*-sulfooxy-2-AAF; 1-SF, 1-sulfooxy-2-AAF; 3-SF, 3-sulfooxy-2-AAF; and *o*-SF, Σ(1-SF + 3-SF).

* In Expt. 1, [^{3}H]NSF (50 nmol, sp. act. 2.0×10^9 dpm/mmol) in Tris–HCl buffer (1.0 ml, pH 7.0) was incubated at 37° for 30 min. The protein concentration was 5.0 mg/ml incubation mixture. After completion of the incubation, carrier compounds (1-SF and 3-SF) were added. The quantities of the sulfuric acid esters were determined by the method of isotope dilution after removal of the proteins, acid hydrolysis of the sulfuric acid esters, and separation of the resulting *o*-amidofluorenols by HPLC as described in Materials and Methods. In Expt. 2, a different preparation of [^{3}H]NSF (sp. act. = 2.2×10^9 dpm/mmol) was used. The incubation conditions and analytical determinations were the same as those of Expt. 1.

† Values are averages ± SD of triplicate determinations.

‡ Proteins were denatured by heating in boiling water for 10 min.

peak, by HPLC followed by acid hydrolysis of the isolated sulfuric acid esters and separation of the resulting 1- and 3-OH-2-AAF by HPLC as described above [4]. Control experiments showed that acid hydrolysis of the aqueous fraction after MBE extraction and separation of the resulting 1- and 3-OH-2-AAF by HPLC gave the same results as the original procedure [4].

Determination of the formation of 4-hydroxy-2-acetylaminofluorene from N-sulfooxy-2-acetylaminofluorene in the presence of serum albumin

In the experiments in which the effect of serum proteins on the solvolysis of [^{3}H]NSF to 4-OH-2-[^{3}H]AAF was investigated, the incubation conditions were those described above. At the completion of the incubation, 4-OH-2-AAF was added as a carrier compound to the incubation mixtures and the incubation mixtures were extracted with MBE. After evaporation of the MBE with a stream of nitrogen, the residue containing 4-OH-2-[^{3}H]AAF was dissolved in MeOH (0.05 ml). The *m*-amidofluorenol was isolated by reverse phase HPLC [4] and purified by additional HPLC on Corasil II (Waters Assoc. Inc.) [4]. The amounts of 4-OH-2-[^{3}H]AAF formed from [^{3}H]NSF were obtained by the method of inverse isotope dilution.

Determination of the o-rearrangement of N-acetoxy-2-acetylaminofluorene to 1- and 3-acetoxy-2-acetylaminofluorene in the presence of serum albumin

N-OAc-2-[^{3}H]AAF (50 nmol, sp. act. 6.6×10^8 dpm/mmol, in 0.02 ml dimethyl sulfoxide) was added to the solution containing the serum proteins under investigation (1.0 ml of 0.05 M Tris buffer, pH 7.0). After incubation for 30 min at 37°, carrier compounds (1- and 3-OAc-2-AAF) were added, and the incubation mixtures were extracted with benzene (2×1.0 ml). Control experiments showed that the acetic acid esters of 1- and 3-OH-2-AAF were hydrolyzed by adding MeOH (3.0 ml) and heating the solution at 50° for 30 min. Under these conditions, hydrolysis of 1-OAc-2-AAF to 1-OH-2-AAF was complete. 3-OAc-2-AAF was hydrolyzed to the extent of 65%. Hydrolysis of the acetic acid esters formed by the *o*-rearrangement of *N*-OAc-2-[^{3}H]AAF was carried out, therefore, at 50° for 30 min. After the solvent had been evaporated under a stream of nitrogen, the residue was taken up in MeOH, and 1- and 3-OH-2-[^{3}H]AAF were separated by reverse phase HPLC [4] or by HPLC on Carbowax 400/Porasil C (Fig. 2). The quantities of 1- and 3-OAc-2-[^{3}H]AAF formed were calculated, by the method of inverse isotope dilution, from the amounts of 1- and 3-OH-2-[^{3}H]AAF isolated.

RESULTS

o-Rearrangement of NSF to 1- and 3-SF in the presence of serum proteins

In agreement with the preliminary observations [4], *o*-rearrangement of NSF to 1- and 3-SF, a relatively minor reaction involving ~5–10% of substrate in the absence of serum albumin, was nearly quantitative in the presence of BSA (Table 1). A catalytic effect on the *o*-rearrangement of NSF was also noted with HSA and RSA (Table 1, Expts 1 and 2). However, the catalytic efficiencies of HSA and RSA were only ~50 and 20%, respectively, of that of BSA (Table 1). In all instances, denaturation by heat abolished the catalytic effect (Table 1). Intactness of the tertiary structure appeared to be a requirement for the catalytic effect of serum albumin. In contrast to serum albumin, serum γ-globulin (bovine or human) did not increase the rate of the *o*-rearrangement of NSF significantly (Table 1). The reasons

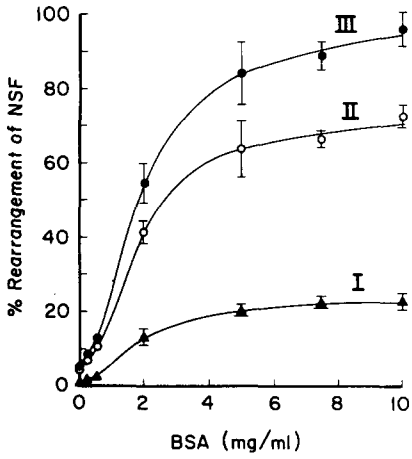

Fig. 3. *o*-Rearrangement of NSF to the sulfuric acid esters of 1- and 3-OH-2-AAF as a function of BSA concentration. I = percent rearrangement of NSF to 1-SF (1-sulfooxy-2-acetylaminofluorene); II = percent rearrangement of NSF to 3-SF (3-sulfooxy-2-acetylaminofluorene); III = percent rearrangement of NSF to *o*-SF [Σ(1-SF + 3-SF)]. Each point is the average of duplicate or triplicate determinations ± SD. Each assay sample contained [^{3}H]NSF (59 nmol, sp. act. 2.2×10^9 dpm/mmol) in 0.02 ml dimethyl sulfoxide. The [^{3}H]NSF was added to solutions of BSA (0–10 mg) in 0.05 M Tris–HCl buffer (1.0 ml, pH 7.0). After incubation for 30 min at 37° carrier compounds (1- and 3-SF, 100–125 nmol) were added, and the mixtures were extracted with MBE (2×1.0 ml). To the aqueous fraction was added 0.25 M H_2SO_4 (1.0 ml). After the acidified solutions had been kept at 90° for 1 hr, 1- and 3-OH-2-[^{3}H]AAF were extracted from the hydrolysate with MBE (2×1.0 ml). The extract was washed with distilled water (1.0 ml), and the MBE was evaporated. The residue was dissolved in MeOH (0.05 ml) and the *o*-amido-[^{3}H]fluorenols were separated by reverse phase HPLC (10 μm Econosil C-18 column, 250×4.6 mm, Alltech Assoc., Inc., Deerfield, IL). Eluent: 55% MeOH: 45% H_2O; flow rate: 1.0 ml/min). The amounts of 1- and 3-[^{3}H]SF were calculated from the quantities of the isolated *o*-amido-[^{3}H]fluorenols by the method of inverse isotope dilution.

why serum globulins are unable to catalyze the *o*-rearrangement of NSF are not known and remain the subject of further investigation.

Under the experimental conditions of this study, the catalytic effect of BSA appeared to be an approximately linear function of BSA concentration from 0.25 to 2.0 mg BSA/ml incubation mixture and approached a maximal value at ~5.0 to 6.0 mg BSA/ml (Fig. 3). The apparent increase from 5.0 mg BSA/ml (84.7 ± 8.4% rearrangement) to 10.0 mg BSA/ml (97.0 ± 4.7% rearrangement) was not statistically significant.

o-Rearrangement of NSF consists of the transfer of the sulfate group from the amide nitrogen to carbon atoms 1 and 3 of the fluorene moiety (Fig. 4). The data of Table 1 and of Fig. 3 show that the sulfate transfer to carbon atom 3 of the fluorene ring was the preferred reaction of the albumin-catalyzed rearrangement. A preferential transfer of the sulfate group is likely due to steric hindrance of transfer to carbon atom 1 on account of the methylene bridge of the fluorene ring. Since sulfate transfer to carbon atom 3 was also predominant in uncatalyzed *o*-rearrangement of NSF (Table 1), the preferential transfer of sulfate to carbon atom 3 appears not to be related to the catalytic effect of serum albumin.

Catalytic effect of purified BSA on the o*-rearrangement of NSF*

Heat denaturation abolished the catalytic effect of serum albumin on the *o*-rearrangement of NSF (Table 1), and measurements of the reaction catalyzed by untreated and fatty acid-free BSA gave identical values. These observations suggested that the catalytic effect required an intact tertiary protein structure and was probably not due to a contaminant(s) of low or high molecular weight. This view was supported by determination of the catalytic activity of BSA that had been subjected to dialysis or anion exchange chromatography. Comparison of the catalytic efficiency of untreated BSA with that of dialyzed or chromatographed BSA showed that the catalytic effect of BSA remained unchanged following dialysis or chromatography (Table 2). Each of the three fractions into which the chromatographed BSA was divided (Fig. 1) exhibited equal catalytic efficiency (Table 2). Since the yield of BSA eluted from the column was nearly quantitative (>99%), the catalytic activity of the three fractions selected for assay was representative of the activity of the entire protein sample applied to the column. The evidence led us to conclude (1) that the capacity of BSA to catalyze the *o*-rearrangement of NSF is evenly distributed over a molecular population with heterogeneous structural characteristics [12–15] and (2) that it is not attributable to a contaminant(s) of low or high molecular weight.

Inverse relation between the albumin-catalyzed o*-rearrangement of NSF and its degradation to 4-OH-2-AAF*

4-OH-2-AAF has been identified as a major product of the solvolytic degradation of NSF [4]. About 50% of NSF was found to be converted to 4-OH-2-AAF previously [4] as well as in the present study (Table 3). In addition, the present investigation showed that the albumin-catalyzed rearrangement proceeded at the expense of the 4-hydroxylation of NSF (Table 3). Thus, the ratio of *o*-SF [Σ(1-SF + 3-SF)] to 4-OH-2-AAF increased from 0.75 at 0.5 mg BSA/ml incubation mixture to 7–20 at 5.0 mg BSA/ml incubation mixture. An inverse relation between the *o*-rearrangement of NSF and its conversion to 4-OH-2-AAF was also observed with HSA. In the presence of serum γ-globulins which exhibited no catalytic effect on the *o*-rearrangement of NSF, the formation of 4-OH-2-AAF was in large excess of the formation of *o*-SF. These data suggest that the extent of the albumin-catalyzed *o*-rearrangement determines the rates of the reactions which have been shown to account for the degradation and disposition of NSF [4]. It remains to be determined whether the albumin-catalyzed *o*-rearrangement of NSF competes effectively with the formation of adducts which result from the interaction of NSF with DNA and which may be a required step in the initiation of hepatocarcinogenesis in the rat [1, 2].

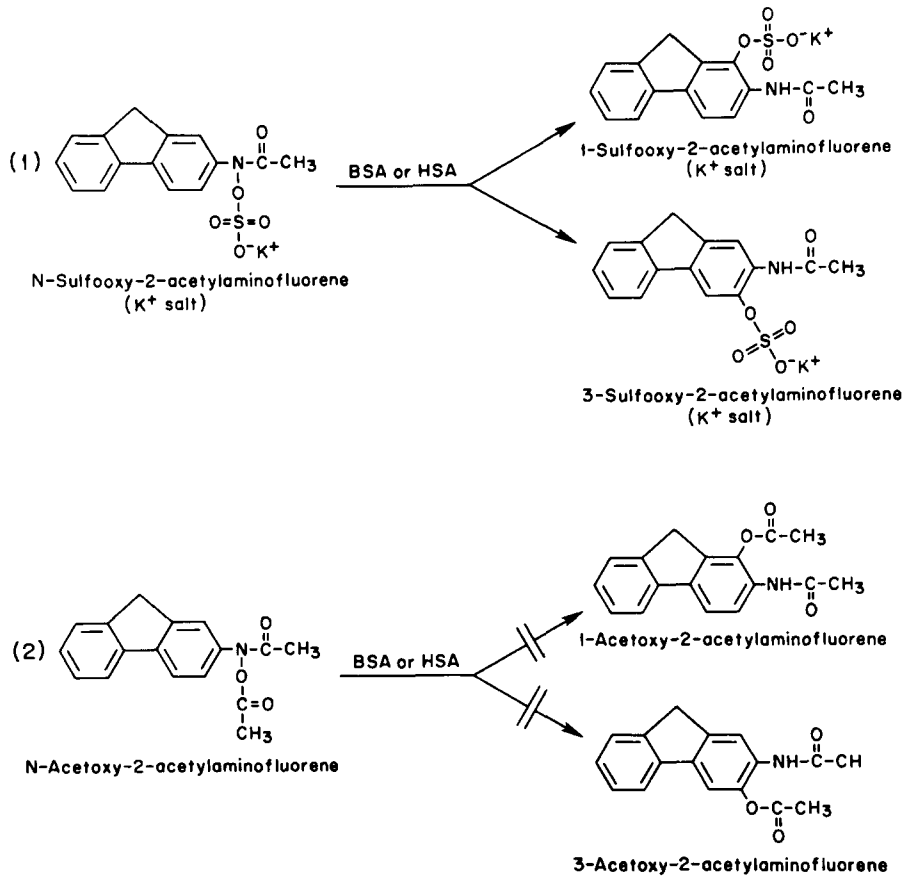

Fig. 4. Representation of the *o*-rearrangement of *N*-sulfooxy- and *N*-acetoxy-2-acetylaminofluorene to the nonmutagenic sulfuric acid and acetic acid esters of 1- and 3-hydroxy-2-acetylaminofluorene and of the selective catalytic effect of bovine and human serum albumin.

Table 2. Comparison of the extent of the *o*-rearrangement of *N*-sulfooxy-2-acetylaminofluorene to sulfuric acid esters in the presence of untreated BSA, dialyzed BSA and BSA fractionated by anion exchange chromatography

Treatment of BSA	Formation of sulfuric acid esters* (nmol) 1-SF	3-SF	*o*-SF	% of NSF rearranged
None	13.3 ± 0.6	32.5 ± 2.1	45.8 ± 2.2	91.6
Dialysis	12.1 ± 3.5	35.3 ± 1.5	47.4 ± 5.0	94.8
Fractionation by chromatography				
Fraction A	12.9 ± 0.8	33.1 ± 0.6	46.0 ± 1.4	92.0
Fraction B	13.5 ± 0.6	32.6 ± 0.1	46.1 ± 0.7	92.2
Fraction C	13.3 ± 0.7	33.7 ± 0.1	47.0 ± 1.7	94.0

BSA was dialyzed or fractionated by anion exchange chromatography as outlined in Materials and Methods and the legend of Fig. 1. Three fractions (A, B and C) were assayed for their catalytic effect on the rearrangement of NSF to sulfuric acid esters. The concentration of BSA was 5.0 mg/ml incubation mixture. Each assay mixture (1.0 ml) contained 50 nmol [^{3}H]NSF, sp. act. 2.0×10^9 dpm/mmol. Conditions of incubation and the analytical procedures of estimating the amounts of sulfuric acid esters resulting from the *o*-rearrangement of NSF are detailed in Materials and Methods and the legend of Fig. 3.

Abbreviations: NSF, *N*-sulfooxy-2-AAF; 1-SF, sulfooxy-2-AAF; 3-SF, 3-sulfooxy-2-AAF; and *o*-SF, Σ(1-SF + 3-SF).

* Values are the averages ± SD of duplicate or triplicate determinations.

Table 3. Evidence of the catalytic effect of serum albumin on the *o*-rearrangement of *N*-sulfooxy-2-acetylaminofluorene to sulfuric acid esters and of decreased solvolysis of *N*-sulfooxy-2-acetylaminofluorene to 4-hydroxy-2-acetylaminofluorene in the presence of serum albumin

Protein in the incubation mixture	Amounts of protein (mg)	Rearranged to *o*-SF	Hydrolyzed to 4-OH-AAF
None	—	10.3 ± 2.0	52.9 ± 5.9
Serum albumin, bovine	0.5	27.4 ± 3.2	36.1 ± 1.2
	5.0	89.4 ± 6.8*	12.9 ± 1.2
	5.0	97.8 ± 1.6*	4.7 ± 2.0
Serum albumin, human	5.0	52.2 ± 4.4	8.4 ± 1.0
Serum γ-globulin bovine	5.0	12.4 ± 0.6	36.4 ± 2.0
Serum γ-globulin, human	5.0	11.3 ± 0.6	36.9 ± 1.4

[^{3}H]NSF (50 nmol, sp. act. 2.0 × 10^9 dpm/mmol) in Tris–HCl buffer (1.0 ml, pH 7.0) was incubated at 37° for 30 min with the proteins in the amounts listed. After completion of the incubation, carrier compounds (1-SF, 3-SF and 4-OH-2-AAF) were added and the incubation mixtures were extracted with MBE (3 × 1.0 ml). The MBE extracts were washed with water (2 × 1.0 ml) and the MBE was evaporated. The residual *m*-amidofluorenol was isolated by reverse phase HPLC [4]. The sulfuric acid esters in the aqueous fraction were isolated by HPLC, subjected to acid hydrolysis, and the resulting *o*-amidofluorenols (1-OH-2-AAF and 3-OH-2-AAF) were separated by HPLC as described under Materials and Methods. The quantities of the sulfuric acid esters and of 4-OH-2-AAF formed from [^{3}H]NSF were determined by the method of isotope dilution. Values are the averages ± SD of duplicate or triplicate determinations. Abbreviations: NSF, *N*-sulfooxy-2-AAF; *o*-SF, Σ(1-sulfooxy-2-AAF + 3-sulfooxy-2-AAF); 4-OH-AAF, 4-hydroxy-2-AAF.

* These values were obtained in two separate experiments.

Table 4. Determination of the *o*-rearrangement of *N*-acetoxy-2-acetylaminofluorene to 1-acetoxy-2-acetylaminofluorene and 3-acetoxy-2-acetylaminofluorene in the presence of serum albumin

Proteins in incubation mixture	*o*-OAc-2-AAF formed* (nmol)	% of *N*-OAc-2-AAF rearranged
None	1.7 ± 0.4	3.4
Serum albumin, bovine	1.0 ± 0.2	2.0
Serum albumin, bovine†	1.7 ± 0.1	3.4
Serum albumin, human	0.6 ± 0.1	1.2
Serum albumin, humans†	1.7 ± 0.2	3.4
Serum γ-globulin, human	1.2 ± 0.2	2.4

N-Acetoxy-2-[^{3}H]AAF (50 nmol, sp. act. 6.6 × 10^9 dpm/mmol) in Tris–HCl buffer (1.0 ml, pH 7.0) was incubated at 37° for 30 min with the proteins listed. The protein concentration was 5.0 mg/ml incubation mixture. At the completion of the incubation, carrier compounds (1-acetoxy-2-AAF and 3-acetoxy-2-AAF) were added, and the mixtures were extracted with benzene (3 × 1.0 ml). Following addition of methanol (3.0 ml) to the benzene layer, the acetic acid esters were hydrolyzed to 1- and 3-hydroxy-2-AAF as detailed in Materials and Methods. After solvent evaporation, 1- and 3-hydroxy-2-[^{3}H]AAF were separated by HPLC as described in Materials and Methods. The quantities of 1- and 3-acetoxy-2-[^{3}H]AAF formed from *N*-acetoxy-2-[^{3}H]AAF were determined by the method of inverse isotope dilution. Abbreviations: *o*-OAc-2-AAF, Σ(1-acetoxy-2-AAF + 3-acetoxy-2-AAF); and *N*-OAc-2-AAF, *N*-acetoxy-2-AAF.

* Values are the averages ± SD of duplicate or triplicate determinations.

† Proteins were denatured by heating in boiling water for 10 min.

Determination of the rearrangement of N-*OAc-2-AAF in the presence of serum albumin*

To determine whether serum albumin catalyzes the *o*-rearrangement of other esters of arylhydroxamic acids or whether serum albumin exhibits substrate specificity, the *o*-rearrangement of *N*-OAc-2-AAF to the acetic acid esters of 1- and 3-OH-2-AAF (1- and 3-OAc-2-AAF) (Fig. 4) was investigated. In contrast to the marked catalytic effect of serum albumin on the *o*-rearrangement of NSF, neither serum albumin nor serum γ-globulin increased the rate of the *o*-rearrangement of *N*-OAc-2-AAF (Table 4). The data suggest that the albumin-catalyzed *o*-rearrangement is restricted to esters of arylhydroxamic acids which contain a readily ionizable leaving group, such as a sulfate. The present evidence on the albumin-catalyzed *o*-rearrangement of fluorenylhydroxamic acid esters is summarized in Fig. 4.

DISCUSSION

Heretofore, enzymatic sulfonation of *N*-OH-2-AAF which results in the formation of NSF has been detected by interaction of NSF with cellular nucleophiles through a bimolecular mechanism [9] and by isolation of the resulting adducts [16–18]. The BSA-catalyzed *o*-rearrangement may provide

another sensitive and specific method for the quantitation of synthetic or enzymatically generated NSF and, possibly, of other sulfate esters of arylhydroxamic acids. Preliminary measurements from this laboratory (unpublished observations) indicate that the amounts of NSF, produced from *N*-OH-2-AAF by fortified cytosol of rat livers and estimated by the BSA-catalyzed rearrangement, were the same as those obtained previously from the isolation of the adduct of NSF with *N*-acetyl-L-methionine, the most reactive of four nucleophiles [9].

Serum albumin has been reported to exhibit "enzyme-like" properties, and there is evidence that esterase activity is an intrinsic property of serum albumin [19–21]. To our knowledge, the present report is the first to show that the intra- or intermolecular shift of the sulfate group of an arylhydroxamic acid ester is catalyzed by serum albumin. The evidence of this study allows no conclusion as to whether there is an active site on the albumin molecule which contains the functional group required for sulfate transfer. Another explanation of the observed catalytic effect is that serum albumin stabilizes NSF, a labile hydrophobic molecule, on an albumin ligand binding site so that *o*-rearrangement, a prevalent reaction of sulfate or acetate esters of arylhydroxamic acids [22], can occur [23–25]. Further work is in progress to elucidate the mechanism by which BSA accelerates the *o*-rearrangement of NSF and, possibly, of sulfate esters of other arylhydroxamic acids.

The results of this study indicate that HSA as well as BSA catalyze the *o*-rearrangement of NSF (Table 1). This finding raises the question whether the albumin-catalyzed reaction contributes to the detoxification of this reactive electrophile, a potential ultimate hepatocarcinogen [1, 2]. NSF would undoubtedly rearrange to inactive sulfuric acid esters of *o*-amidofluorenols in the blood stream since albumin is the predominant blood protein. However, formation as well as disposition of NSF takes place primarily in the liver, and because of its instability NSF is unlikely to reach the circulatory system in major amounts. The following considerations, which are based on available data in human tissue, suggest that NSF may undergo albumin-catalyzed *o*-rearrangement in hepatic tissue. The adult human liver (approx. wt = 1600 g [26]; 1.4 mg albumin/g liver [27]) contains about 2240 mg of albumin on the basis of these data. The average content of water in the liver is about 71.1% of its weight [28–30]. Accordingly, the amount of water in the average liver is approximately 1138 ml (0.711 × 1600 *g*) and the average concentration of albumin in liver is equal to ~2 mg of albumin/ml. In the present study, about 60% of NSF underwent the albumin-catalyzed *o*-rearrangement at a comparable concentration (Fig. 3). Since the catalytic activity of HSA was 50% of that of BSA (Table 1), approximately 30% of NSF, if it were formed in human tissue, might be subject to *o*-rearrangement.

As shown in this study, the extent of the albumin-catalyzed *o*-rearrangement determines the rate of the concurrent solvolytic degradation of NSF to 4-OH-2-AAF. It has been reported that the metabolic disposition of NSF includes enzymatic reduction to 2-AAF [4] as well as interaction with cellular nucleophiles which results in the formation of stable adducts [2, 4]. Both steps have been linked to the carcinogenic action of NSF [2, 4]. One may conjecture that the albumin-catalyzed *o*-rearrangement of NSF enhances the detoxification of NSF by decreasing the enzymatic reduction of NSF and its interaction with cellular receptors. In support of this view are preliminary data from this laboratory which indicate that the formation of 2-AAF from *N*-OH-2-AAF by hepatic cytosol, fortified to generate NSF, is only a minor reaction in the presence of BSA, when the formation of *o*-SF [Σ (1-SF + 3-SF)] predominates.

Acknowledgements—The work reported in this investigation was supported by funds from the Veterans Administration. The authors thank I. Rutks and K. Arndt for technical assistance, and R. Kolanczyk and Dr. R. Derr for valuable suggestions in editing and revising the manuscript.

REFERENCES

1. Miller EC and Miller JA, The metabolic activation and reactivity of carcinogenic amines and amides. In: *Chemical and Viral Oncogenesis. Proceedings of the 11th Cancer Congress, Amsterdam* (Eds. Bucalossi P, Veronesi U and Casanelli N), Vol. 2, pp. 3–8. Excerpta Medica, Amsterdam, 1975.
2. Miller EC and Miller JA, Mechanisms of chemical carcinogenesis. *Cancer* **47**: 1055–1064, 1981.
3. Miller JA, Carcinogenesis by chemicals. An overview. *Cancer Res* **30**: 559–576, 1970.
4. Smith BA, Springfield JR and Gutmann HR, Solvolysis and metabolic degradation, by rat liver, of the ultimate carcinogen, *N*-sulfonoxy-2-acetylaminofluorene. *Mol Pharmacol* **31**: 438–445, 1987.
5. Rayshell M, Ross J and Werbin H, Evidence that *N*-acetoxy-*N*-acetyl-2-aminofluorene crosslinks DNA to protein by a free radical mechanism. *Carcinogenesis* **4**: 501–507, 1983.
6. Saint-Ruf G, Loukakou PE and Spodheim-Maurizot M, C-Alkylation of *N*-acetoxy-*N*-acetylaminofluorene: effects on its reaction with DNA *in vitro*. *Cancer Biochem Biophys* **7**: 89–99, 1984.
7. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
8. Smith BA, Gutmann HR and Springfield JR, Interaction of nucleophiles with the enzymatically-activated carcinogen, *N*-hydroxy-2-acetylaminofluorene, and with the model ester, *N*-acetoxy-2-acetylaminofluorene. *Carcinogenesis* **6**: 271–277, 1985.
9. Smith BA, Springfield JR and Gutmann HR, Interaction of the synthetic ultimate carcinogens, *N*-sulfonoxy- and *N*-acetoxy-2-acetylaminofluorene, with nucleophiles. *Carcinogenesis* **7**: 405–411, 1986.
10. Gutmann HR, Malejka-Giganti D and McIver R, Identification of carcinogenic acetates of fluorenylhydroxamic acids by high-pressure liquid chromatography. *J Chromatogr* **115**: 71–78, 1975.
11. Weisburger EK and Weisburger JH, *ortho*-Hydroxy derivatives of the carcinogen, 2-acetylaminofluorene. *J Org Chem* **19**: 964–972, 1954.
12. Foster JF, Some aspects of the structure and conformational properties of serum albumin. In: *Albumin Structure, Function and Uses* (Eds. Rosenor VM, Oratz M and Rothschild MA), pp. 53–84. Pergamon Press, Oxford, 1977.
13. Noel JFK and Hunter MJ, Bovine mercaptalbumin and non-mercaptalbumin monomers. Interconversions and

structural differences. *J Biol Chem* **247**: 7391–7406, 1972.
14. Darcel CLQ and Kaldy MS, Further evidence for the heterogeneity of serum albumin. *Comp Biochem Physiol* **85B**: 15–22, 1986.
15. Janatova J, Fuller JK and Hunter MJ, The heterogeneity of bovine albumin with respect to sulfhydryl and dimer content. *J Biol Chem* **243**: 3612–3622, 1968.
16. DeBaun JR, Miller EC and Miller JA, *N*-Hydroxy-2-acetylaminofluorene sulfotransferase: its probable role in carcinogenesis and protein-(methion-*S*-yl) binding in rat liver. *Cancer Res* **30**: 577–595, 1970.
17. Maher VM, Miller EC, Miller JA and Szybalski W, Mutations and decrease in density of transforming DNA produced by derivatives of the carcinogens, 2-acetylaminofluorene and *N*-methyl-4-aminoazobenzene. *Mol Pharmacol* **4**: 411–426, 1968.
18. van den Goorbergh JAM, Meerman JHN, de Wit H and Mulder GJ, Reaction of 2-acetylaminofluorene-*N*-sulfate with RNA and glutathione: Evidence for the generation of two reactive intermediates with different reactivities towards RNA and glutathione. *Carcinogensis* **6**: 1635–1640, 1985.
19. Casida JE and Augustinsson K, Reaction of plasma albumin with 1-naphthyl *N*-methylcarbamate and certain other esters. *Biochim Biophys Acta* **36**: 411–426, 1959.
20. Wilde CE and Kekwick RGO, The arylesterase of human serum. *Biochem J* **91**: 297–307, 1964.
21. Eto M, Oshima Y and Casida JE, Plasma albumin as a catalyst in cyclization of diaryl *o*-(α-hydroxyl)tolyl phosphates. *Biochem Pharmacol* **16**: 295–308, 1967.
22. Gassman PG and Granrud JE, Synthesis and rearrangement of methane sulfate esters of *N*-hydroxyacetanilides. A model for penultimate carcinogens. *J Am Chem Soc* **106**: 1498–1499, 1984.
23. Taylor RP, Enzyme-like activities associated with albumin. In: *Albumin Structure, Function and Uses* (Eds. Rosenor VM, Oratz M and Rothschild MA), p. 188, Pergamon Press, Oxford, 1977.
24. Kauzmann W, Some factors in the interpretation of protein denaturation. *Adv Protein Chem* **14**: 1–63, 1959.
25. Kuntz ID, Tertiary structure of carboxypeptidase. *J Am Chem Soc* **94**: 8568–8572, 1972.
26. Boyd E, Growth, including reproduction and morphological development. In: *Biological Handbooks*, pp. 346–348. Federation of American Societies for Experimental Biology, Washington, 1962.
27. Peters T Jr, Serum albumin. In: *The Plasma Proteins* (Ed. Putnam FW), Vol. 1, pp. 161–163. Academic Press, New York, 1975.
28. Tompsett SL, Copper and "inorganic" iron content of human tissues. *Biochem J* **29**: 480–486, 1935.
29. Bruckmann G and Zondek S, Iron, copper and manganese in human organs of various ages. *Biochem J* **33**: 1845–1857, 1939.
30. Widdowson EM, McCance RH and Spray CM, The chemical composition of the human body. *Clin Sci* **10**: 113–125, 1951.