The procedure generally employed to affect such functionalizations[3–10] suffer from the drawback that they employ strong bases to generate anions prior to reaction with electrophiles. The presence of esters, ketones, aldehydes, etc., in the same molecules would result in preferential attack at the carbon atoms α to these more electrophilic carbonyl groups. BAN et al.[11,12] have developed an alternative procedure involving the use of MEERWEIN reagent[13–15] to generate the enaminoether of the amide which can then be alkylated. This reagent, however, alkylates alcohols, phenols, carboxylic acids, amines[13,14]. More recently, observation of intermolecular condensation reactions of Vilsmeier complexes by attack of the enamine on the immonium intermediates have been made, but attempts to use these as synthetically useful reactions for functionalization of amides have met with very limited success[16].

We now report substitution reactions in high yield at carbon atoms α to amide and imide carbonyl groups. The method employed involves the prior formation of the Vilsmeier intermediate of the amide (or imide) with phosphorus pentachloride in an aprotic solvent. This complex is then allowed to react with a suitable electrophile for a few hours to afford the desired functionalized amide on aqueous work-up.

Thus *N*-acetyl piperidine (5) was treated with phosphorus pentachloride in warm benzene for 10 min to afford the corresponding chloroenamine (6). To this was then added an equivalent amount of bromine in dioxane and the mixture was stirred at 30° for 4 h. On aqueous work-up, the product (7) was isolated as a white crystalline material in 80% yield, m.p. 38–40 °C.

Similarly, when the Vilsmeier intermediate (6) was allowed to react with an equivalent amount of benzaldehyde or *p*-nitrobenzaldehyde for 4 h in refluxing benzene, (8) and (9) were obtained in 80 and 90% yield respectively.

Treatment of the Vilsmeier complex (6) with diethyloxalate in refluxing benzene for $1^1/_2$ h afforded the corresponding acetylated acid (10) in 45% yield on aqueous work-up.

When alkylation was attempted with epichlorohydrin, the Vilsmeier complex (6) afforded only the intermolecular acylation product (11) in 60% yields.

In order to examine the possibility of similar activation of imide carbonyls, the imide (12) was treated with excess phosphorus pentachloride in benzene for 4 h when complete conversion to a new faster moving substance was detected by TLC. Aqueous work-up, extraction with ethyl acetate and concentration afforded colourless crystals, m.p. 169–71°. The IR-spectrum showed peaks at 1710 cm^{-1} (S) and 1790 cm^{-1} (W). The UV-spectrum afforded maxima at 224, 274 and 291 nm in expectation with the normal indolic chromophore. The mass spectrum afforded a prominent molecular ion at 276 with the isotope peak of chlorine at 278. Other major peaks appeared at 115, 116, 130, 114, 164, 177, 241 and 258 nm. Structure (14) was therefore assigned to the product.

In view of these reactions, it appears that this procedure for activating amides and imides may be significantly superior to the methods previously described, both in its scope as well as selective applicability. Extension of this method to other reactions involving both attack of various nucleophiles at the activated carbonyl groups and attack of other electrophiles at α-carbon atoms is currently being investigated.

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Ambivalent Effect of Protein Binding on Computed Distributions of Metal Ions Complexed by Ligands in Blood Plasma

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Summary. Although the absolute concentrations of metal complexes in blood plasma are controlled by protein binding, the percentage distribution of transition metal ions amongst low molecular weight ligands is not. Thus, computer simulations which omit protein equilibria can nevertheless afford reliable information about such metals in the biofluid.

Although the vast majority of transition metal ions in blood plasma are protein bound, low molecular weight complexes play an essential physiological role[2–4]. In particular, they may alter the bioavailability of the metal by mediating in its exchange between macromolecules[5,6] and also by facilitating membrane penetration[7–10]. Moreover, the free aquo-ions of metals such as Fe(III) and probably Cu(II) exist in plasma at such low concentrations that it is extremely unlikely that they, rather than their low molecular weight complexes, participate in bioinorganic reactions. The nature and relative concentrations of these complexes are thus of considerable interest.

Current analytical methods are incapable of measuring individual concentrations of low molecular weight complexes in plasma. This is due to the multicomponent nature of the system as well as to their extremely low concentrations. Further, the complexing reactions are

labile and close to equilibrium so they are susceptible to disturbances by chemical probes. For these reasons, computer programs are used to calculate the desired distribution from a knowledge of the total concentration of the components, the pH of the solution and the appropriate equilibrium constants. This kind of simulation was pioneered by SILLÉN who demonstrated how valuable it could be in his study of seawater[11]. PERRIN introduced the concept to blood plasma[12,13]. Subsequently, other models of plasma which attempted to accommodate the most important metal protein interactions also appeared[14–16].

Opponents of such computer simulations argue that current models are too simplistic to represent the biological system satisfactorily. Whilst efforts to approximate the protein binding relationships are a step in the right direction, their effectiveness remains controversial. Even protagonists of computer approaches have to concede that at best it is possible to include only a very limited selection from all of the metal protein equilibria which ought to be considered. Thus, it is a widely accepted conclusion that the details of the metal ion distribution amongst low molecular weight ligands remain uncertain.

The purpose of this communication is to report that in the low molecular weight fraction of plasma the percentage of a transition metal attached to specific ligands can be calculated by computer simulation without a knowledge of metal protein binding constants. This percentage is almost totally independent of the extent to which proteins bind the transition metal in question. On the other hand, the absolute concentrations of the free metal ions and therefore of all their complex species are controlled by protein binding equilibria.

These conclusions arise as follows. Consider the formation of low molecular weight complexes by transition metals in plasma. Owing to very low free metal ion concentrations, the amount of complex produced is always negligible in comparison with the amount of ligand present. Complexing reactions thus have no significant effect on the mass balance relationships for each ligand. Consequently, the free ligand concentrations are also not significantly affected, i.e. they are 'concentration buffered'. Under these circumstances, the concentration of each complex species is solely dependent on the concentration of the free metal ion. The one is directly proportional to the other. As this is true for all mononuclear species, the total concentration of each metal in the low molecular weight fraction is also directly proportional to the free metal ion concentration. Thus, the percentage of metal appearing in a given species is constant, regardless of the exact free metal ion concentration that exists in equilibrium with proteins.

Table 1. The percentage distribution of the metal ions Cu(II), Fe(III), Mn(II) and Zn(II) amongst predominant low molecular weight ligands in human blood plasma as found by computer simulation

Complex	Percentage of the total metal in the low molecular weight fraction
Cu histidinate cytinate$^-$	21
Cu histidinate cystinate H	17
Cu histidinate$_2$	11
Cu histidinate threoninate	8
Cu histidinate valinate	5
Cu histidinate lysinate H^+	5
Cu histidinate alanate	4
Cu histidinate serinate	4
Cu histidinate phenylalanate	3
Cu histidinate glycinate	3
Fe citrate OH^-	99
Mn carbonate H^+	22
Mn citrate$^-$	10
Mn carbonate	2
Zn citrate cysteinate^{3-}	43
Zn cysteinate$_2^{2-}$	19
Zn cysteinate histidinate^{--}	12

Table 2. Concentration limits applied to the model of the metal ion distribution in plasma

'Aquated' metal ion	Estimated free conc.	Free conc. limits	Estimated total low molecular weight metal complex concentration
Ca^{2+}	1.13 mM	(experimentally measured value from the literature)	300 μM
Cu^{2+}	$10^{-18} M$	$10^{-19} M$–$10^{-12} M$	$10^{-12} M$–$10^{-9} M$
Fe^{3+}	$10^{-23} M$	$10^{-24} M$–$10^{-18} M$	–
Pb^{2+}	$10^{-14} M$	$10^{-16} M$–$10^{-10} M$	–
Mg^{2+}	520 μM	510 μM–550 μM	120 μM
Mn^{2+}	$10^{-12} M$	$10^{-15} M$–$10^{-8} M$	–
Zn^{2+}	$10^{-9} M$	$10^{-12} M$–$10^{-8} M$	$10^{-7} M$–$10^{-6} M$

Rough estimates of the free metal ion concentrations were obtained from such metal protein binding constants as had been reported in the literature. The values employed by the model were varied substantially around these estimates, subject to constraints imposed by solubility and total low molecular weight complex concentrations (these were also estimated from reports in the literature).

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The most important low molecular weight complexes formed by Cu(II), Fe(III), Mn(II) and Zn(II) in plasma, as found by computer simulation are listed in Table 1. The computer model that calculated these percentages included almost 5000 species that could be produced in the presence of seven metal ions and 40 ligands at $-\log [H^+] = 7.4$. Although the details of this work are to be published elsewhere (manuscript in preparation[17]), the present purpose is served by noting that the percentage distribution recorded is independent of the free metal ion concentrations chosen for the model. This is valid to a precision of one percent when computed for concentrations within the ranges shown in Table 2.

The similarity between the metal ion distributions published here and those reported previously[13–15] deserves some comment. Although the agreement is certainly not perfect, it is surprisingly good in view of the disparities that exist with regard to the number of kinds of metal ion and the number of complexes the models contain. This is also a consequence of the fact that the percentages depend so strongly on the total concentration and on the protonation constants of the ligands in the system. The formation of complexes by one transition metal has essentially no influence on the percentage distributions of other metal ions. Thus, it is possible to obtain a reliable distribution for one transition metal without including all the others in the model.

Of course, models are only as good as the parameters upon which they depend. As many of the formation constants, especially for the ternary complexes, remain to be determined, this type of investigation is subject to continuous improvement. However, it is true that with only a few exceptions, the formation constants of the species appearing in Table 1 have been measured. Moreover, the vitally important ligand pK_as have all been determined, mostly under physiological conditions of temperature and ionic strength. Hence, whilst the actual percentages recorded may be changed in the future and whilst it is possible that other complexes may turn out to be significant, in our opinion, the broad picture will remain. In particular, this applies to the ratio between the concentrations of complexes whose formation constants have been adequately established. Of even greater importance, the idea that information about transition metal ion complexes in plasma can be obtained from simulations which omit protein equilibria provides encouragement both to measure the formation constants still outstanding and to expand existing models of blood and other biofluids.

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Subcellular Localization of *O*-Acetylserine Sulfhydrylase in Spinach Leaves[1]

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Summary. A combination of differential centrifugation and isopycnic sucrose density gradient centrifugation of extracts from spinach leaves (*Spinacia oleracea* L.) shows that about 20% of the O-Acetylserine sulfhydrylase are associated with chloroplasts. No appreciable amounts of O-Acetylserine sulfhydrylase band with mitochondrial and peroxisomal marker enzymes.

From the quantitative standpoint, the most important function of sulfate reduction is to produce cysteine. There is evidence from different organisms that L-cysteine is produced by sulfhydrylation of *O*-acetyl-L-serine (OAS) with sulfide: *O*-acetyl-L-serine + sulfide → L-cysteine + acetate. The reaction is catalyzed by *O*-acetylserine sulfhydrylase, abbr. OAS-S (*O*-acetyl-L-serine acetate lyase [adding hydrogensulfide], EC. 4.2.99.8). The enzyme has been reported in bacteria[2], fungi[3,4] and several higher plants[4–7].

Results of different authors[7–9] indicate that in higher plants the enzyme is localized in the cytoplasm. This is in contrast to reports that isolated chloroplasts can form cysteine[10–12,15]. It seemed desirable, therefore, to clarify whether the enzyme might be associated, at least in parts, with organelles such as chloroplasts, mitochondria and peroxisomes.

The extraction and isolation of organelles from spinach leaves (*Spinacia oleracea* L., var. Nobel) was achieved according to ROCHA and TING[13] by means of a sucrose based extraction medium and a combination of differential centrifugation and isopycnic sucrose density gradient centrifugation. The method consists of 3 differential centrifugation steps: 250 g for 90 sec (250 g crude ex-

Table 1. Percent organelle cross contamination of spinach leaf organelles in a 40–75% (w/v) linear sucrose density gradient

Organelle fraction	Isopycnic density (g/cm³)	Contaminating organelle			
		Peroxisomes %	Mitochondria %	Intact chloroplasts %	Broken chloroplasts %
Peroxisomes	1.255–1.26	–	1.5	< 1	< 1
Mitochondria	1.20	3	–	1.5	2.5
Intact chloroplasts	1.226	2.5	5	–	
Broken chloroplasts	1.178	2	19.5	< 1	–