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285. Human Milk Lysozyme: Unpublished Data Concerning the Establishment of the Complete Primary Structure; Comparison with Lysozymes of Various Origins[1])

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(16. IX. 71)

Summary. Details concerning the establishment of the complete primary structure of human milk lysozyme (previously published in a preliminary note) are presented. The chymotryptic peptides obtained from the reduced alkylated enzyme were purified and their amino acid sequences determined chiefly by the '*Edman*-dansylation' procedure, and in two cases by partial acid or peptic hydrolyses. The tryptic peptides are alined into a single chain containing 129 amino acid residues, on the basis of overlapping peptides. Two labile glutamine residues easily converted into glutamic acid residues were characterized. Human milk lysozyme is compared with other human lysozymes (from normal and leuchaemic individuals) prepared by our group. The structure proposed is identical with the sequence of human leuchaemia lysozyme (from the urine of a patient with chronic monocytic leuchaemia) reported by *Canfield*. Human milk lysozyme is also near by related to several bird egg-white lysozymes (and bovine α-lactalbumin): identical positions of Cys and Trp residues and of the residues essential for the catalytic activity or involved in some hydrogen bonds; several identical regions, especially in the β-sheet region; between 71 and 77 identical amino acid residues. It is suggested that by an insertion and a deletion in the sequence of human milk lysozyme, sequences homologous to those of bird lysozymes can be obtained.

1. Introduction. – The establishment of the primary sequences of the N-terminal moiety (72 amino acid residues) and of the C-terminal end (23 amino acid residues) of human milk lysozyme (EC 3.2.1.17) (129 residues) was reported in detail in 1969 by *Jollès & Jollès* [2], who proposed simultaneously a tentative structure of the enzyme. The complete sequence was shortly published by the same authors in 1971 in a general review devoted to the evolution of proteins [2a]. In the present paper, evidence will be provided (a) for the sequence of the unique tryptic peptide which was not yet described in detail (residues No. 70–97 of the enzyme [2]); (b) for the alignment of all the tryptic peptides into a single chain, from chymotryptic overlapping peptides. Human milk lysozyme is compared with other human lysozymes as well as with lysozymes from various origins.

2. Materials and methods. – 2.1. Human milk lysozyme was prepared according to *Jollès & Jollès* [3] from pooled milk by ion-exchange chromatography on Amberlite CG-50.

2.2. Reduction, alkylation, tryptic hydrolysis, and separation of the tryptic peptides: for these procedures see [2].

[1]) 80th communication on lysozymes; 79th communication, [1].

2.3. Chymotryptic hydrolysis and purification of the chymotryptic peptides. The peptides obtained after chymotryptic hydrolysis (2%, *w/w* of enzyme; pH 7.8; 37°; 24 h) were separated on a Sephadex G-25 column (200 × 2 cm) with 30% acetic acid as eluent. The 7 fractions collected were submitted to preparative paper electrophoreses (*Whatman* No 1) at pH 6.5 (pyridine:H_2O: acetic acid, 100:900:4, *v/v*) and at 50 volts per cm. When necessary, the peptides were further purified by paper chromatography; development with solvent A (*n*-butanol:pyridine:acetic acid:H_2O, 15:10:3:12, *v/v*) and elution with 30% acetic acid.

2.4. Determination of the primary structure of the peptides. The methods employed have been discussed in detail by *Jollès & Jollès* [2] [4].

3. Results. – 3.1. *Detailed study of the tryptic peptide No. 12* (the number of the peptide corresponds to the number of the peak characterized during the chromatography of the tryptic hydrolysate on Dowex 1 × 2 [2]): *residues No. 70–97 of the enzyme*. The structure of this long peptide was established by sequencing, chiefly by the '*Edman*-dansyl' technique, the fragments obtained by chymotryptic digestion (details and alignment of the fragments see in Table 1).

Table 1. *Establishment of the structure of tryptic peptide 12* [2]: *residues 70–97 of human milk lysozyme*

Amino acid composition: Asp_4, Thr_1, Ser_2, Glu_1, Pro_1, Gly_1, Ala_7, Val_2, $SCMCys_3$, Ile_1, Leu_3, His_1, Lys_1

Method	Isolated substances
Edman-dansyl	Thr-Pro-Gly-Ala-Val
Chymotryptic digestion (24 h)	1. (Thr, Pro, Gly, Ala, Val)-Asn
	2. (Thr, Pro, Gly, Ala, Val, Asn, Ala, SCMCys, His)-Leu
	3. [a] Ala–SCMCys–His–Leu
	4. [a] Ser-SCMCys-Ser-Ala—Leu (54%) (70%)
	5. Ser-(SCMCys, Ser, Ala, Leu, Leu)
	6. [a] Leu-Gln-Asp-Asn-Ile-Ala-Asp-Ala-Val—Ala [b] (10%) (40%)
	7. (SCMCys, Ala)-Lys
Carboxypeptidases A + B (24 h)	Ala-Lys

Structure: Thr-Pro-Gly-Ala-Val-Asn-Ala-SCMCys-His-Leu-Ser-SCMCys-Ser-Ala-Leu-Leu-Gln-Asp-Asn-Ile-Ala-Asn-Ala-Val-Ala-SCMCys-Ala-Lys

—> Residue determined by the '*Edman*-dansyl' technique.

—> Residue determined by the '*Edman*-dansyl' technique and as PTH-derivative.

<— Residue characterized after digestion with carboxypeptidase A (24 h).

[a] The chymotryptic peptides 3, 4 and 6, obtained from tryptic peptide 12, correspond to the chymotryptic peptides C5h, C5g, C2c, respectively, obtained directly from reduced human milk lysozyme (see Table 2).

[b] For details see paragraph 3.2.3 (C2c, chymotryptic peptides).

3.2. *Chymotryptic peptides obtained from reduced alkylated human milk lysozyme.* By filtration of the chymotryptic digest of this lysozyme on Sephadex G-25 7 fractions (C1–C7) were characterized. The chymotryptic peptides contained in these fractions were purified by preparative paper electrophoresis and, when necessary,

paper chromatography. Their complete sequences, accounting for all the amino acids present in the molecule of the lysozyme (see Table 2) were established by usual methods. For 4 peptides, however, some comments are necessary.

Table 2. *Mobility m values (at pH 6.5), Rf in solvent A and detailed structures of all the chymotryptic peptides of human milk lysozyme characterized in the 7 fractions obtained by filtration on Sephadex G-25*

Peptide	m [a]	Rf	Structure
C1	−0.20	0.20	SCMCys-Asn-Asp-Gly-Lys-Thr-Pro-Gly-Ala-Val-Asn-Ala--SCMCys-His-Leu
C2a1	+0.30	0.12	SCMCys-Ala-Lys-Arg-Val-Arg-Asp-Pro-Gln-Gly-Ile-Arg--Ala-Trp
C2a2	+0.30	0.20	Lys-Arg-Leu-Gly-Met-Asp-Gly-Tyr
C2b	−0.30	0.10	SCMCys-Asn-Asp-Gly-Lys-Thr-Pro-Gly-Ala-Val-Asn
C2c	−0.50	0.50	Leu-Gln-Asp-Asn-Ile-Ala-Asp-Ala-Val-Ala
C3a	+0.40		Arg-Asn-Arg-SCMCys-Gln
C3b	+0.20		Asn-Arg-Asp-Val-Arg-Gln-Tyr
C4	−0.25	0.15	Asn-Ala-Gly-Asp-Arg-Ser-Thr-Asp-Tyr
C5a	+0.55	0.60	Lys-Val-Phe
C5b	+0.50	0.53	Ala-Arg-Thr-Leu
C5c	+0.40		Arg-Gly-Ile-Ser-Leu
C5d	+0.25	0.22	Asn-Thr-Arg-Ala-Thr-Asn-Tyr
C5e	0	0.53	PyrGlu [b])-Ile-Asn-Ser-Arg-Tyr (ninhydrin negative)
C5f	−0.35	0.32	Val-Gln-Gly-SCMCys-Gly-Val
C5g	−0.40	0.47	Ser-SCMCys-Ser-Ala-Leu
C5h	−0.45	0.40	Ala-SCMCys-His-Leu
C5i	−0.60		Glu-Arg-SCMCys-Glu-Leu
C6a	+0.70		Ala-Lys-Trp
C6b	+0.40		Ser-Arg-Tyr
C6c1	0	0.30	Ala-Asn-Trp
C6c2	0	0.40	Val-Ala-Trp
C6c3	0	0.60	Gly-Ile-Phe
C6d	−0.45		Glu-Ser-Gly-Tyr
C6e	−0.50		Met-SCMCys-Leu
C7	0		Trp

[a]) $m = 0$ for Gly; $m = +1$ for Arg; $m = -1$ for $CySO_3H$.
[b]) PyrGlu: pyrrolidonecarboxylic acid.

3.2.1. *Peptide C2a1* constitutes the overlapping peptide which allows to link to each other the following short tryptic peptides: 12 + 1b + 2 + 5d + 7b [2]. Its structure was established by partial acid hydrolysis (0.03 N HCl; 24 h; 105°) which provoked a split at the N- and C-terminal sides of the Asp residue. The following main products were characterized after preparative paper electrophoresis at pH 6.5: Asp; Arg-Val-Arg ($m = +1$); Pro-Glu-Gly-Ile-Arg-Ala ($m = 0$). This latter peptide furnished a glutamic acid by partial hydrolysis, but in the original tetradecapeptide C2a1 a glutamine residue was characterized (m at pH 6.5: +0.3; 2 acidic residues: SCMCys and Asp, and 4 basic residues; see also tryptic peptide 5d [2]).

3.2.2. In *peptide C2b*, the second and third amino acids were not only characterized by the '*Edman*-dansyl' procedure but also as PTH-amino acids; thus the place of the asparagine residue could be determined: SCMCys-Asn-Asp-Gly-...

3.2.3. *Peptide C2c* (for the establishment of its structure see Table 1, chymotryptic digestion product 6) contains 3 Asx (Asp or Asn) and 1 Glx (Glu or Gln) residues.

As its mobility at pH 6.5 is -0.50, 2 of these residues must be amidated in the isolated peptide. The first and second Asx residues were identified as Asp and Asn residues, respectively, by their PTH-derivatives. As a prolongated action of chymotrypsin did not shorten the peptide, the third Asx residue should be Asp. Indeed, chymotrypsin did split human milk lysozyme at the C-terminal side of all the Asn residues (the small chymotryptic split products are not all mentioned in Table 2) with two exceptions: (a) Asn residue preceded or followed by an acidic residue (Asp); (b) Asn residue preceded or followed by an aromatic acid residue (Tyr); in this latter case, the split occured at the C-terminal side of the aromatic acid residue. These observations allowed to conclude that the Glx residue of peptide C2c ($m = -0.50$) corresponds to a Gln residue; furthermore by the '*Edman*-dansyl' procedure we identified Glu accompanied by the artifact usually observed when a Gln residue occurs in the native peptide.

After peptic digestion (24 h; 5% formic acid) of peptide C2c the following products (among others) were separated by paper chromatography: Leu-Glu (m at pH 6.5: -0.9) and Asp-Asn (m at pH 6.5: -0.9). Thus in an acidic medium the glutamine residue No. 86 is rather labile and is converted into a glutamic acid residue.

The C-terminal amino acid of peptide C2c is Ala; this amino acid occurs rarely in such a situation in peptides resulting from chymotryptic digestion.

3.2.4. When *peptide C5e* was prepared by filtration on Sephadex G-25 with 30% acetic acid as eluent, it was neutral at pH 6.5 and was not coloured by ninhydrin on paper. No N-terminal dansyl derivative could be detected, unless the peptide was pretreated with 1N NaOH. These data suggest that peptide C5e has a N-terminal Gln residue converted to pyrrolidonecarboxylic acid during the purification. This conclusion is strengthened by the following observation: when the chymotryptic digest of human milk lysozyme was purified directly on Dowex 1×2 (basic and neutral pyridine – collidine – acetic acid buffers), peptide C5e was eluted together with basic peptides. All these results are consistent with a glutamine residue at position No. 58 of human milk lysozyme. Having a doubt concerning its stability we have reinvestigated the electrophoretic behaviour at pH 6.5 of the tryptic peptide 15 [2]: two mobilities, $m = -0.26$ [1] and $m = 0$ were found. This observation suggests that glutamine residue No. 58 is capable of being partly converted into glutamic acid.

3.3. *The complete primary structure of human milk lysozyme* (Table 3). The chymotryptic peptides containing basic amino acids were used in reconstructing the complete amino acid sequence of human milk lysozyme. Evidence for the overlaps between tryptic peptides is indicated in Table 3.

4. Discussion. – 4.1. *Comparison with other normal human lysozymes.* The group of *Jollès* [13] obtained a great number of chromatographically pure lysozymes from *normal* human tissues and secretions (milk, tears, saliva, placenta, spleen, serum, leucocytes) by ion-exchange chromatography on Amberlite CG-50: all these enzymes had the same chromatographic behaviour; the main active peak, preceded by a small active fraction ($\leqslant 5\%$), was eluted at the same place when the same column was employed. Multiple forms were not observed as in the case of the lysozymes isolated from duck egg-white [6]. It is suggested that all normal human lysozymes have the primary structure of human milk lysozyme.

Table 3. *Primary structure of human milk lysozyme. Comparison with hen* [5], *duck II* [6], *guinea-hen* [7], *turkey* [8], *and japanese quail* [9] *egg-white lysozymes and bovine α-lactalbumin* [10]; *only replacements are indicated*

To optimize homologous relationships, an insertion and a deletion occur in human milk lysozyme [2] [2a] [11] [12]. For the numbers of the tryptic peptides, see [2]. -⊖-: deletion

Peptides: ←3c→←9a→←1c→←
←C5a→←C5i→←C5b→←C2a2

Human milk	Hum	1	Lys-	Val-	Phe-	Glu-	Arg-	Cys-	Glu-	Leu-	Ala-	Arg-	Thr-	Leu-	Lys-	Arg-	Leu-	Gly-
Hen	H	1				Gly						Ala	Ala	Met			His	
Duck II	D	1			Tyr	Ser						Ala	Ala	Met				
Guinea-hen	G	1				Gly						Ala	Ala	Met			His	
Turkey	T	1			Tyr	Gly						Ala	Ala	Met				
Quail	Q	1			Tyr	Gly						Ala	Ala	Met			His	
α-lactalbumin	L	1	Glu	Gln	Leu	Thr	Lys			Val	Phe		Glu			-⊖-	-⊖-	Asp

Peptides: 8→←7a
→←C5c→←C6cl→←C6e→←

Hum	17	Met-	Asp-	Gly-	Tyr-	Arg-	Gly-	Ile-	Ser-	Leu-	Ala-	Asn-	Trp-	Met-	Cys-	Leu-	Ala-
H	17	Leu		Asn				Tyr			Gly			Val		Ala	
D	17	Leu		Asn				Tyr			Gly			Val		Ala	
G	17	Leu		Asn				Tyr			Gly			Val		Ala	
T	17	Leu		Asn				Tyr			Gly			Val		Ala	
Q	17	Leu		Lys		Gln		Tyr			Gly			Val		Ala	
L	15	Leu	Lys			Gly		Val			Pro	Glu		Val		Thr	Thr

Peptides: →←9b→←5b
C6a→←C6d→←C5d→←

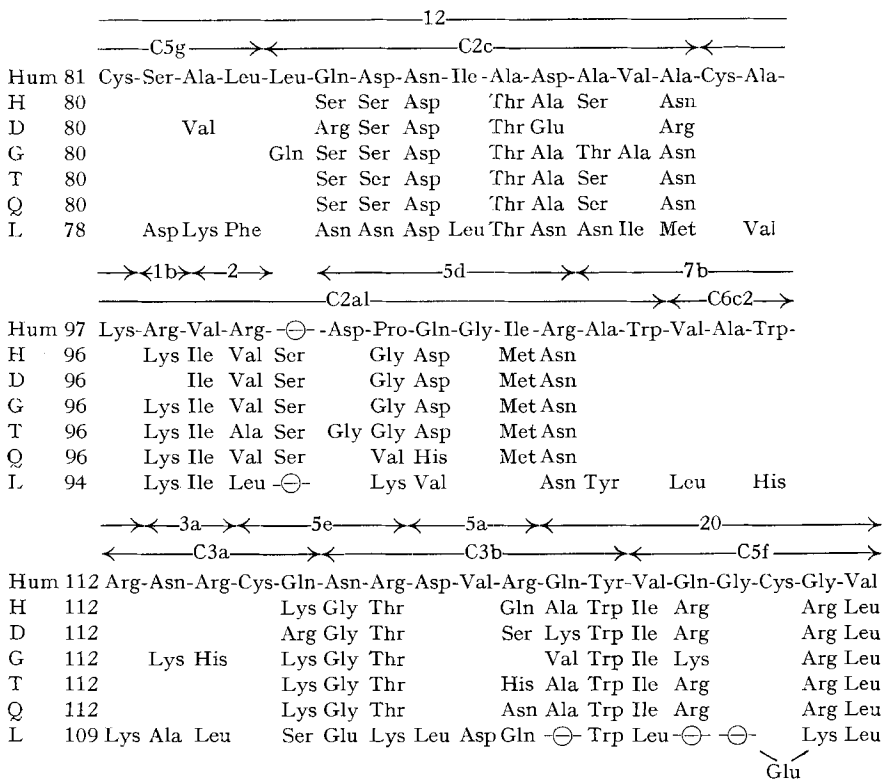
Hum	33	Lys-	Trp-	Glu-	Ser-	Gly-	Tyr-	Asn-	Thr-	Arg-	Ala-	Thr-	Asn-	Tyr-	Asn-	Ala-	Gly-
H	33		Phe			Asn	Phe			Gln				Arg	Asp	Thr	-⊖-
D	33	Asn	Tyr			Ser	Phe			Gln				Arg		Thr	-⊖-
G	33		Phe			Asn	Phe		Ser	Gln				Arg		Thr	-⊖-
T	33		Phe			Asn	Phe			His				Arg		Thr	-⊖-
Q	33		Phe			Asn	Phe			Gln				Arg		Thr	-⊖-
L	31	-⊖-	Phe	His				Asp		Glu		Ile	Val	Glu		-⊖-	-⊖-
					Thr												

Peptides: →←15→←
C4→←C6c3→←C5e→←C7→

Hum	49	Asp-	Arg-	Ser-	Thr-	Asp-	Tyr-	Gly-	Ile-	Phe-	Gln-	Ile-	Asn-	Ser-	Arg-	Tyr-	Trp-
H	48	Asn	Gly							Leu						Trp	
D	48		Gly							Leu	Glu					Trp	
G	48		Gly						Val	Leu						Trp	
T	48		Gly							Leu						Trp	
Q	48		Gly							Leu						Trp	
L	46	Asn	Gln						Leu					Asn	Lys	Ile	

Peptides: 14→←12
←C2b→←C5b→←

Hum	65	Cys-	Asn-	Asp-	Gly-	Lys-	Thr-	Pro-	Gly-	Ala-	Val-	Asn-	Ala-	Cys-	His-	Leu-	Ser-
H	64		Asp	Asn		Arg				Ser	Arg		Leu		Asn	Ile	Pro
D	64		Asp	Asn						Ser	Lys				Gly	Ile	Pro
G	64					Arg				Ser	Arg		Leu		Asn	Ile	Pro
T	64					Arg				Ser	Lys		Leu		Asn	Ile	Pro
Q	64					Arg				Ser	Arg		Leu		Asn	Ile	Pro
L	62		Lys	Asn	Asp	Gln	Asp		His	Ser	Ser		Ile		Asn	Ile	



4.2. *Comparison with lysozymes of leuchaemia patients.* From tissues or secretions of leuchaemia patients (leucocytes and serum of patients with chronic myelogenous leuchaemia; urine of patients with monocytic and monomyelocytic leuchaemias) *Jollès et al.* have characterized two main active fractions by the above mentioned chromatographic procedure [14]; these fractions had a slightly different immunological behaviour [15]. The primary structure of human leuchaemia lysozyme isolated by a *different technique* from the urine of a patient with chronic monocytic leuchaemia was recently described by *Canfield et al.* [11]. Their sequence is identical with ours of human milk lysozyme.

4.3. *Presence of two labile glutamine residues in human milk lysozyme.* We have characterized two labile glutamine residues which are easily converted into glutamic acid residues (No. 58 in peptide 15 [2] or C5e; No. 86 in peptide C2c).

4.4. *Close structural homology between human and several bird egg-white lysozymes.* As shown by Table 3 human milk lysozyme can be ranged among the group of lysozymes including the enzymes from many bird egg-whites (hen [5], duck [6], guinea-hen [7], turkey [8], quail [9]). All these enzymes have considerable homology. In view to optimize homologous relationships, an insertion and a deletion were suggested [2] [2a] [11] to occur in the sequence of human lysozyme when compared to bird lysozymes. Their most probable positions seem to be residue No. 48 (insertion) and after residue No. 100 (deletion), for considerations of homology (present paper and

[11]) and following *X*-ray crystallographic data presented by *Blake & Swann* [12]. All the lysozymes mentioned are made of 129 amino acid residues, 71 to 77 of them being identical (Table 3). The half-cystine and tryptophan residues as well as the two acidic residues essential for the catalytic activity (Glu No. 35; Asp No. 52 for bird and No. 53 for human lysozymes) [16] appear in identical positions. The same holds for residues involved in hydrogen bonds playing a rôle in holding the folded chain together (Ser No. 24, Trp No. 28, Ser No. 36, Asn No. 39). The most constant regions among the various lysozymes of Table 3 are situated between (a) residues No. 49–72, and for bird lysozymes residues No. 48–71, respectively (in part β-sheet region), where the only acceptable substitutions are Ile for Val, Phe for Leu, Tyr for Trp and Lys for Arg; (b) residues No. 107–112 (sequence Ala-Trp-Val-Ala-Trp-Arg). In general, the N-terminal ends of the enzymes present more homology than the C-terminal parts. Despite the common elements in the primary structures of all these lysozymes, studies devoted to their action on various substrates have demonstrated important differences [17]; furthermore no immunological cross-reactivity could be found between native human milk lysozyme and several bird egg-white lysozymes [18]. It is worth pointing out that bovine α-lactalbumin is a protein closely related to the lysozymes indicated in Table 3. The suggestion of *Brew et al.* [10] that these molecules evolved from a common ancestral gene seems thus to be confirmed.

4.5. *Existence of different classes of lysozymes.* The group of lysozymes constituted by the human and some bird egg-white enzymes is entirely different from a) goose egg-white lysozyme [11] [19]; b) phage lysozymes [20]; c) plant lysozymes [21]. They form *different classes* of lysozymes [13] with different molecular weights ranging from 14,000 to 28,000, different structures, different immunological and kinetical properties. Their common point remains however their capacity of hydrolysing glycosidic linkages in bacterial cell walls.

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286. The Reaction of Methyl Tosylate with Halide Ions in Pyridine-Dimethylformamide

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(11. VIII. 71)

Summary. The reactivity of the lithium halides in 83.3% pyridine-dimethylformamide changes from $Cl^- > Br^- > I^-$ to $Br^- > I^- > Cl^-$ with increasing concentration of the salt from 0 to 0.35 M. This behaviour is explained by ion pairing which reduces the concentration of reactive free nucleophiles. Equilibrium constants K for ion pair dissociation and rate constants k_2 for the reaction of the free nucleophiles were determined from the variation of the observed rate constant with the total halide concentration and from conductivity measurements.

The normal order of nucleophilic reactivity of the halide anions with $I^- > Br^- > Cl^-$ is based on measurements in water or other protic solvents [1]. This order can be reversed if the medium is changed to a dipolar aprotic solvent of high dielectric constant [2]. The rationalizations used in the past for nucleophilic reactivity, based on polarizability [3], oxidation potentials [4] and other physical properties are of little value considering the reactivity changes upon transfer from protic to aprotic solvents. The most important factor governing nucleophilic reactivity now appears to be solvation of the nucleophile [2]. The stability of a given nucleophile in a particular solvent with respect to a reference solvent can be expressed by the solvent activity coefficient [2] [5]. Rates of many bimolecular substitution reactions correlate reasonably well with the solvent activity coefficients of the attacking nucleophiles, whereas solvent activity coefficients of organic substrates and transition states are usually of minor importance [2] [6]. Among the halide anions, chloride is the most stable in protic, and the least stable in aprotic solvents. Accordingly, it is the least reactive in protic and the most reactive in aprotic reaction media.

In aprotic solvents of low dielectric constant the situation appears to be more complicated. Thus, in liquid sulfur dioxide, bromide is more reactive than chloride, an anomaly still awaiting explanation [7], and in acetone the observed reactivity sequence of the halide anions corresponds to that obtained in water and not, as would be expected, to that for dipolar aprotic solvents [8]. The behaviour of the halide ions in acetone is easily understood if incomplete dissociation of the inorganic salts in this