Kinetic Analysis of Covalent Binding between N-Acetyl-L-Cysteine and Albumin through the Formation of Mixed Disulfides in Human and Rat Serum *in Vitro*

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Purpose. Covalent binding between N-acetyl-L-cysteine (NAC) and albumin was evaluated kinetically by conducting *in vitro* experiments.
Methods. After ^{14}C-NAC was incubated with human or rat serum, the solution was analyzed by anion-exchange HPLC. The albumin-bound ^{14}C-NAC was quantified by measuring the radioactivity in the albumin fraction.
Results. Ultraviolet chromatograms and/or radiochromatograms indicated the presence of a stable covalent bond between ^{14}C-NAC and either human or rat albumin. By analyzing the time dependence of this protein binding in serum, the first-order binding and dissociation rate constants (k_{on} and k_{off}) were obtained. The serum was treated in a CO_2 incubator to avoid oxidative interference, and the initial rates were determined separately. The k_{on} values obtained were 0.33 (h^{-1}) and 0.48 (h^{-1}) for human and rat serum, respectively. L-Cysteine was required to initiate the dissociation of ^{14}C-NAC bound to albumin. Following the addition of appropriate amounts of L-cysteine, the k_{off} values were determined to be 0.30–1.0 h^{-1} and 0.54–1.4 h^{-1} for human and rat serum, respectively.
Conclusions. The k_{on} and k_{off} values obtained for rat serum were in good agreement with the *in vivo* plasma protein binding kinetics of NAC in rats, indicating the reliability of this *in vitro* method for evaluating protein binding. No species differences in protein binding kinetics were found between human and rat serum.

KEY WORDS: N-acetyl-L-cysteine; serum albumin; covalent binding; mixed disulfide; kinetic analysis.

INTRODUCTION

In general, because drug–protein binding is an extremely rapid reaction (1), the binding process itself is usually neglected when the pharmacokinetics of a drug are considered. However, if the drug–protein binding process shows time dependence, it may affect the pharmacokinetics of the drug itself. Therefore, the kinetic evaluation of such drug–protein binding is an important issue in commercial drug development. In fact, in the case of the anticancer agent UCN-01 (7-hydroxystaurosporine), it has been shown that the time-dependent dissociation of UCN-01 from serum α_1-acid glycoprotein is a critical factor that drastically limits drug clearance in human subjects (2,3).

In an earlier study, we reported that the thiol-containing drug N-acetyl-L-cysteine (NAC) binds to plasma protein(s) in a time-dependent manner via a mixed disulfide covalent bond when NAC is administered intravenously to rats and that the elimination of NAC in plasma is rate-limited by the dissociation of the drug from plasma protein(s) (4). Although the contribution of covalent protein-binding kinetics to the pharmacokinetics of NAC was judged to be low because most of the NAC was metabolized to L-cysteine in the rat before covalent binding occurred, the kinetic analysis contributed to our overall understanding of the pharmacokinetics of NAC.

However, when the various thiol-containing drugs, almost all of which are known to bind to proteins covalently (5–9), are considered, it becomes clear that their covalent protein-binding kinetics have not been sufficiently elucidated, with the exception of a few basic *in vitro* studies (8,9). In addition, the relationships between the binding parameters obtained in such *in vitro* studies and the actual binding kinetics *in vivo* are poorly understood. If a reliable method for the evaluation of covalent protein-binding kinetics could be established, it would be expected to be very useful in clarifying the pharmacokinetics of thiol-containing drugs.

The present study was therefore conducted to address the issue of establishing a kinetic evaluation method for the covalent protein binding of NAC *in vitro,* and the *in vitro* kinetic parameters obtained were compared with *in vivo* values in order to verify the reliability of the method. In addition, both human and rat sera were used in order to identify species differences in the protein-binding kinetics of NAC.

Among the many proteins found in serum, albumin is the most abundant and contains a free thiol function that can bind thiol-reactive compounds or metal ions (10). This free thiol function of the albumin molecule is located at the 34th cysteine residue (Cys-34) of the amino acid sequence (11,12). In the present study, albumin was selected as the target protein for evaluation because it was thought likely that the covalent protein-binding kinetics in serum through mixed disulfide formation would be strongly influenced by the binding kinetics to albumin.

MATERIALS AND METHODS

Materials

N-Acetyl-L-cysteine was purchased from Sigma Chemical Co. (St. Louis, MO). N-Acetyl-L-[1-^{14}C] cysteine ([^{14}C]NAC) was synthesized at Amersham Pharmacia Biotech Ltd. (Buckinghamshire, UK) and was purified before use. Briefly, [^{14}C]NAC was treated with dithiothreitol to reduce the degradation product (disulfide form of [^{14}C]NAC) and then was injected into a HPLC system using a Crownpak® CR (+) column (Daicel Chemical Industries, Ltd., Osaka, Japan). [^{14}C]NAC was developed by perchloric acid solution, pH 2.0, and monitored by UV absorption at 210 nm. The recovered [^{14}C]NAC fraction was confirmed to be radiochemically pure and then was freeze-dried. L-Cysteine was purchased from Katayama Chemical Industries Co., Ltd. (Osaka, Japan). Human and rat serum albumin (fraction V

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ABBREVIATIONS: NAC, N-acetyl-L-cysteine; Cys-34, 34th cysteine residue of the amino acid sequence of albumin; 4VP-EG-Me column, N-methylpyridinium polymer-based column; MA, mercaptoalbumin; NA, nonmercaptoalbumin; SS, disulfide bond.

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powder, fatty acid-free) were purchased from Seikagaku Corporation (Tokyo, Japan) and Sigma Chemical Co., respectively. Human serum (all male donors) was purchased from Irvine Scientific Sales Co., Inc. (Santa Ana, CA). All other chemicals or solvents were of analytic or HPLC grade.

Animals were treated in a manner consistent with the *Principles of Laboratory Animal Care* (NIH publication #85-23, revised 1985). Rat blood was obtained from the postcaval vein of male Sprague-Dawley rats purchased from Charles River Japan (Kanagawa, Japan), and the serum (plasma) was obtained by centrifugation.

The *N*-methylpyridinium polymer was synthesized as previously described (13) and packed into a column at the Laboratory of Biofunctional Chemistry, Faculty of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan.

HPLC Analysis of Albumin

An *N*-methylpyridinium polymer-based column (4VP-EG-Me column, 4-mm I.D. × 250-mm length) was used in the anion-exchange HPLC analysis of albumin. The mobile phases used were A (50 mM Tris-acetate buffer, pH 7.0) and B (the same buffer containing 500 mM CH_3COONa), and these were delivered to the column at a rate of 0.4 ml/min. Linear gradient mode analysis was performed using a buffer composition of A/B = 100/0 at 0 min and A/B = 0/100 at 36 min. The analysis was performed isocratically using buffer ratios of A/B = 60/40 for human serum and A/B = 65/35 for rat serum. The column was operated at room temperature (approximately 23°C). The elution of proteins was monitored by measuring absorption at 280 nm.

Radioactivity Determination

The radioactivity in the column eluent was measured by either the fraction method or the flow scintillation method.

In the fraction method, the column eluent was collected in vials every 30 s, and a scintillation cocktail (ACS-II, Amersham) was added. The radioactivity was then determined using a liquid scintillation counter.

In the flow scintillation method, the column eluent was continuously delivered into the flow scintillation analyzer (500TR, Packard, Meriden, CT). The scintillation cocktail (Ultima-Flo™ AP, Packard) was mixed with the eluent at 1.2 ml/min in a 0.5-ml solid-type detection cell. The radioactivity was obtained as the peak area of the chromatogram.

Confirmation of Covalent Bond Formation between Albumin and NAC

First, 20 mg of human or rat albumin was dissolved in 1 ml of 1.5 mM [^{14}C]NAC solution (0.067 M phosphate buffer, pH 7.4, 57 kBq/μmol NAC). This solution was incubated at 37°C, and a 10-μl aliquot was used for 4VP-EG-Me column analysis (gradient mode) after filtration through a hydrophilic filter (0.45 μm) at various time points. The reaction medium was then repeatedly ultrafiltrated (M.W. cutoff = 30,000), and buffer was added to remove the protein-unbound and noncovalently protein-bound NAC. Finally, the protein solution retained on the filter membrane was subjected to 4VP-EG-Me analysis, and the radioactivity was determined.

Determination of the Time Course of the Covalent Binding of NAC to Albumin in Serum

Equilibrium Determination Method

Rat serum and either a 2 or 6 mM [^{14}C]NAC solution (approximately 280 kBq/μmol NAC) were mixed in a ratio of 95/5 (v/v) and incubated at 37°C for 24 h. Aliquots were diluted with 0.1 M phosphate buffer (pH 7.2) two times, and a 10-μl aliquot was then used in the 4VP-EG-Me analysis (gradient mode) at various time points. The amount of [^{14}C]NAC covalently bound to albumin was calculated from the radioactivity determined. The protein-unbound [^{14}C]NAC was simultaneously determined by postcolumn derivative HPLC analysis (14). Briefly, following the removal of the proteins from the serum by precipitation with perchloric acid and centrifugation, the supernatant was treated with dithiothreitol to convert the disulfide form of [^{14}C]NAC to the reduced form. The supernatant was then subjected to C_{18} column analysis, and the thiol function was detected quantitatively by mixing the column eluent with an iodoplatinate-containing solution. However, it should be noted that the NAC determined by the above method included noncovalently protein-bound [^{14}C]NAC because this compound readily dissociates from serum proteins during perchloric acid treatment. Therefore, the noncovalently protein-bound [^{14}C]NAC was included in the "protein-unbound NAC" in the present study. Serum L-cysteine concentrations were determined simultaneously.

Initial Rate Determination Method

The binding process was determined as follows. Following preincubation of human or rat serum in the CO_2 incubator (37°C, 95% CO_2/5% O_2) for more than 2 h, 1.9 ml of serum was mixed with 0.1 ml of 1 mM [^{14}C]NAC solution (approximately 560–570 kBq/μmol NAC). This solution was again incubated in the CO_2 incubator. Aliquots were sequentially diluted with 0.1 M phosphate buffer (pH 7.2) twice, and a 10-μl aliquot was then subjected to 4VP-EG-Me analysis (isocratic mode).

The dissociation process was determined as follows. A mixture of serum and [^{14}C]NAC was incubated in the CO_2 incubator for more than 12 h in order to form a sufficient quantity of bound materials. The mixture was then repeatedly ultrafiltrated (M.W. cutoff = 30,000), and buffer was added to remove the protein-unbound and the noncovalently protein-bound NAC. Finally, the protein solution was concentrated (to approximately one-fifth of the original volume) and recovered. The untreated serum was also ultracentrifugated, and the non-protein-containing filtrate was recovered. The reconstituted serum for k_{off} determination was then prepared by mixing the concentrated protein solution with the non-protein-containing filtrate. Although that mixing process generated a small artificial difference of albumin-bound [^{14}C]NAC concentrations between the reconstituted serum samples (see Fig. 6), we neglected such a small difference because it is not a critical factor in the kinetic analysis.

Then, 120 μl of the reconstituted serum was mixed with 5 μl of L-cysteine solution and incubated in the CO_2 incubator, and 10-μl aliquots were sequentially subjected to 4VP-EG-Me analysis (isocratic mode).

The amount of covalently albumin-bound [^{14}C]NAC was

calculated from the radioactivity as determined using the flow-scintillation method.

The procedure for the initial rate determination method is shown diagrammatically in Fig. 1.

Calculation of k_{on} and k_{off} Values

The apparent first-order binding and dissociation rate constants (k_{on} and k_{off}) for [^{14}C]NAC with albumin were calculated as follows.

In the equilibrium determination method, the differential equations shown below were input into the computer program, and nonlinear least-squares fitting analysis was performed using the serum concentrations of the protein-unbound and albumin-bound [^{14}C]NAC.

$$d[NAC_{unbound}]/dt = -k_{on}[NAC_{unbound}] + k_{off}[NAC_{alb-bound}]$$

$$d[NAC_{alb-bound}]/dt = k_{on}[NAC_{unbound}] - k_{off}[NAC_{alb-bound}]$$

In the initial rate determination method, the common logarithm of the concentration of albumin-bound [^{14}C]NAC in serum was plotted against the incubation time. The ascending (binding) and descending (dissociation) correlation lines were then obtained by the least-squares method. The k_{on} and k_{off} values were calculated as 2.303 × (absolute value of the slope of the correlation line).

RESULTS AND DISCUSSION

The 4VP-EG-Me column, which is a strong anion-exchange polymer-based column developed by Sugii et al., is capable of separating human albumin into mercaptoalbumin (MA) and nonmercaptoalbumin (NA) fractions with relatively high resolution (15,16). The difference between MA and NA represents the structural alteration of the thiol function of Cys-34; i.e., MA contains an intact thiol group, and NA contains the oxidized form of the thiol, which is further divided into the mixed disulfide form with intrinsic low-molecular-weight thiol compounds (NA_{SS}) and the oxidized form such as sulfinic acid (NA_{OXY}) (8). It is possible that a slight difference in electronic charge distribution derived from a three-dimensional structural alteration may exist between MA and NA, and this difference might be detected by 4VP-EG-Me polymer (16,17). In addition, Narazaki et al. showed that the thiol-containing drug-bound form of NA ($NA_{SS,\,DRUG}$) can be separated from the abovementioned intrinsic albumin subtypes (8).

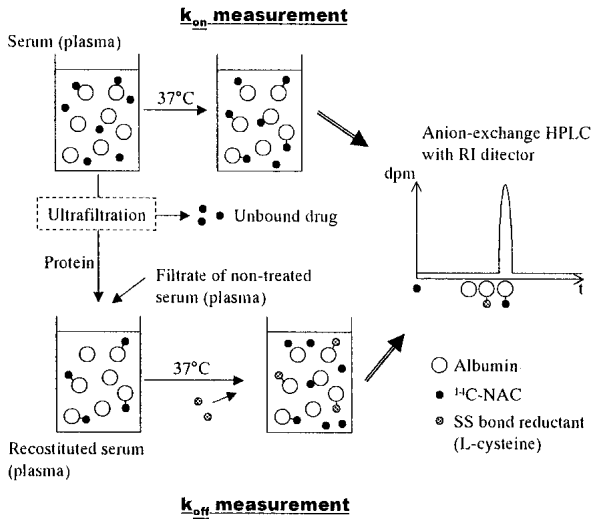

Fig. 1. Diagram showing the initial rate determination method for the time-dependent protein binding and dissociation of NAC and albumin in serum.

In the present study, the UV chromatogram of human albumin treated with [^{14}C]NAC for 24 h at 37°C in buffer showed the formation of a new peak on the back of the peak corresponding to NA_{OXY}, and the peak corresponding to the cysteine-bound form of NA ($NA_{SS,\,CYS}$) was absent (Fig. 2). Such changes in the chromatogram were also observed in a previous study by Narazaki et al. in which albumin was treated with the thiol-containing ACE inhibitor captopril (8). This indicates that [^{14}C]NAC substituted the L-cysteine derived from the $NA_{SS,\,CYS}$ included in the purified albumin reagent and that the NAC-bound form of NA ($NA_{SS,\,NAC}$) was newly formed. In addition, the radioactivity determination of the medium, from which the protein-unbound and noncovalently-bound [^{14}C]NAC were removed, showed that the radioactivity peak corresponded to the newly formed peak on the UV chromatogram (Fig. 2). This also indicates the formation of a stable complex of human albumin and [^{14}C]NAC. Although some extent of radioactivity was also detected in the tail fraction (Fig. 2), it has been reported that such fraction corresponds to the polymerized albumin (15). As a result, a slight amount of [^{14}C]NAC might be bound to the polymerized albumin.

On the other hand, it has already been shown that the 4VP-EG-Me column is not able to separate rat albumin into its subtypes (15). This was confirmed in the present study, and

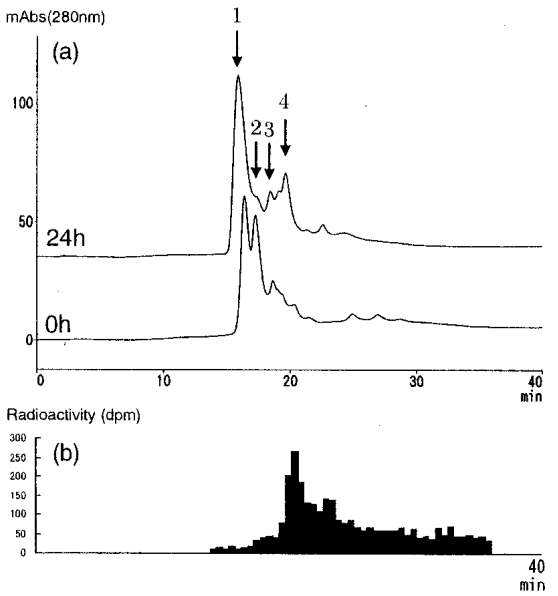

Fig. 2. UV chromatogram **(a)** and radiochromatogram **(b)** of human albumin treated with [^{14}C]NAC. Approximately 300 μM albumin and 1.5 mM [^{14}C]NAC containing buffer were incubated at 37°C. **Arrows 1–3** indicate the intrinsic albumin subtypes: **1,** mercaptoalbumin (MA); **2,** the cysteine-bound form of nonmercaptoalbumin ($NA_{SS,\,CYS}$); and **3,** the oxidized form of nonmercaptoalbumin (NA_{OXY}). **Arrow 4** indicates the NAC-bound form of nonmercaptoalbumin ($NA_{SS,\,NAC}$). The radiochromatogram was obtained after removal of the protein-unbound and noncovalently protein-bound [^{14}C]NAC from the medium by ultracentrifugation.

the formation of rat $NA_{SS,\,NAC}$ could not be confirmed from the UV chromatogram (Fig. 3). However, the radiochromatogram showed that [^{14}C]NAC bound to rat albumin in a stable manner, suggesting that $NA_{SS,\,NAC}$ was also formed in the case of rat albumin.

The UV chromatogram of serum was almost identical to that of purified albumin despite the fact that serum contains a variety of proteins (data not shown). Figure 4 shows the time course of the concentrations of the protein-unbound [^{14}C]NAC (which actually contains the noncovalently protein-bound form) and the albumin-bound [^{14}C]NAC in rat serum incubated with 100 or 300 μM [^{14}C]NAC under atmospheric conditions. The increase in the albumin-bound [^{14}C]NAC was clearly proportional to the decrease in the protein-unbound [^{14}C]NAC, and the sum of these concentrations remained constant at 80% to 90% of the original amount of [^{14}C]NAC added. This indicates that nearly all of the stable (covalent) protein binding of [^{14}C]NAC observed in rat serum can be accounted for by binding to albumin. The apparent first-order binding and dissociation rate constants (k_{on} and k_{off}) obtained at the equilibrium shown in Fig. 4 were determined to be 0.11–0.13 h^{-1} and 0.046–0.12 h^{-1}, respectively (Table I).

On the other hand, in our previous study performed in the rat *in vivo,* the administered NAC bound to plasma protein(s) at rates of $k_{on} = 0.23\ h^{-1}$ and $k_{off} = 0.57\ h^{-1}$ (4). Thus, the *in vitro* parameters obtained by the equilibrium method suggest that the dissociation of [^{14}C]NAC from albumin is markedly suppressed compared with the situation *in vivo.* The concentration of albumin-bound [^{14}C]NAC in serum continued to increase for a period of up to 12 h (Fig. 4), indicating that the disulfide bonds were being formed continuously. This suggests that the serum was converted to an oxidative environment under long-term incubation and that the thiol/disulfide equilibrium leaned toward disulfide formation. The thiol/disulfide equilibrium is known to be affected by various environmental factors such as pH (8,9) and the presence of dissolved oxygen (18), fatty acids (19), metal ions (20), and other thiol-containing compounds (21). The pH is a principal factor affecting both the rate and extent of disulfide formation: the formation is facilitated when the pH is high. In the serum incubated under atmospheric conditions, the pH was much higher than that of fresh serum (data not shown). In addition, it is considered that the *in vitro* experimental system does not include disulfide reduction systems, such as cystine reductase (EC 1.6.4.1). Thus, it is likely that these factors excessively facilitate the disulfide formation in the incubated serum.

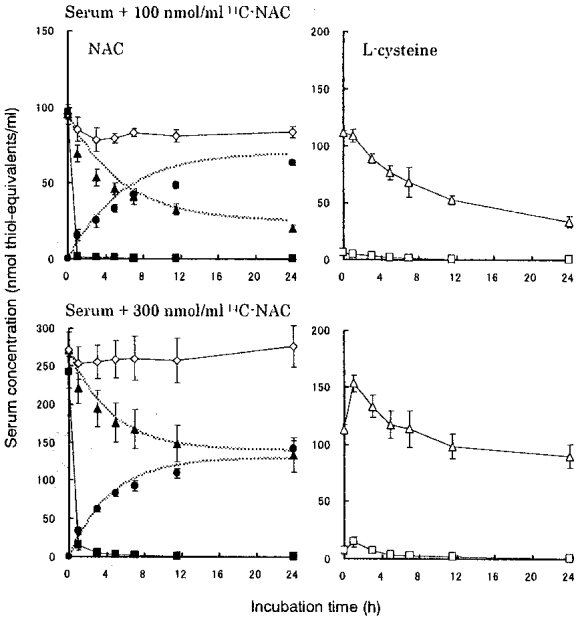

Fig. 4. Concentrations of various forms of NAC **(left panels)** and L-cysteine **(right panels)** in rat serum treated with 100 or 300 μM [^{14}C]NAC under atmospheric conditions. The forms of NAC and L-cysteine are indicated by the following symbols: **square,** reduced form; **triangle,** protein-unbound form (reduced form + low-molecular-weight disulfide form); **circle,** albumin-bound form; and **diamond,** sum of albumin-bound and protein-unbound forms (calculated value). **Thick gray lines** indicate the fitting lines obtained by nonlinear least-squares analysis. Mean ± SD, n = 3.

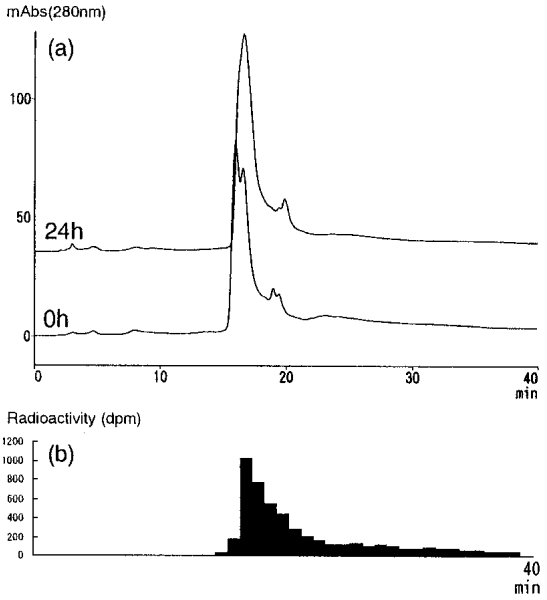

Fig. 3. UV chromatogram **(a)** and radiochromatogram **(b)** of rat albumin treated with [^{14}C]NAC. The analytic conditions are the same as those indicated in Fig. 2.

For the above reasons, the serum samples were incubated in a CO_2 incubator, and the initial binding and dissociation rates were determined separately to minimize the influence of oxidative alteration of the serum. In the CO_2 incubator, the gas composition and pH of the serum remained similar to those of fresh serum. Figure 5 shows the increase in the albumin-bound [^{14}C]NAC concentrations in human and rat serum in the presence of 50 μM [^{14}C]NAC. Although the increased rate of albumin-bound [^{14}C]NAC decreased slightly with the progress of incubation, an almost linear correlation was observed between the logarithm of concentration and the incubation time. The k_{on} values obtained were 0.33 ± 0.06 h^{-1} for human serum and 0.48 ± 0.07 h^{-1} for rat serum (Table I).

Dissociation of [^{14}C]NAC from albumin in the reconstituted serum was not observed with incubation alone (Fig. 6). It is likely that the dissociation proceeds through a thiol–disulfide exchange reaction (21). In serum, L-cysteine is the most abundant thiol-containing compound with a total concentration of approximately 200 μM, which is at least 10 times higher than other thiol compounds such as glutathione, ho-

Table I. Kinetic Parameters of the Protein-Binding of NAC to Albumin in Serum

Serum	Determination method	NAC (nmol/ml)	Cys (nmol/ml)	Protein binding rate constant (h^{-1})	
				k_{on}	k_{off}
Rat	Equilibrium	100	—	0.13 ± 0.02	0.046 ± 0.006
		300	—	0.11 ± 0.01	0.12 ± 0.00
Rat	Initial rate	50	—	0.48 ± 0.07	—
		13[a]	30	—	0.17[b]
		13[a]	100	—	0.54[b]
		13[a]	200	—	1.1[b]
		13[a]	300	—	1.4[b]
Human	Initial rate	50	—	0.33 ± 0.06	—
		6-22[a]	30	—	0.00 ± 0.03
		6-22[a]	100	—	0.30 ± 0.19
		6-22[a]	200	—	0.71 ± 0.15
		6-22[a]	300	—	1.0 ± 0.2

[a] Determined values of the albumin-bound [^{14}C]NAC in the reconstituted sera. Other values indicate added concentrations of [^{14}C]NAC and Cys to sera.
[b] Average values for triplicate determinations. Other values indicate mean ± SD (n = 3).

mocysteine, and cysteinylgricine (14,22–24). The data shown in Fig. 4 indicate that L-cysteine was almost completely oxidized to the disulfide form in serum *in vitro;* i.e., it was estimated that the thiol functions, which could substitute for the albumin-bound [^{14}C]NAC, were practically nonexistent in the reconstituted serum during the k_{off} determination. In comparison with this *in vitro* situation, the concentration of the reduced form of L-cysteine in serum *in vivo* is maintained at approximately 10–20 μM (14,25). This difference in the thiol function status is probably related to functional differences in the disulfide reduction system, as mentioned above, between *in vitro* and *in vivo* conditions.

Thus, we determined the dissociation of [^{14}C]NAC from albumin by adding L-cysteine to the reconstituted serum to initiate a thiol–disulfide exchange reaction. In both human and rat reconstituted sera, the dissociation of [^{14}C]NAC was observed continuously during the 36-min incubation period after the addition of 100–300 μM L-cysteine (Fig. 6). The k_{off} values obtained in the above range of L-cysteine concentrations were 0.3–1.0 h^{-1} for human serum and 0.54–1.4 h^{-1} for rat serum (Table I). In the case of 100 μM L-cysteine added to rat reconstituted serum, the k_{off} (0.54 h^{-1}) is almost the same as that obtained in our previous *in vivo* study (0.57 h^{-1}). Although we have not determined the reduced form of L-cysteine in the reconstituted serum, it was estimated to be about 12 μM when 100 μM L-cysteine was initially added because L-cysteine partially converted to its disulfide form. This condition may mimic to the *in vivo* condition. The k_{off} value and its response to the added concentration of L-cysteine (dose-response) were almost same between rat and human sera. This suggests that the actual dissociation rates of NAC from albumin in the serum *in vivo* are almost same in rat and human because their serum cysteine concentrations are adequately approximated.

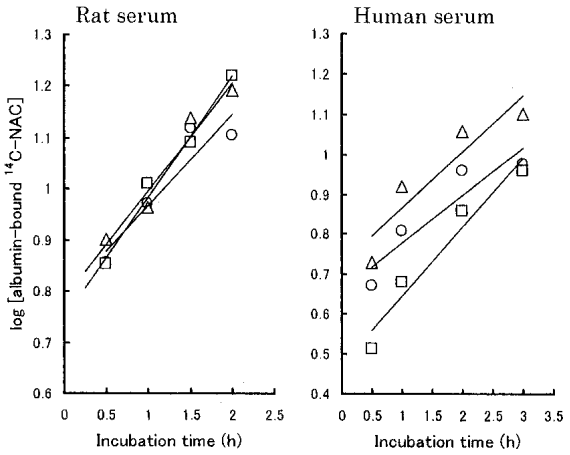

Fig. 5. Initial increase in albumin-bound [^{14}C]NAC concentrations in rat and human serum treated with 50 μM [^{14}C]NAC in a CO_2 incubator. Each symbol indicates a consecutively determined value in an individual sample (n = 3). Each line indicates the correlation obtained by the least-squares method.

Thus, no major differences in protein-binding kinetics were observed between human and rat serum in the present study. On the other hand, the pharmacokinetic parameters of NAC differ between humans and rats *in vivo;* i.e., the elimination half-life of total plasma NAC is approximately 2 h in rats (4), whereas it is approximately 6 h in humans (26). In the case of rats, it has been shown that the total plasma drug elimination is rate-limited by the dissociation of NAC from plasma protein(s) (4). In contrast, the results of the present study suggest that the protein (albumin) dissociation of NAC is not a rate-limiting factor in the relatively slow elimination of NAC from human plasma. On the other hand, some rare allergic side effects such as urticaria and dermatitis have been reported in patients receiving NAC therapy (27), but no remarkable toxic effects were observed in preclinical toxicity studies in rats (in-house data). Covalent drug–protein binding is thought to induce these allergic side effects through hapten formation (28). Although the allergic side effects of NAC in humans may be caused by covalent protein binding, the species differences in toxic effects cannot be fully accounted for by the albumin-binding kinetics of NAC observed in the present study. The issue of whether the pharmacologic effects of NAC, such as its mucolytic and antioxidative effects, depend on NAC itself or on its metabolites, such as L-cysteine or

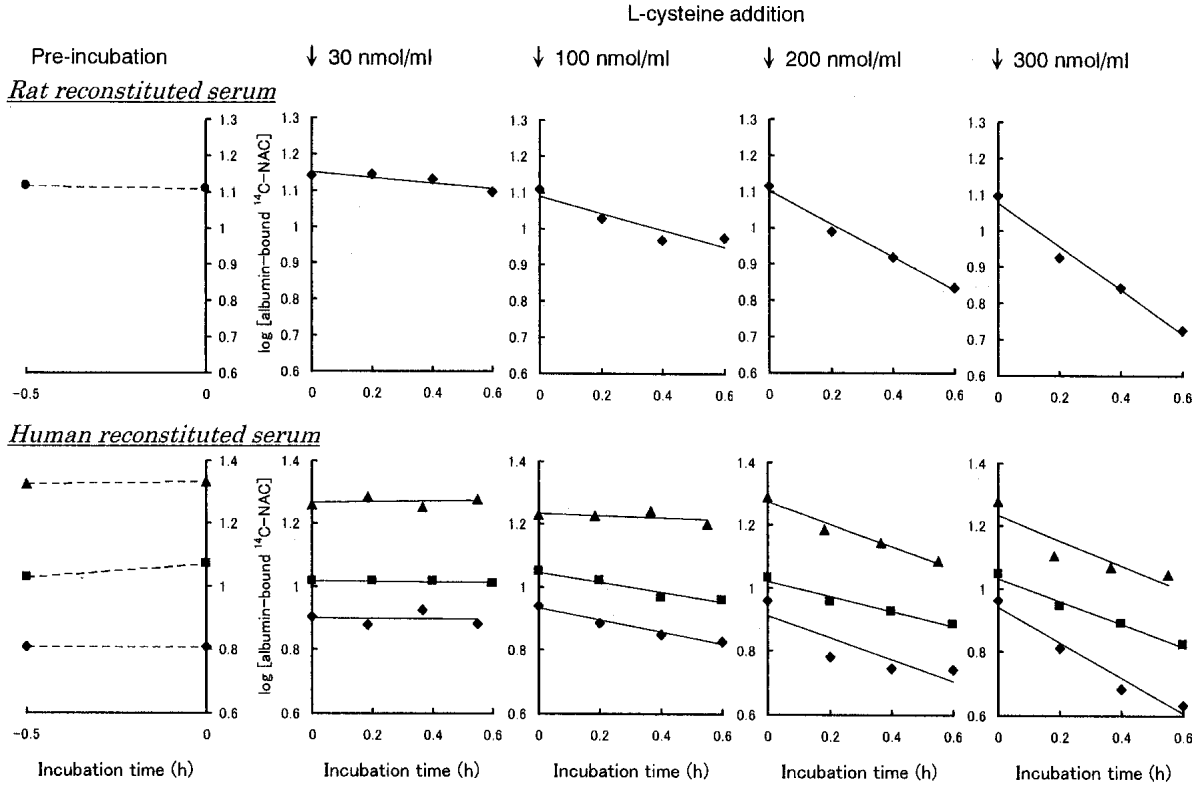

Fig. 6. Albumin-bound [^{14}C]NAC concentrations in rat and human reconstituted serum before and after the addition of L-cysteine. For rat serum, determinations were performed in triplicate, and mean values are indicated. For human serum, each symbol indicates a consecutively determined value in an individual sample. Each **solid line** indicates the correlation obtained by the least-squares method.

glutathione, has not been elucidated. Therefore, unfortunately, the relationships between the protein-binding kinetics and the pharmacologic effects of NAC remain unclear. However, time-dependent protein binding can also be interpreted to mean "drug storage associated with a time difference," and it must therefore be involved in drug efficacy to some extent.

It is possible that the protein-binding evaluation method described here may also be applicable to the analysis of other thiol-containing drugs or compounds, taking into consideration the universality of the disulfide bond. It is expected that the findings of the present study will prove valuable in future investigations concerning time dependence as it relates to drug–protein binding.

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