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REVIEW

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GENERAL LIGAND AFFINITY CHROMATOGRAPHY

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Introduction

Recent developments in enzyme purification technology have raised the status of the area from purely empirical to one displaying considerable rationale. Old fashioned enzyme purification techniques such as the precipitation methods have, now, given way to chromatographic methods. Precipitation techniques are realised by changing the pH, temperature[1,2] and/or salt concentration[3], or in the presence of organic solvents[4,5] or water soluble high molecular weight polymers, such as polyethylene glycol[6-8]. More refined purification procedures are those employing chromatographic techniques. In the latter case the separation of a mixture of compounds is achieved by passing a flow of liquid (mobile phase) through a non-moving solid medium (stationary phase) saturated with liquid. Hopefully this will result in a differential migration of the compounds according to their Partition Coefficient K. If C_s is the concentration of the solute in the stationary phase and C_m that in the mobile phase, then $K = C_s/C_m$. If $K \leqslant 1$ partition favours the solute being in the mobile phase giving rise to partition chromatographic techniques such as thin layer chromatography, gas liquid chromatography and gel filtration[9]. In the latter method the separation of the substances depends on the molecular size. If $K >> 1$ most of the substance has been taken up by the stationary phase, therefore, giving rise to adsorption chromatography. Three types of adsorption chromatography have attracted considerable attention in enzyme purification: ion-exchange[10-13], hydrophobic[14-17] and affinity chromatography[17-20]. During the last decade or so affinity chromatography has revolutionised enzyme purification technology. The present review will focus attention upon this modern technique.

I. The Principle of Affinity Chromatography

The isolation of macromolecules such as proteins, polysaccharides and

nucleic acids by conventional methods on the basis of small differences in their physicochemical properties within the individual groups, is generally difficult and time consuming. However, one of the most characteristic properties of biological macromolecules is their ability to bind other molecules specifically and reversibly. Accordingly, enzymes form complexes with substrates, inhibitors, cofactors and other structurally related compounds. Antibodies bind specific antigens and nucleic acids bind complementary nucleic acids, etc. The exploitation of such reversible specific complexes formed by biological macromolecules is the basis for their purification by the method known as affinity chromatography.

The concept of affinity chromatography is realised by covalently attaching one of the interacting species (e.g. a competitive inhibitor of the enzyme to be isolated) to a water-insoluble support (e.g. agarose) and packing the support into a chromatographic column. In principle, when a solution containing the other interacting species (e,g. the enzyme that exhibits affinity for the immobilised inhibitor) is passed through the column, only this particular enzyme that displays affinity for the immobilised species will be adsorbed whereas other molecules which show no recognition for the insolubilised species will pass through unretarded. The adsorbed enzyme can then be desorbed by introducing into the solution free substrate which will compete with the immobilised inhibitor.

II. Nomenclature in Affinity Chromatography

A number of different terms have been proposed for this technique in order to emphasize the purely biologically specific interactions involved and to distinguish it from chromatography exploiting non-specific ion-exchange or hydrophobic interactions. The technique has been termed «biospecific adsorption chromatography»[21], «bioselective adsorption chromatography»[22], «bioaffinity chromatography»[23], «ligand specific chromatography»[24] and «biospecific affinity chromatography»[25]. The term affinity chromatography is widely used in accordance with the recommendation of the committee for the standardization of nomenclature in affinity chromatography[26].

Aside from various terms for the technique, the individual components involved have been named differently. The polymer to which one of the interacting species is covalently bound has been termed «solid support»[27], «carrier»[24] and «matrix»[25]. The species attached to the matrix has been called «effector»[28], «affinant»[29] or «ligand»[27], whereas the substance to be isolated may be called «affiner partner»[28] or «ligate»[30]. The terms «ligand» and «complementary enzyme or substance», respectively, will be used here. The molecule that separates the ligand from the matrix is termed «arm», «extension arm» or «spacer»[31]. In order to attach the spacer or the ligand to the matrix one must first introduce the necessary chemically reactive group to the matrix by a process known as activation. The latter reactive group is known as «connector substance» or «connector», «active group» or «reactive

group»[25]. The process of binding the ligand to the matrix has been named as «coupling», «insolubilisation» or «immobilisation»[31].

III. Historical Background to Affinity Chromatography

The concept of separating macromolecules by means of biospecific interactions with immobilised substances is not new. Starkenstein (1910)[32] reported the isolation of α-amylase by means of adsorption onto insoluble substrate (starch), while, Willstatter and co-workers (1923)[33] succeeded in an appreciable purification of lipase by adsorption onto powdered stearic acid.

The principle of affinity chromatography as realised today using a covalently bound ligand on the matrix, was first exploited by Campbell and co-workers (1951)[34] in the isolation of rabbit anti-bovine serum albumin antibodies using an immunoadsorbent synthesised by coupling bovine serum albumin to diazotized p-aminobenzylcellulose. Affinity chromatography was first employed in the isolation of enzymes by Lerman (1953)[35] who isolated tyrosinase on various p-azophenol-substituted cellulose columns. Later on, Arsenis and McCormick (1964, 1966) purified liver flavokinase on flavin substituted cellulose[36] and flavin mononucleotide-dependent enzymes on flavin phosphate substituted cellulose[37].

Within the last decade or so considerable interest has been aroused in the development of affinity adsorbents based on biological specificity. Cuatrecasas and co-workers (1968)[38] introduced agarose as a matrix and the insertion of a spacer molecule between the ligand and the matrix[27]. The latter developments in the methodology of affinity chromatography along with the cyanogen bromide activation method of polysaccharide matrices by Axen and co-workers (1967)[39] were decisive for the dramatic expansion of this chromatographic technique and stimulated the extensive use of affinity chromatography in the isolation of enzymes, proteins, antibodies, nucleic acids, receptors and many other biological substances[17,19,20].

IV. Designing an Affinity Adsorbent

Matrix, spacer and ligand constitute the three main components of an affinity system. Suitable coupling methods are required to combine these three constitutents, furthermore, each individual component should exhibit certain properties so that the overall system possesses the desired features necessary for efficient operation.

The Matrix

Although there are many polymers capable of forming gels, very few are

suitable for affinity chromatography since an ideal matrix should meet certain requirements[38] :

(i) It must be insoluble, hydrophilic with a high degree of porosity.
(ii) The gel particles should be uniform, spherical and rigid.
(iii) It must be physically and chemically stable and chemically inert.
(iv) The matrix should possess functional groups for coupling.

In practice, a matrix possessing all these ideal properties does not yet exist and the various matrices available to the biotechnologist display properties close to the ideal matrix to different extents.

Agarose (Sepharose) is a linear natural polysaccharide consisting of alternating 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose residues[40]. The linear polymer forms a left-hand turn paralleled double helix[41] where the interior cavity is occupied by water molecules that participate in a hydrogen bonding system that contributes to the stability of the structure. The lack of covalent cross-linkages compromises the stability of the gel and this is the main drawback of agarose as a matrix, when used at temperatures above 40 ^{0}C or below 0 ^{0}C or at extreme pH values. Nevertheless, the relatively large voids present in the polymer's aggregated form (network) endow agarose with an important property. The stability of agarose may be further improved by cross-linking. The latter can be achieved either by employing cross-linking reagents such as epichlorohydrin or 2,3-dibromopropan-1-ol[42,43], or during the activation step. This is a necessary procedure that must be followed in order to covalently link the ligand onto the matrix. The cyanogen bromide activation method is the most commonly used for polysaccharide matrices and was first introduced to affinity chromatography by Axen and co-workers[39]. Fig. 1 illustrates the probable course of events. The imidocarbonate formed may be cyclic where the two hydroxyls belong at the same monosaccharide residue, or, it may be acyclic where the two hydroxyls belong to different polysaccharide chains. The latter would lead to matrix cross-linking improving its stability. However, the introduction of isourea linkages possessing cationic groups of $pK_a \sim 10$, when coupling aliphatic amines, results in significant non-specific adsorption of proteins[44,45]. This problem can be eliminated by employing alkylhydrazides[46,47] which would result in isourea linkages with pK_a values of around 4[45]. Furthermore, the isourea linkages are not very stable in the long term, depending upon the pH and the composition of the irrigant[48]. Alternatively, activation may be effected by other means: treatment of the polysaccharide matrix with bis-epoxides or epichlorohydrin[49]; oxidation with sodium periodate[50,51]; treatment with divinylsulphone[52], benzoquinone[53], s-trichlorotriazine (cyanuric chloride) or dichloro-s-triazines[54], 2,4,6-trifluoro-s-chloropyrimidine (FCP)[31] or carbonyldiimidazolide[55].

Other natural polymers are less popular as matrices in affinity chromatography. Cellulose frequently displays considerable nonspecific adsorption and the fibrous and non-uniform structure impairs flow rate properties, furthermore, penetration by large protein molecules occurs. Dextrans (Sephadex),

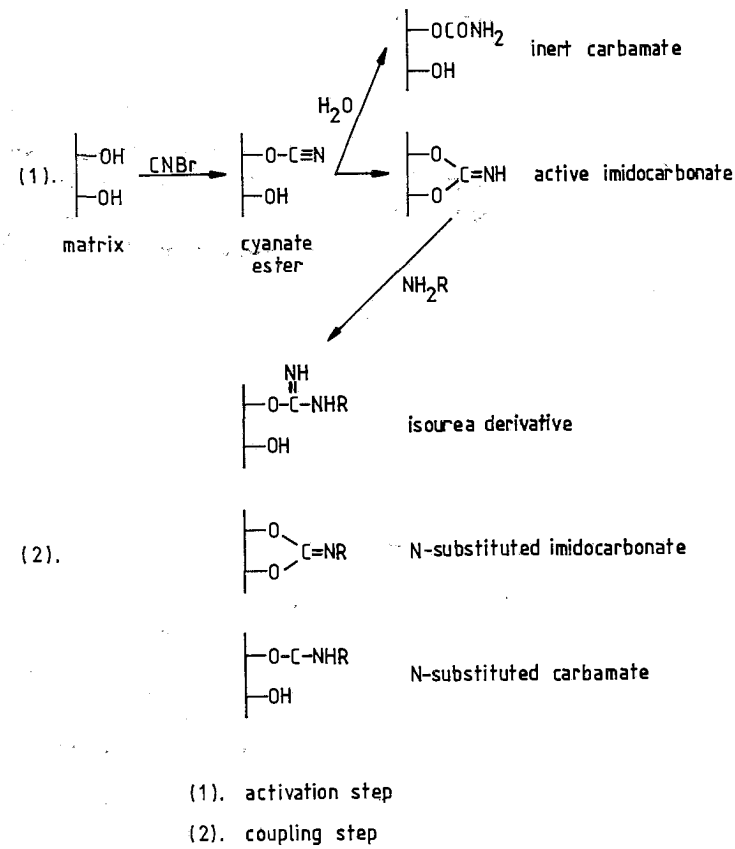


FIG. 1 : *Chemical activation of polysaccharides by means of cyanogen bromide and chemical coupling of a ligand NH_2R to cyanogen bromide activated matrix.*

although they combine a hydrophilic character with low non-specific adsorption, are too soft for column procedures[25] and their degree of substitution is relatively low. Furthermore, activation leads to a considerable degree of further cross-linking of the gel making it ineffective for affinity chromatography even for enzymes with low molecular weight.

Besides the natural polysaccharides used, other synthetic gels have been employed as matrices. Co-polymerisation of acrylamide with the cross-linking agent N,N′-methylenebisacrylamide forms a gel comprising crosslinked polyacrylamide chains[56]. This gel is not subject to microbial or enzymatic attack and has no ionic groups, thus, it does not exhibit ion-exchange interactions, however it does display some non-specific aromatic interactions. The functional groups present permit a versatility in derivatisation and ligand attachment[57] but its low degree of porosity endows this synthetic polymer with a serious drawback. Other synthetic matrices employed are poly(hydroxylalkylmethacrylate)(Spheron)[58], ethylene-maleic anhydride copolymer

(EMA)[59], poly-p-aminostyrene[60] and semi-synthetic polyacrylamide-agarose gels (Ultrogel)[47]. Finally, inorganic carriers, such as porous glass, are resistant to microbial attack and have a very rigid structure, the bed volumes do not alter with solvent or ionic strength changes and, furthermore, they offer sharp exclusion limits. Non-specific adsorption, however, can be rather high but this can be overcome by coating the glass surface with a monolayer of covalently bound dextran[31].

Nevertheless, it is accepted that agarose displays properties most closely resembling those of the ideal matrix and is the best compromise as a matrix[61]. Furthermore, since Hijerten succeeded in preparing agarose in beaded form[62] this natural polysaccharide has been extensively employed in affinity chromatography[63].

The Spacer

For successful application of affinity chromatography one must make certain that the experimental ligand-macromolecule interactions are as similar as possible to those in free solution. Accordingly, the matrix should have a high degree of porosity so that the macromolecule can diffuse through the matrix network without any exclusion effects imposed by the size of the matrix pores, and the ligand should be placed away from the matrix backbone so that the interaction with the complementary macromolecule will not be hindered by the matrix microenvironment. The latter may be achieved by inserting a spacer molecule between the matrix backbone and the ligand.

The usefulness of the spacer concept in affinity chromatography was first shown by Cuatrecasas and co-workers[38] in the purification of a-chymotrypsin on Sepharose-bound D-tryptophan methylester, a competitive inhibitor ($K_i = 10^{-4}$M) of a-chymotrypsin. When this inhibitor is directly coupled to Sepharose unsatisfactory resolution of the enzyme results, whereas dramatically stronger adsorption of the enzyme occurs if a 6-carbon chain is interposed between the matrix and the ligand[38]. However, for ligands which are relatively strong inhibitors the spacer effect may not be as profound as with those with lower affinity; for example, staphylococcal nucelase binds on the immobilised strong competitive inhibitor 3′-(4-aminophenyl phosphoryl) deoxythymidine 5′-phosphate ($K_i = 10^{-6}$M) without any spacer requirements[64].

When one is choosing a spacer two parameters must be taken into account, the length and the nature of the spacer. Hipwell and co-workers[65] investigated the effect of the spacer length on the binding of several dehydrogenases to N^6-(ω-aminoalkyl)-AMP-Sepharose and they found that the binding strength increased with the spacer length until an optimum spacer length had been reached. Similarly, Lowe and co-workers[66] performed experiments with several dehydrogenases and kinases versus ω-aminoalkyl-NAD^+-Sepharose and ω-aminoalkyl-ATP-Sepharose, respectively. They found

(Fig. 2) that the binding was very weak for spacers up to 4 methylene groups

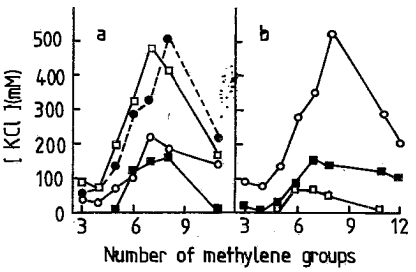


FIG. 2 : *The effect of arm length on the binding of nucleotide-dependent enzymes to immobilised nucleotides. (a) Rabbit skeletal muscle lactate dehydrogenase (□), pig heart muscle lactate dehydrogenase (•), malate dehydrogenase (o), glucose-6-phosphate dehydrogenase (■); immobilised NAD^+. (b) Hexokinase (□), 3-phosphoglycerate kinase (■), glycerokinase (o); immobilised ATP. The KCl concentration (mM) required to effect elution of the bound enzyme is a measure of binding. Reproduced from C.R. Lowe* at al., Biochem. J., **133,** *499 (1973).*

(0-0.5 nm) whereas the binding strength increased substantially on elongation of the spacer from 4 to 8 methylene groups (0.5-1.0 nm) reaching a maximum at about 8, but then declined when arms containing more than 8 methylene groups were used. It may be possible that the employment of a spacer of at least 4 methylene groups (0.5 nm) is necessary for the nucleotide ligand to traverse a barrier imposed by the micro-environment associated with the matrix whereas the decreased binding observed with longer, than the optimum, spacers may be explained if we assume a folding of the hydrocarbon chain back along itself[66] although this may not be the case[67]. Similar results were obtained when lactate dehydrogenase was tested versus 8-(ω-aminoalkyl)-AMP-Sepharoses; a spacer of about 6 methylene groups was required here[68] and this seems to be the optimum length in general[67]. Lowe and co-workers[66] noticed that enzymes with lower affinity for the ligand required at least five methylene groups before any interaction was observed and this is in agreement with observations by Cuatrecasas[64] that the length of the spacer is more important for low affinity systems. Furthermore, not only the affinity strength but also the molecular size of the macromolecule is critical in determining the optimum spacer's length. Accordingly, low molecular weight enzymes display good accessibility for the immobilised ligands and the length of the extension arm is not critical[66,69]. Thus the length of the spacer is more important for low affinity systems and/or for high molecular weight enzymes. Accordingly, the employment of large size spacers such as denatured albumin[70], poly (DL-alanine)[71], polylysine and poly(lysyl-alanine)[72] was introduced in large macromolecules' separations in order to increase the accessibility towards the ligands.

The nature of the spacer is another important parameter when designing an affinity adsorbent. It has become evident in recent years that true biospecific adsorption is a rare phenomenon and is often accompanied by non-specific interactions. In fact in some cases operational chromatography is only possible when non-specific phenomena [14,73,74] are present to reinforce the interaction between ligand and enzyme. Thus, the binding strength of several dehydrogenases was increased as the hydrophobicity of the spacer was increased [47,68,75]. The poor results obtained when hydrophilic spacers were used may be explained in terms of physical unavailability of the spacer-ligand complex as a result of hydrogen bonding with the hydrophilic matrix backbone [68]. In this context one cannot rule out conformational differences between spacer-ligand complexes of different hydrophobicity/hydrophilicity. Although it seems that hydrophobic spacers do reinforce ligand-macromolecule interactions, ionic effects may add to these interactions. For example, several dehydrogenases were shown to bind tighter to N^6-(6-aminohexyl)-AMP-Sepharose (isourea $pK_a \sim 10$) than to N^6-(hydrazidohexyl)-AMP-Sepharose (isourea pK_a 4.2) [47]. These observations underline the contribution of the isourea positive charge in strengthening the ligand-enzyme interactions.

The choice of a spacer of a particular length and nature will depend upon the nature of the ligand as well. Thus, lactate dehydrogenase requires a spacer of about 0.8 nm long when oxamate was employed as a ligand [76], a spacer 1.1 nm long when AMP linked through its N^6 position was employed as a ligand and a spacer 1.5 nm long when AMP linked through its C8-position was employed as a ligand [77].

The procedure followed in synthesising the matrix-spacer-ligand conjugate, in orthodox and specise science, is to first prepare the spacer-ligand complex by conventional organic chemistry and subsequently immobilise it to a properly activated matrix. Although this procedure requires considerable labour in isolating, purifying and characterising the compounds of each individual synthetic step it is, however, a worthwhile effort since the reward of having precisely defined affinity systems would be to interpret with confidence the experimental results.

The Ligand

The ligand may be of two main types, the «specific ligands» and the «group-specific» or «general ligands». The former class includes ligands with very narrow specificity while the latter class includes ligands which display affinity for a group of related macromolecules. The high specificity of specific ligands endows them with the serious drawback that a new adsorbent must be prepared for each different substance to be purified, which is time consuming and uneconomical. On the other hand, a group-specific adsorbent, once prepared, can be employed for a whole group of substances. Although these

ligands offer a compromise in specificity, however, this can be overcome if specific elution techniques are employed [17], i.e. formation of a binary complex between free ligand and adsorbed enzyme, formation of a ternary complex, employment of specific, for example, NAD^+-adducts, use of negative elution, photolysis, electrophoretic desorption, inhibition of ligand-enzyme interaction and selective clearage of the matrix-ligand bond.

There are several group-specific ligands used in affinity chromatography for the isolation of macromolecules [17,19,31]: coenzymes (e.g. pyridine nucleotides and nucleotides), nucleic acids and polynucleotides, lectins, organomercurials, amino acids, protein A and dystuffs. Among them, nucleotides and their analogues and dystuffs have received dramatic attention. This is not surprising since of the 2000 or so enzymes found in the cell over 30% participate in reactions involving NAD^+, $NADP^+$, ATP, FAD, coenzyme A or other nucleotides [78,79]. Especially, immobilised adenine nucleotides and adenine-based coenzymes have been the subject of excellent reviews [78-81], in addition, the use of non-adenine nucleotides in affinity chromatography has been summarised [17].

The knowledge of the enzymatic three-dimentional structure in conjunction with the enzyme-ligand (nucleotide) interactions is useful in designing suitable affinity adsorbents. In this way one may obtain information of what groups of the ligand are not so important in the enzyme-ligand interactions and thus can be chemically derivatised to immobilise the ligand. Following this logic we alter least the immobilised nucleotide-enzyme interactions. However, in this context one cannot rule out individual variations or the lack of pre-accumulated knowledge and, thus, ligands immobilised through different positions should be tested. In fact, it is well established that affinity adsorbents prepared by immobilising nucleotides through different positions exhibit different abilities to bind the same complementary enzyme [47,82-84].

The sequence in preparing immobilised ligands is to chemically attach, for example, a diaminoalkane spacer to the ligand and to then immobilise the ligand-spacer complex to a properly activated matrix via the free amino group of the spacer. Immobilisation can be achieved through the purine base, ribose moiety, phosphate moiety, pyrimidine base or the nicotinamide moiety (Fig. 3).

Purine base substitutions are realised by (i) direct nucleophilic attack by diaminoalkanes at the C6 position of 6-Cl-IMP [85], 6-mercapto-IMP [86] and 6-methylsulphonyl-IMP [87] to produce the corresponding N^6-(n-aminoalkyl)-nucleotide. Also, the N^6-purinyl amino group of NAD^+ can react with succinic anhydride to form the corresponding succinamide-NAD^+ derivative and its terminal carbonyl group can then be condensed with diaminoalkames in a carbodiimide promoted reaction [88]. Direct carbamoylation of the N^6-amino group of AMP, ADP or ATP by hexamethylene diisocyanate generates the corresponding 6-ureido purine derivative [89], or, the N^6-amino group may be directly attacked on reaction with formaldehyde to yield the corresponding N^6-hydroxymethyl derivative which is then converted to N^6-(carboxyethyl)-

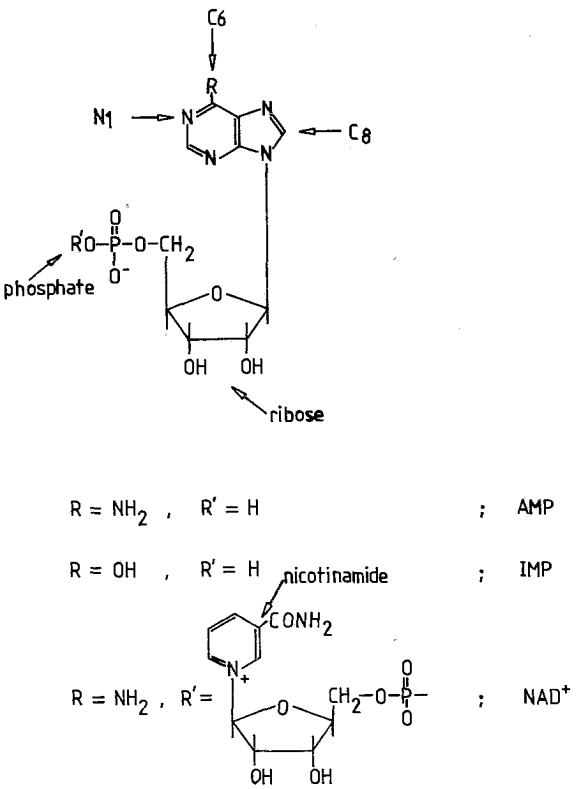


FIG. 3 : *Positions of dirivatisation of nucleotides and nicotinamide coenzymes for subsequent immobilisation.*

thiomethyl nucleotide derivative on reaction with 3-mercaptopropionic acid[90]. In both of the above cases the terminal free carboxyl group of the spacer may be condensed with diaminoalkanes via a carbodiimide reaction. However, the chemical lability of NAD^+ necessitated the use of alternative procedures. Thus, direct alkylation of the N1 position of the purine base with iodoacetic acid yields the corresponding 1-carboxymethyl derivative which is then converted to the N^6-carboxymethyl derivative via an alkaline Dimroth rearrangement[91-93]. The terminal carboxyl group can then react with a diamino-containing spacer in a carbodiimide reaction, as usual. Of the different substitution methods quoted above for the N^6-position of the purine base, the direct nucleophilic attack with diaminoalkanes that produces N^6-alkyl-derivatives and the alkylation at the N1 position followed by Dimroth rearrangement that yields N^6-(N-alkyl)carbamoyl-derivatives produce more stable linkages between spacer and ligand than the linkages of the N^6-alkyl-thiomethyl-[90] and N^6-acyl-[88] derivatives.

(ii) Bromination at the C8 position of the purine base followed by a nucleophilic displacement of the halogen by a suitable nucleophile-spacer

such as diaminoalkanes[94,95,96], cysteamine[97] or 3-mercaptopropionic acid[98].

There are no reports on the use of nucleotides or coenzymes immobilised via the C2 position of the purine base in affinity chromatography.

Ribose substitutions and immobilisation via the vicinal hydroxyls may be achieved by two methods: (i) Periodate oxidation of the nucleotide generates aldehyde groups at the 2′ and 3′ position of the ribose moiety which may then react either with an alkyldihydrazide[46,51], or with alkyldiamines to form the corresponding Schiff base. The latter is then reduced to a stable alkylamino linkage. (ii) Condensation of the diol of the ribose moiety with ethyl levulinate to the corresponding acetal ester of the nucleoside. The latter is then phosphorylated at its 5′-position and subsequently the acetal ester is hydrolysed to the corresponding free carboxylic acid. This may be coupled to an alkyldiamino spacer via a carbodiimide promoted condensation[17,96].

Phosphate substitutions are now achieved by formation of assymmetrically substituted pyrophosphates. For this purpose one can follow either the anion displacement method[99,100] or the imidazolide method[94,100-103].

Pyrimidine base substitutions are performed, so far, by two procedures: the first involves direct transamination between the exocyclic N^6-amino group of the pyrimidine ring with an alkyldiamine by a bisulphite-induced reaction[104]. The second method involves direct halogenation of the 5-position of the ring followed by nucleophilic displacement by spacer molecules such as cysteine or glutathione[105]. However compounds prepared by the second method have not beeen tested in affinity chromatography, so far.

Nicotinamide substitutions may be achieved by exchanging the whole of the nicotinamide ring with the appropriate pyridine analogue to produce the corresponding NAD^+-analogue, in the presence of NADase[106].

However, besides the significance of the position of immobilisation of the the various nucleotide ligands in the enzyme-ligand interaction, other parameters may play important role:

(i) Immobilised ligand concentration : Experimental work suggests that the binding strength increased with increasing ligand concentration[82]. However, it is remarkable that after a critical ligand concentration is exceeded the adsorbent lost its selectivity and acted as an unpecified ion-exchanger[107]. Furthermore, adsorbents with high ligand concentration may cause difficulties in eluting the macromolecule[108], thus, ligand concentration must be cerfully controled. A ligand concentration of about 2-5 μmol/g moist gel is commonly employed and the enzyme only *ca.* 0.1% of that actually utilises[82].

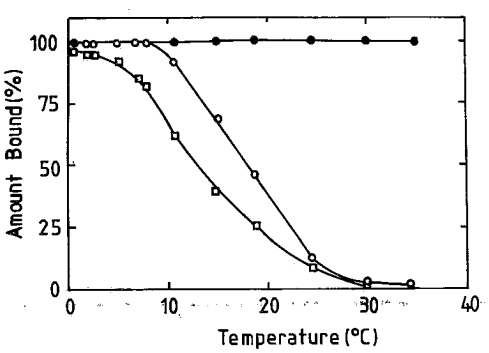
(ii) Bed geometry and dynamics : The strength of the enzyme-ligand interaction is a complex function related to bed geometry, ligand concentration ([L]) and total amount of ligand (T_L)[109]. Lowe *et al.* (1974)[109] demostrated that the binding strength of the nucleotide-dependent enzymes tested, on immobilised AMP, increased with increasing bed length and approached a maximum limiting value; [L] and T_L were kept constant and the bed diameter altered inversely according to bed's length at a 4/60 ratio. Similarly, when [L] and bed diameter were kept constant and the T_L was

proportional to bed's length, the binding strength increased linearly with bed length [109]. Contrarily, when T_L and bed diameter were kept constant and [L] varied inversely to bed's length, binding strength increased inversely to bed's length [109]. However, such bed geometry effects are rather critical for low affinity systems.

Since the ligand-enzyme interaction is a time-dependent process, it would be expected that equilibrium time and flow rate would influence that interaction. One would expect that prolonged equilibrium times would increase binding strength, resulting in better resolution, and low flow rates would allow better diffusion of the enzyme in the gel's network [109,110]. Interestingly, such bed dynamic effects were, in practice, not always in line with theoretical considerations and proved not to be important factors for some cases [18,38,109]. However, to the author's experience, opperational affinity chromatography could only be achived, in a preparative scale with IMP dehydrogenase onto immobilised IMP and AMP ligands, after a 20-30 min equilibrium time had been exeeded [17,96,111].

(iii) Enzyme concentration : The enzyme-ligand interaction is almost idependent of the initial free enzyme concentration, providing that the enzyme is applied at a suitable flow rate and at sub-saturating amounts [18].

(iv) Temperature : The adsorption of an enzyme to a stationary phase is generally exothermic and according to the Le Chatelier principle, elevated temperatures will move the equilibrium to the direction of free enzyme [112]. In practice, the increasing temperature affects substantially the amound of enzyme bound to affinity adsorbent (Fig. 4). The reduced binding observed



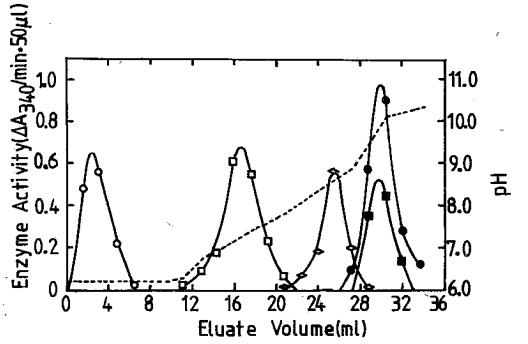


FIG. 4 : *Effect of temperature on the capacity of N^6-(6-aminohexyl)-AMP immobilised to Sepharose. Yeast alcohol dehydrogenase (o), glycerokinase (□), ligand at 1.5 µmol/ml; glycerokimase (•), ligand at 4.0 µmol/ml. Reproduced from M.J. Harvey* et al., Eur. J. Biochem., **41**, *353 (1974).*

FIG. 5 : *The resolution of a mixture of dehydrogenases on N^6-(6-aminohexyl)-AMP immobilised to Sepharose, by a pH gradient. The equilibration pH was 6.0 and the pH gradient was varied from 6.0 to 10.0. Bovine serum albumine (o), malate dehydrogenase (•), glucose-6-phosphate dehydrogenase (□), lactate dehydrogenase (■), yeast alcohol dehydrogenase (◇). Reproduced from C.R. Lowe* et al., Eur. J. Biochem., **41**, *347 (1974).*

has been exploited to effect resolution of enzyme mixtures by raising the temperature linearly[112].

(v) PH : Deviation from an enzyme's pH optimum influences both the velocity of its reaction and the affinity for its ligand[113]. Fig. 5 shows the exploitation of the pH influence on the enzyme-ligand interaction, in the resolution of a mixture of dehydrogenases on immobilised AMP by a pH gradient.

(vi) Dielectric constant : Any agent that alters the enzyme conformation is potentially able to effect elution of the enzyme from its immobilised ligand. For example, lactate dehydrogenase may be eluted from AMP adsorbent with linear gradients of organic agents such as ethylene glycol, dioxane, dimethylformamide or urea[114]. Such effects could be correlated to changes of the affinity of the enzyme for its ligand and, also, may contribute to eliminate non-specific hydrophobic adsorption to spacer arm assemblies[114].

So far we have seen the rational approach in synthesising specific affinity adsorbents suitable for macromolecule separations and, also, the various parameters that one must take into account. However, recent studies[17,115,116] regarding the mode of interaction of *E. coli* IMP dehydrogenase with IMP and AMP affinity ligands, revealed that specifically synthesised ligands do not always produce the anticipated ligand-enzyme interaction. Such information is important for affinity chromatography which employs a rational design of affinity adsorbents.

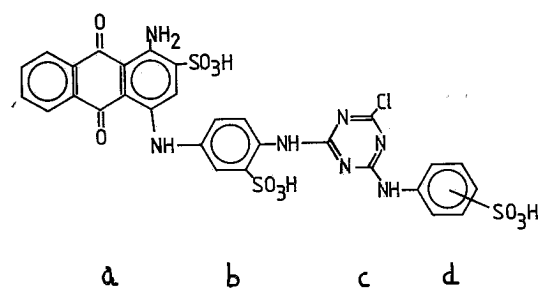


FIG. 6 : *Structure of a widely used triazine dye; Cibacron Blue F3G-A or Procion Blue H-B.*

Besides the extensive use of nucleotides and pyridine nucleotides as general ligand chromatographic media, triazine dyes are, also, increasingly exploited in affinity chromatography. However, these sulphonated polyaromatic reactive molecules are relatively new tools in general ligand enzyme purification technology.

A typical structure of a triazine dye[117] comprises the following: a terminal sulphonated benzene ring (Fig. 6d) linked via a -NH-bridge to the triazine ring (Fig. 6c) containing one or two chlorine atoms depending on whether the dye belongs to the H- or MX- range, respectively, and the chromophore (Fig.

6ab) which is always linked to the trazine ring via a -NH- bridge. However, the structures of the majority of reactive dyes remains a closely guarded secret by the two main suppliers ICI (Procion range) and CIBA (Cibacron range). The triazinyl moiety substituted with one (Fig. 6c) or two chlorines is responsible for the high reactivity of the dyestuffs. Accordingly, triazine dyes can be immobilised to polysaccharide matrices [118,119] by means of nucleophilic displacement of the chlorine(s) [117] by the matrice's hydroxyls.

A large body of information has been accumulated over the last decade or so on the interaction of enzymes and proteins with the anthraquinone dye Cibacron Blue F3G-A (Procion Blue H-B, C.I. 61211). This dye selectively interacts with pyridine nucleotide-dependent dehydrogenases, kinases, CoA-dependent enzymes, hydrolases, polynucleotide-dependent enzymes, restriction endonucleases, synthetases and a number of blood proteins including serum albumin, clotting factors, lipoproteins, complement factors and interferon [17,120,121], and thus, has been used to purify biological molecules by means of affinity chromatography. Furthermore, recently Procion Red HE-3B is also increasingly employed in enzyme purification technology [119-124] as well as other triazinyl dyes [118,119,121,125].

Several studies have been performed, mainly with Cibacron Blue F3G-A, to establish the basis for these selective interactions [17,121]; kinetic inhibition studies of free dyes [119,126-132], adsorption spectrum differences of the dye caused in the presence of enzyme [133-138], induced circular dichroism studies [139,140], X-ray crystallographic studies [141], irreversible inactivation studies of nucleotide-dependent enzymes with various triazine dyes [138,142-144], post-immobilisation chemical modification studies of triazine dyes [145] and metal-ion promoted binding of several enzymes to triazine dyes [138,146].

It has been tentatively concluded that at least a part of the dye's structure (Fig. 6abc) mimics the naturally occuring biological heterocycles, such as nucleoside phosphates, NAD^+, CoA and folic acid, in terms of overall shape, charge and aromaticity [140], and accurately reflects coenzyme binding to the complementary enzyme [131,141,147]. Therefore, triazinyl dyes may be considered as nucleotide substitutes and used as pseudo-affinity general ligands for a large number of enzyme purification cases. Furthermore, it has been recently found that dyes may differentiate between different nucleotide-binding sites of the same enzyme [119]; this information can be exploited to design specific elution techniques [119].

V. Applications of Affinity Chromatography

In principle the technique can be applied whenever a specific interaction occures between any two molecules.

Affinity chromatography has been traditionally applied in separations and purifications of biological macromolecules and in particular for proteins that recognise nucleotide-coenzymes and triazine dyes as ligands [17,19,20,63,79,81,120,121,144].

The main powerful inherent advantages of affinity chromatography over conventional classical purification methods are its selectivity and simlicity of completion of work in a short time. Although the synthesis of general ligand affinity adsorbents may, sometimes, be laborious and uneconomical, however, this can be readily compensated by the fact that the adsorbents can be used succesfully for a number of different purification cases. Furthermore, the introduction of triazine dyes as general ligand media, along with their extremely low cost and ease of immobilisation [17,20], provided affinity chromatography with one more advantage over other techniques. Only some typical examples of the effectiveness of nucleotide and triazine dye ligands will be suffice.

Human serum lactate dehydrogenase could be purified, in a large scale, from crude extracts on agarose-bound N^6-(6-aminohexyl)-AMP, followed by elution with a NAD-pyruvate adducts, affording a 17,00-fold increase in specific activity in a single step [148]. Similarly, human lactate dehydrogenase could be purified 10,000-fold and 145-fold respectively from erythrocyte and liver extracts by chromatography on 8-immobilised AMP [149]. Purification to homogeneity of IMP dehydrogenase from crude extracts of *E. coli* provides another example of large scale purification. The enzyme was chromatographed on AMP [111] and IMP [96] affinity adsorbents, followed by elution with AMP and IMP gradients respectively, resulting in pure protein in one chromatographic step.

Other examples where triazine dye ligands are involved may be cited. Purification to homogeneity, on a preparative scale, of 3-hydroxybutyrate dehydrogenase in two succesive chromatographic steps on Procion Red H-3B and Procion Blue MX-4GD dye columns, afforded 70% yield [150]. This two-step affinity procedure substitutes a conventional purification method involving eight steps with an overall 9% yield [151]. The same ligand system may be used to purify malate dehydrogenase [150]. Furthermore, three t-RNA synthetases; methionyl-, tryptophanyl- and tyrosyl- were purified to homogeneity on immobilised Procion Green HE-4BD, Brown MX-5BR and Orange MX-G, respectively [125]. Conventional purification methods involved three further columns for tryptophanyl-tRNA synthetase and at least four for tyrosyl- and methionyl-tRNA synthetase [125].

Affinity chromatography has also been utilised in other, than enzyme, macromolecule separations, for example, in immunoseparations [152-155] and nucleic acid separations [156-158] where complementary antibodies and nucleic acids were used as ligands respectively, and separations of hormone-binding proteins [159] and membrane-bound receptor proteins [160-162]. Furthermore, affinity chromatography has been exploited in the isolation of supramolecular structures such as Influenza virus [163], lymphocytes [164,165] and other cells [166-168].

Besides its traditional use in separations and purifications, affinity chromatography may be employed in other purposes:

(i) Isoenzyme resolution : All five isoenzymes of lactate dehydrogenase have been resolved on immobilised N^6-(6-aminohexyl)-AMP, where elution

was effected with a concave gradient of NADH [169]. Similarly, horse liver alcohol dehydrogenase isoenzymes were resolved on the same adsorbent, following specific elution with NAD^+ and cholic acid mixture [170]. Malate dehydrogenase isoenzymes were separated, also, on the same adsorbent. The cytoplasmic enzyme passed through the column unretarded whilst the mitochondrial malate dehydrogenase was bound and subsequently eluted in a NADH gradient [171]. Finally, creatine kinase isoenzymes of green sunfish were resolved on Blue Sepharose CL-6B [172].

(ii) Removal of contaminants : Commercially available crude pyruvate kinase preparation containing lactate dehydrogenase can be purified to lactate dehydrogenase-free preparation on immobilised N^6-(6-aminohexyl)-AMP [173]. A similar procedure was used to remove an impurity of mitochondrial malate dehydrogenase from commercial preparation of pig heart cytoplasmic enzyme [171]. Furtheremore, an NAD adsorbent was used to remove various dehydrogenases associated with a preparation of cytochrome C oxidase [174]. Finally, Sepharose-bound Cibacron Blue F3G-A was capable of absorbing human serum albumin yielding albumin-depleted plasma [175,176], whereas, other dye adsorbents were utilised to remove contaminants from near homogeneous proteins [177].

(iii) Resolution of mutant proteins : Defective β-galactosidase forms produced from mutant strains of *E. Coli* have been studied by affinity chromatography [178], whereas inactive mutant forms of *E. coli* IMP dehydrogenase from guaB mutants have been purified on 8-(6-aminohexyl)-AMP following elution with a linear gradient of AMP [111]. Finally, resolution of wild type from mutant enzymes and subunits of protein aggregates, on immobilised Procion dyes, have been reported [179].

(iv) Concentrate diluted solutions : Diluted solutions of IgG may be concentrated on Sepharose-immobilised protein-A [180].

(v) Resolution of chemically modified forms frorm native proteins : Active site-modified staphylococal nuclease may be separated from inactive enzyme on agarose-bound deoxythymidine-3-(p-aminophenylphosphate)-5′-phosphate [181], whereas resolution of functionally inactive $NADP^+$-isocitrate dehydrogenase from active form on immobilised triazine dyes was reported [182].

(vi) Estimation of dissociation constants : Such information can be obtained either by elution analysis [183] or by frontal analysis [184]; the methods may be used to determine the dissociation constant of free and immobilised figand. However, since the interaction ligand-enzyme may, sometimes, be complicated as, for example, between lactate dehydrogenase and immobilised N^6-(6-aminohexyl)-AMP where a complex biphasic time dependance is observed [185], direct quantitative measurments may be difficult to make. A more qualitative approach to the estimation of dissociation constants is that reported by Brodelius and Mosbach (1976) [186] where a standard curved of dissociation constants, known from literature data, for various lactate dehydrogenases *versus* the concentration of NADH reqired to effect elution from

N^6-(6-aminohexyl)-AMP-agarose, was employed to determine the dissociation constant of NADH for various other unknown lactate dehydrogenases.

(vii) Studies on the binding of ligands to enzymes : The compulsory ordered mechanism of lactate dehydrogenase in which the pyridine nucleotide binds first, was confirmed by affinity chromatography[76]. Similarly, hexokinase only binds to immobilised ATP in the presence of the specific cosubstrate glucose. This supports an ordered reaction sequence where glucose is added first followed by MgATP[47]. Contrarily, the equal effectiveness of all ADP and glucosamine adsorbents tested for hepatic glucokinase, suggest that the kinetic mechanism for the binding of glucose and ATP to the enzyme is random and idependent[187].

(viii) Studies on ternary complex formation : It has been possible to demonstrate that lactate dehydrogenase bound to immobilised AMP will elute at lower NAD^+ concetrations when either lactate or pyruvate is included in the NAD^+ gradient[188]. Similar results were obtained with L-threonine dehydrogenase[189] and malate dehydrogenase[188] when L-threonine or L-malate/oxalacetete were included in the NAD^+ gradients. Such results support the formation of a ternary enzyme·NAD^+·substrate complex.

(ix) Studies on the nature and topography of binding sites : Isocitrate dehydrogenase displays no affinity for agarose-bound ADP[190] and $NADP^+$[92] substituted at the N^6 position, suggesting that this position is essential for the coenzyme binding to the enzyme. However, in many other cases N^6-substituted coenzymes may be used as efficient affinity adsorbents. Such information can establish whether gross difference in topography exist for different enzymes.

Malate dehydrogenase from two different sources, pig heart and *B. subtilis,* displayed a preferred specificity for N^6- and C8-immobilised AMP adsorbents respectively. It appears, therefore, that for the same enzyme but of different species, minor differences may exist and produce significant changes in the specificity for differently immobilised ligands.

Immobilised coenzymes may, also, be utilised to dinstiguih between catalytic and effector sites, as with pyridine nucleotide transhydrogenase[191], or to establish whether nucleotides may bind at the same or different sites of a particular enzyme, as with $NAD(P^+)$-dependent D-galactose dehydrogenase[192].

Finally, information concerning the relative affinity of coenzyme fragments for the same enzyme binding site, may be gained by affinity chromatography. Pig heart lactate dehydrogenase and horse liver alcohol dehydrogenase have been shown that bind strongly to all fragments greater than AMP and very weak to lower fragments[192]. Such studies in cojunction with studies on the ionic, hydrophobic and hydrogen bonding interactions between coenzyme fragments and enzyme, may make possible to map the topography of the coenzyme binding site.

(x) Biomedical applications : Affinity chromatography is proving to be

useful for the selective removal of unwanted material from the blood of patients by means of extracorporeal blood treatment. It is essential, however, to make sure that the adsorbent is blood compatible and prevented from releasing contaminants into the patient's blood. This is achieved when articicial cells are employed.

A number of synthetic immunoadsorbents may be used to remove specific antigens and antibodies from the blood, whereas bilirubin can be also removed by extracorporeal hemoperfusion through agarose-bound human serum albumin. Other substances associated with clinical toxicity, such as digitoxin, cholic acid and salicylate, may also be removed.

It is worth mentioning that the affinity concept has found application in many relevant biochemical techniques [17,31], such as :

Covalent chromatography
Hydrophobic chromatography
Charge transfer and metal-chelate affinity chromatography
Affinity density perturbation
Affinity electrophoresis
Affinity partition
Affinity precipitation
Affinity electrodes
High performance liquid affinity chromatography
Affinity histochemistry.

Περίληψη

Χρωματογραφία Συγγενείας

Τήν τελευταῖα δεκαετία ἡ Χρωματογραφία Συγγενείας (Affinity Chromatography) ἔχει συμβάλλει ἀποφασιστικά στόν καθαρισμό καί ἀπομόνωση βιολογικῶν μακρομορίων καί ἔχει μονοπωλήση τήν προτίμηση τῶν ἐρευνητῶν σάν τεχνική στόν τομέα αὐτό.

Ἡ ἀρχή τῆς τεχνικῆς αὐτῆς βασίζεται στή δυνατότητα σχηματισμοῦ ἀντιστρεπτῶν βιοσυμπλόκων, ὅπως π.χ. μεταξύ ἐνζύμου καί συνενζύμου· τό τελευταῖο ἀκινητοποιῆται (immobilised) συνήθως ἐπάνω σέ ἕνα κατάλληλο ἀδιάλυτο ὑδρόφιλο πολυμερές, τό «ὑπόστρωμα» (matrix), καί ἀπό τήν θέση αὐτή ἀναγνωρίζει ἐκλεκτικά, ἀνάμεσα σέ ἄλλα, τό ἀντίστοιχο ἔνζυμο τό ὁποῖο καί δεσμεύει δυναμικά. Στήν προκειμένη περίπτωση τό συνένζυμο ὀνομάζεται «συγγενῆς» (affinant, ligand) τό δέ ἔνζυμο «συμπληρωματικός (complementary) συγγενῆς ἤ βιομόριο». Ὁ συγγενῆς ἀκινητοποιῆται χημικά μέ ἕνα «βραχίονα» (arm, spacer) στό ὑπόστρωμα τό ὁποῖο ἔχει προηγουμένως ἐνεργοποιηθεῖ (activated) χημικά.

Τά βασικά συστατικά ἑνός συστήματος χρωματογραφίας συγγενείας εἶναι τρία: τό ὑπόστρωμα, ὁ βραχίονας καί ὁ συγγενῆς, καί τά ὁποῖα

ἐνώνονται μεταξύ τους μέ διάφορες μεθόδους ὀργανικῆς χημείας. Συνήθως σχηματίζουμε πρῶτα τό σύμπλοκο συγγενῆ-βραχίονα καί κατόπιν τό ἀκινητοποιοῦμε στό ἐνεργοποιημένο ὑπόστρωμα.

Τό ὑπόστρωμα πρέπει νά πληρῆ ὁρισμένες ἰδιότητες τίς ὁποῖες ὅμως τά διάφορα ὑποστρώματα πληροῦν σέ διαφορετικό βαθμό. Ἡ ἀγαρόζη (agarose, Sepharose) εἶναι εὐραίως διαδεδομένη ἀλλά καί ἄλλα φυσικά πολυσάκχαρα ὅπως κυτταρίνη (cellulose) καί δεξτρᾶνες (dextrans, Sephadex), καί συνθετικά πολυμερῆ δυνατόν νά χρησιμοποιηθοῦν. Οἱ φυσικές καί χημικές ἰδιότητες τοῦ ὑποστρώματος εἶναι βασικοί παράγοντες γιά τήν ἐπιτυχία τῆς τεχνικῆς. Ὁ βραχίονας ἀπαιτεῖ ἰδιαίτερη προσοχή ὡς ἀναφορά τό μῆκος του καί τήν πολικότητά του. Τέλος, ὁ συγγενῆς καί ὁ τρόπος ἀκινητοποιήσεώς του εἶναι καθοριστικῆς σημασίας γιά τήν τεχνική διότι ἐπηρεάζει ἄμμεσα τήν ἐπιτυχία ἀλληλεπίδρασης (interaction) συγγενῆ — συμπληρωματικοῦ βιομορίου.

Ὁ συγγενῆς δυνατόν νά εἶναι ὑψηλῆς εἰδικότητας (specific ligand), ὡς ἀναφορά τό συμπληρωματικό βιομόριο, ἤ δυνατόν νά εἶναι εἰδικός γιά μία ὁμάδα βιομορίων (group specific ligand ἤ general ligand). Στή δεύτερη κατηγορία συμπεριλαμβάνονται καί τά νουκλεοτίδια (nucleotides) καί δραστικές χρωστικές (reactive dyes, triazine dyes) μέ τά ὁποῖα καί ἀσχολούμαστε στήν παροῦσα ἐργασία.

Ὁπωσδήποτε ἡ χρωματογραφία συγγενείας χρησιμοποιῆται ἰδιαίτερα ἐκτεταμένα καί ἀποτελεῖ τήν πλέον ἐκλεπτισμένη καί εἰδικευμένη τεχνική διαχωρισμοῦ καί καθαρισμοῦ βιολογικῶν (μακρο)μορίων καί δομῶν ὅπως π.χ. πρωτεϊνῶν, ἐνζύμων, (πολυ)πεπτιδίων, γλυκοπρωτεϊνῶν καί πολυσακχαριτῶν, λιποπρωτεϊνῶν, πολυνουκλεοτιδίων καί νουκλεϊκῶν ὀξέων, ἀντιγόνων καί ἀντισωμάτων, ὁρμονῶν καί ὑποδοχεῖς ὁρμονῶν, κυττοπλασματικῶν ὀργανιδίων, ἰῶν καί κυττάρων, ἡ βασική της δέ ἀρχή βρίσκει ἐφαρμογές σέ παρεμφερεῖς τεχνικές τῆς βιοχημείας.

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THE DETERMINATION OF MILK SERUM SOLIDS-NOT-FAT IN FORTIFIED MILK USING THE CRYOSCOPE

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Summary

Fortified and unfortified lowfat and nonfat milks were analysed for freezing point and milk serum solids-non-fat. Data were statistically evaluated for possible correlation. Results indicate that freezing point can be used effectively as a quality control tool for monitoring amounts of added milk solids-not-fat in fortified milk products.

Experience in our laboratory has demonstrated that solids-not-fat determinations on individual batches of Nuform, Balance and Fortified Fat Free milk products can be completed in less than two minutes with precision and accuracy comparable to that of the Mojonnier method.

Key words : Cryoscope, Milk serum solids-not-fat, Milk solids-not-fat.

Introduction

The freezing point of cow's milk has been recognized as one of its most constant values, normally varying between -0.530^{0}C and -0.566^{0}C on individual cow samples[1,2]. It is known that the freezing point of a liquid is governed by the number of molecules in solution and not the weight of the molecules[3,4]. The freezing point of milk, like that of any aqueous system, also depends on the concentration of water – soluble constituents. The lower freezing point of milk than that of water (1.86 ^{0}C) is caused (75-80%) by the lactose and chlorides in solution[5]. In addition a complementary relationship exists between lactose and sodium chloride in milk such that the freezing point is maintained within a narrow range.

Freezing point has been used for many years as a tool for detecting added water in milk. The principle is based upon studies that show that as the milk is diluted by the addition of water, its freezing point tends to approach that of water. It follows then, that the reverse should be true; i.e., the freezing point depression should increase as the solids in solution are increased. Since

freezing point depression is dependent upon the molecular concentration of the milk serum, it is unaffected by variations in fat content.

Until Baer and Lowenstein published their work in September, 1977[6], little research had been done on the relationship of freezing point depression to added nonfat milk solids. Their studies demonstrated that a high correlation does indeed exist between freezing point and added solids-not-fat when using nonfat milk and specific levels of fortification with nonfat dry milk. It was felt that an adaptation of their technique might become a useful quality control tool, and this paper describes our approach to the subject and our findings and conclusions.

Method and Materials

Seventy-two samples of fortified lowfat and nonfat milk were prepared using skim milk, raw standardized whole milk and condensed skim milk so as to contain milkfat and milk solids-not-fat levels corresponding to the specifications of the three commercially available fortified lowfat products; Balance, Nuform and Silouet. Solids-not-fat levels were varied over a wide range in order to give us a broad picture of the effectiveness of the test. The samples were analyzed for milkfat by the Automated Light Scattering Method (Foss Milkotester; AOAC 16.059 - 16.064, calibrated to Roese-Gottlieb; AOAC 16.055). Total solids were determined by the Mojonnier method[7]. Milk serum solids-not-fat (MSNNF) were then calculated using the following ebuation :

$$\% \text{ MSSNF} = \frac{\text{Total Solids - Milkfat}}{\text{100 - Milkfat}} \times 100$$

Freezing points were determined by using the Advanced Cryomatic Cryoscope Model 4CII (AOAC 16.089 - 16.092) obtained from Advanced Instruments, Inc., Needham Heights, Massachusetts.

Results and Discussion

Of the seventy-two samples tested, thirty-one had fat and solids levels corresponding to Nuform; twenty-nine had levels corresponding to Balance, and twelve had levels comparable to Silouet Fortified Nonfat. The samples were tested for MSSNF and freezing point (Table I), and the test results were plotted (Fig. 1). Performing a statistical regression on the data, the slope and the intercept of the most probable straight line through the cloud of points (Fig. 1) was determined. With this regression equation we were able to forecast an estimated MSSNF value for each sample using its freezing point.

TABLE I : Freezing Point vs. Milk Serum Solids-Not-Fat

Case No.	Freezing Point (-C^0)	MSSNF %	Case No.	Freezing Point (-C^0)	MSSNF %
1	0.532	8.88	37	0.590	9.77
2	0.536	8.92	38	0.603	9.88
3	0.573	9.50	39	0.613	10.07
4	0.578	9.64	40	0.614	10.10
5	0.576	9.52	41	0.616	10.12
6	0.585	9.69	42	0.614	10.16
7	0.601	9.89	43	0.626	10.17
8	0.604	9.95	44	0.615	10.18
9	0.610	10.11	45	0.622	10.21
10	0.617	10.14	46	0.616	10.19
11	0.615	10.16	47	0.623	10.29
12	0.622	10.19	48	0.627	10.28
13	0.626	10.30	49	0.617	10.24
14	0.631	10.31	50	0.625	10.38
15	0.642	10.33	51	0.635	10.39
16	0.521	10.31	52	0.635	10.41
17	0.623	10.34	53	0.630	10.43
18	0.628	10.40	54	0.647	10.51
19	0.625	10.41	55	0.635	10.56
20	0.624	10.46	56	0.642	10.60
21	0.626	10.47	57	0.648	10.60
22	0.638	10.49	58	0.654	10.64
23	0.639	10.52	59	0.629	10.30
24	0.635	10.56	60	0.629	10.35
25	0.645	10.57	61	0.539	8.98
26	0.641	10.60	62	0.542	8.99
27	0.646	10.68	63	0.586	9.79
28	0.650	10.72	64	0.587	9.81
29	0.650	10.78	65	0.594	9.86
30	0.659	10.89	66	0.604	9.95
31	0.662	10.89	67	0.607	9.99
32	0.536	9.02	68	0.610	10.05
33	0.539	9.07	69	0.625	10.23
34	0.576	9.58	70	0.625	10.26
35	0.580	9.66	71	0.643	10.54
36	0.579	9.68	72	0.649	10.59

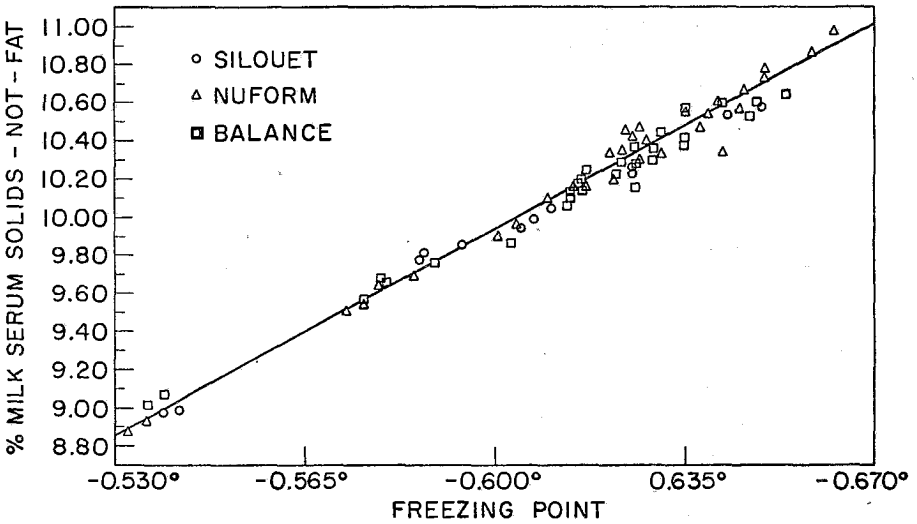

FIG. 1 : *Milk Serum Solids-Not-Fat (Mojonnier) vs. Freezing Point.*

These estimated values were compared with the observed values (Table II) and statistically analyzed (Table III). Figure 2 is a graphic comparison, prepared by computer, illustrating a comparison between the observed and estimated MSSNF values.

Statistical analysis indicated an extremely high correlation between freezing point and MSSNF (0.989). Replicate freezing point determinations by the cryoscope rarely vary by more than $\pm 0.002^0$C, indicating that the analyses are very reproducible.

Regression analysis yielded the following equation :

$$\% \text{ MSSNF} = 14.98611\,(|\text{F.P.}|) + 0.93545$$

On the basis of this equation, it can be seen that for every 0.10% increase in MSSNF the freezing point will be depressed by 0.0067^0. Conversely, for every 0.001^0 change in the freezing point, a 0.015% change in the level of MSSNF is indicated. These observations agree with those of Baer and Lowenstein. However, since their study dealt only with fortified nonfat milk in which the milkfat levels were both constant and negligible, Baer and Lowenstein did not make special note of the fact that the estimate reflects specifically the level of milk serum solids-not fat rather than solids-not-fat (SNF). In order to convert our estimate of MSSNF to SNF, the milkfat in the sample must be determined and the following equation employed :

$$\% \text{ SNF} = \frac{\% \text{ MSSNF}\,(100 - \% \text{ Fat})}{100}$$

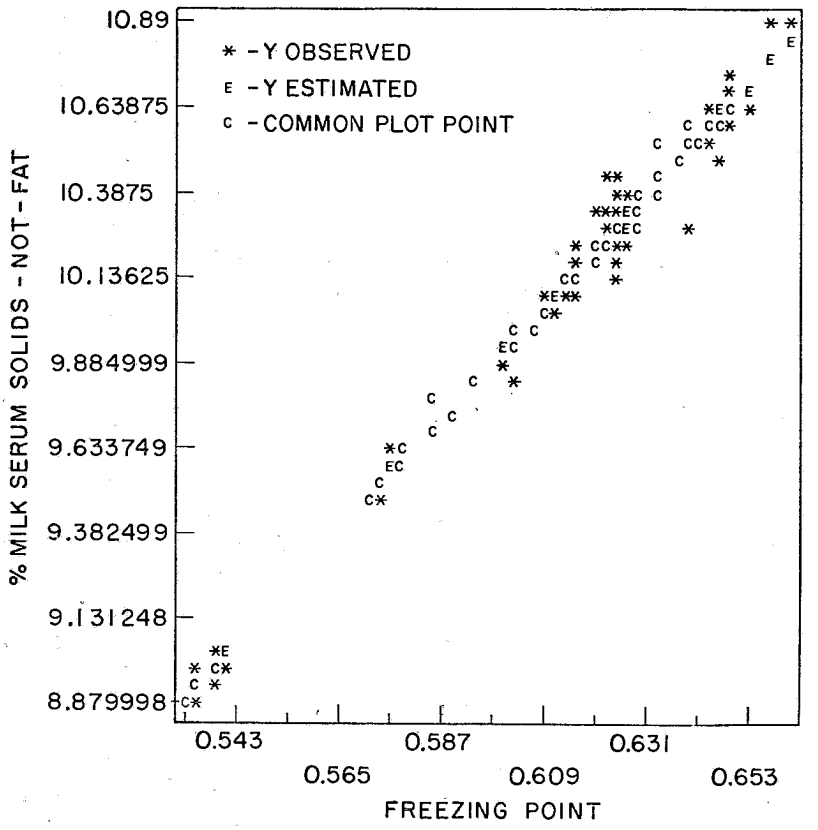

FIG. 2 : *A comparison of the estimated and observed milk serum solids-not-fat levels in seventy-two samples of lowfat fortified milks.*

We had been apprehensive about factors beyond our control that might alter the freezing point/MSSNF relationship in raw milk supply. It is well known that seasonal variations in the composition of milk can be expected. The most dramatic change occurs in the spring and early summer when there is a sharp decrease in total solids. Changes in milkfat content account for the major portion of this variation[8,9]; however, nonfat solids variations in the sera significantly affect the total solids content of the milk[10]. Furthermore, these changes are not signaled by corresponding fluctuations in freezing point[11]. As demonstrated by Stubbs and Elsdon[12], the average freezing point of a large population (1000) of cows' milks varies by less than $\pm 0.002\,^{0}C$ in the course of a year.

It was clear that what was needed was a method by which the regression equation could be adjusted so as to reflect the changing MSSNF levels. Empirical adjustment of the intercept appears to solve the problem. Triplicate determinations of milkfat content, total solids by Mojonnier and freezing

TABLE II : Observed vs. Estimated MSSNF

Case No.	Observed	Estimated	Residual	Case No.	Observed	Estimated	Residual
1	8.88	8.908	-0.028	37	9.77	9.777	-0.007
2	8.92	8.968	-0.048	38	9.88	9.972	-0.092
3	9.50	9.522	-0.022	39	10.07	10.122	-0.052
4	9.64	9.597	0.043	40	10.10	10.137	-0.037
5	9.52	9.567	-0.047	41	10.12	10.167	-0.047
6	9.69	9.702	-0.012	42	10.16	10.137	0.023
7	9.89	9.942	-0.052	43	10.17	10.317	-0.147
8	9.95	9.987	-0.037	44	10.18	10.152	0.028
9	10.11	10.077	0.033	45	10.21	10.257	-0.047
10	10.14	10.182	-0.042	46	10.19	19.167	0.023
11	10.16	10.152	0.008	47	10.29	10.272	0.018
12	10.19	10.257	-0.067	48	10.28	10.332	-0.052
13	10.30	10.317	-0.017	49	10.24	10.182	0.058
14	10.31	10.392	-0.082	50	10.38	10.302	0.078
15	10.33	10.557	-0.227	51	10.39	10.452	-0.062
16	10.34	10.242	0.098	52	10.41	10.452	-0.042
17	10.34	10.272	0.068	53	10.43	10.377	0.053
18	10.40	10.347	0.053	54	10.51	10.631	-0.121
19	10.41	10.302	0.108	55	10.56	10.452	0.108
20	10.46	10.287	0.173	56	10.60	10.557	0.043
21	10.47	10.317	0.153	57	10.60	10.646	-0.046
22	10.490	10.497	-0.007	58	10.64	10.736	-0.096
23	10.52	10.512	-0.008	59	10.30	10.362	-0.062
24	10.56	10.452	0.108	60	10.35	10.362	-0.012
25	10.57	10.601	-0.031	61	8.98	9.013	-0.033
26	10.60	10.542	0.058	62	8.99	9.058	-0.068
27	10.68	10.616	0.064	63	9.79	9.717	0.073
28	10.72	10.676	0.044	64	9.81	9.732	0.078
29	10.78	10.676	0.104	65	9.86	9.837	0.023
30	10.89	10.811	0.079	66	9.95	9.987	-0.037
31	10.89	10.856	0.034	67	9.99	10.032	-0.042
32	9.02	8.968	0.052	68	10.05	10.077	-0.027
33	9.07	9.013	0.057	69	10.23	10.302	-0.072
34	9.58	9.567	0.013	70	10.26	10.302	-0.042
35	9.66	9.627	0.033	71	10.54	10.572	-0.032
36	9.68	9.612	0.068	72	10.59	10.661	-0.071

TABLE III : Statistical Analysis

a) Regression Analysis

Intercept	0.93545
Regression coefficient	14.98611
Standard error of regression coefficient	0.267
Computed T-value	56.081
Correlation coefficient	0.989
Standard error of estimate	0.071
Average difference of estimate	0.000

b) Analysis of Variance

Source of Variation	Degree of Freedom	Sum of Squares	Mean Square	F Value
Attributable to regression	1	15.786	15.786	3145.076
Deviation from regression	70	0.351	0.005	
Total	71	16.137		

point are made routinely on fortified lowfat milks, and the actual versus the estimated MSSNF values are compared. The regression equation is then adjusted, if necessary, by employing the new intercept as determined by the following formula :

$$I = \frac{100(S - F)}{100 - F} - (14.986 \times F.P.)$$

Where : I = intercept
S = total solids by Mojonnier
F = milkfat content
F.P. = freezing point absolute value

The action of monitoring the actual MSSNF levels in the milk supply has the added advantage of helping to explain seasonal variations in ingredient usage.

It has proven to be a simple matter to incorporate these new test procedures into our quality control program. Backup testing by Mojonnier during the trial period increased our confidence in testing production samples.

The reproducibility of the test has been impressive during routine quality control operations and its speed of great values to the processing group.

Provided that an adequate amount of data is accumulated on milkfat and solids for a manufacturing location, new intercept values can be determined for different milk pools. This makes the method readily adaptable to any processing plant.

It is known that the instrumentation operates very well in the laboratory; perhaps the system could be adapted to an «on-line» installation capable of adjusting milk solids non-fat levels automatically.

Περίληψη

Κρυοσκοπικός Προσδιορισμός τοῦ Ἄνευ Λίπους Ξηροῦ Ὑπολείμματος Γάλακτος

Ἡ περιεκτικότης γάλακτος εἰς ἄνευ λίπος ξηρό ὑπόλειμμα προσδιορίσθη διά τῆς μεθόδου Mojonnier. Τό σημεῖο πήξεως ἐπίσης ὑπολογίσθηκε διά κρυοσκοπίου (Πίναξ I).

Στατιστική ἀνάλυσις τῶν ἀποτελεσμάτων (ἁπλή εὐθύγραμμη συμμεταβολή) ἔδωσε τήν κάτωθι ἐξίσωση :

$$\% \text{ M.S.S.N.F.} = 14{,}98511\,(\text{IF.P.I}) + 0{,}93545$$

ὅπου : % M.S.S.N.F. = % ἄνευ λίπους ξηρόν ὑπόλειμμα γαλακτικοῦ πλάσματος.
F.P. = σημεῖον πήξεως γάλακτος (σ.π.).

Ἀνάλυσις τῆς ἐξισώσεως δεικνύει ὅτι διά κάθε 0,1% αὔξηση τοῦ M.S.S.N.F. τό σ.π. ἐλαττοῦται κατά $0{,}0067^0$. Ἄνευ λίπους ξηρόν ὑπόλειμμα γάλακτος δύναται νά προσδιορισθῆ χρησιμοποιόντας τήν ἐξίσωση :

$$\% \text{ S.N.F.} = \frac{\% \text{ M.S.S.N.F.}\,(100 - \% \text{ B.F.})}{100}$$

ὅπου : % S.N.F. = % ἄνευ λίπους ξηρόν ὑπόλειμα γάλακτος.
% B.F. = % λίπος γάλακτος.

Στατιστική ἀνάλυση τῶν μετρήσεων διά Mojonnier καί τῶν ὑπολογισθέντων ἄνευ λίπους ξηροῦ ὑπολείμματος χρησιμοποιόντας τό σ.π. δεικνύει ἁπλή εὐθύγραμμη συμμεταβολή τῶν δύο τιμῶν μέ ἐξαιρετικά ὑψηλή συχνότητα (98,9 %).

Ἡ μέθοδος εἶναι ἁπλή, γρήγορη καί ἀκριβής καί δύναται νά χρησιμοποιηθῆ εἰς ἐργαστήρια ποιοτικοῦ ἐλέγχου γάλακτος μέ ἤδη ὑπάρχοντα ὄργανα ἀναλύσεως.

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EMF MEASUREMENTS IN NON-AQUEOUS MEDIA. (a) STANDARD ELECTRODE POTENTIAL DETERMINATION OF THE SILVER-SILVER CHLORIDE ELECTRODE IN NON-AQUEOUS ALCOHOLIC SOLUTIONS AT 15,20 AND 30 °C. (b) ION PRODUCT OF ALIPHATIC ALCOHOLS AT 15, 20 AND 30 °C.

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Summary

The standard emf of the silver-silver chloride electrode in EtOH, PrOH, i-PrOH, BuOH and i-BuOH solutions at 15, 20 and 30 °C, has been determined and its thermal coefficient computed.

The ion product of EtOH, PrOH, i-PrOH, BuOH and i-BuOH at 15,20 and 30 °C has been evaluated. The experiments were carried out by an electrometric method, where a cell without transferrence, consisted of a platinum and a silver-silver chloride electrode, has been used.

Key words : Standard emf, silver-silver chloride electrode, hydrogen electrode, non-aqueous solvents, ion product, thermal coefficient.

Abbreviations

R : C_2H_5-, C_3H_7-, i-C_3H_7-, C_4H_9-, i-C_4H_9-,
M : Li, Na,
I : ionic strength
D : dielectric constant
d_0 : density of the pure solvent
A′ : $A.d_0^{1/2}$
B′ : $B.d_0^{1/2}$
α : mean distance of closest approach
k : $\frac{2.3.RT}{F}$

EtOH : C_2H_5OH
PrOH : C_3H_7OH
i-PrOH : i-C_3H_7OH
BuOH : C_4H_9OH
i-BuOH : i-C_4H_9OH

Introduction

Whereas the ionization constant (K) of water has been determined with great accuracy and at a large extent of temperatures, small number of measurements concerning the determination of the ionization constants of solvents other than water, has been reported. This could be attributed to the big experimental difficulties which arise at the handling of non-aqueous solvents. Considering the first aliphatic alcohols, only the K of MeOH has been determined at a series of temperatures (0-45°).[1] The K of EtOH has been estimated at 20°,[3] and at 25°.[2,3] and of PrOH and i-PrOH at 20 and 25°.[3]

The method used in all cases was an electrometric one. It can be seen that the above mentioned measurements do not cover the subject of the acidic character of these solvents. In this work is presented a more systematic study of the ionization constant of aliphatic alcohols. The K of MeOH has not been redeterminated, as the temperature range covered, is large. The measurements in EtOH, PrOH i-PrOH at 20° have been repeated. That way a comparison is possible which leads to conclusions about the experimental difficulties and the precautions taken. The ionization constant determinated concerned the EtOH, PrOH, i-PrOH, BuOH, i-BuOH at 15,20 and 30 °C.

The first part of this work is consisted of the determination of the standard electrode potential (E*) of the silver-silver chloride electrode in each solvent and temperature.

The results obtained by different investigators appear in Table I. It is

TABLE I : Standard electrode potential of the silver-silver chloride electrode in non-aqueous aliphatic alcohols

E^*_m (volt)	t °C	solvent	ref.
-0.0098	25	MeOH	1
-0.0099	25	MeOH	5
-0.04462	25	EtOH	6
-0.0365	25	EtOH	7
-0.0883	25	EtOH	8
+0.02190	25	EtOH	9
-0.08138	25	EtOH	10
-0.079	25	EtOH	4
-0.0723	25	EtOH	3
-0.065	20	EtOH	3
-0.102	25	PrOH	3
-0.092	20	PrOH	3
-0.122	25	i-PrOH	3
-0.0995	20	i-PrOH	3

apparent that a serious discrepancy exists as to the value of E* in EtOH.

Various systematic errors arise in the emf measurements of non-aqueous solutions. Concerning the EtOH, the most important of them, seems to be the influence of traces of water, and for that reason appears a large number of measurements in this solvent. It is reported that to attain an accuracy of 0.1 mV in E*, the molality of water should be less than 2.10^{-6} m, practically unachievable[4]. Other systematic errors can be, the oxidation of solvent by the air (which has an opposite effect on the emf than the influence of traces of water) and, the reaction of EtOH with HCl. The emf of other non-aqueous cells does not depend so seriously on the traces of water. The oxidation of the solvent by the air, and the poisoning of the electrode(s) by the solvent, are other effects which may affect the accuracy of the results. Thus it seems that the difference between precision and accuracy in non-aqueous emf measurements, often is large.

From the above mentioned it is obvious that a redetermination of E* was necessary, as the results depend highly on the way of execution of the experiment, on the kind of solvent and on the precautions taken to avoid the possible errors. So it was determined the E* for the already mentioned alcohols at 15,20 and 30 °C. From these values can be calculated the temperature coefficient of the standard emf of the silver-silver chloride electrode in these solvents; the only available estimate appears to be in EtOH [11].

Theoretical part

(a) Determination of the standard emf (molal), of the silver-silver chloride electrode, in HCl-abs.ROH solutions. The cell used was :

$$Pt,H_{2(p\,atm)} \mid HCl_{(m)},ROH \mid AgCl \mid Ag$$

The measured emf was corrected to one atmosphere partial pressure of the hydrogen gas. This value was called the «observed emf», and was related to the molality of the solution, with the equation

$$E = E^* - \frac{RT}{F} \ln m_{H^+} m_{Cl^-} \gamma_{H^+} \gamma_{Cl^-} \qquad (1)$$

$$E = E^* - \frac{2RT}{F} \ln m_{\pm} - \frac{2RT}{F} \ln \gamma_{\pm} \qquad (1a)$$

The mean ion activity coefficient is given by the Debye-Hückel extended type equation

$$-\log \gamma_{\pm} = \frac{A\,(I\,d_0)^{1/2}}{1 + \alpha B\,(I\,d_0)^{1/2}} - \beta I \qquad (2)$$

which for a single electrolyte and dilute solution reduces to

$$-\log \gamma_{\pm} = \frac{A (m d_0)^{1/2}}{1 + \alpha B (I d_0)^{1/2}} - \beta m \qquad (2a)$$

A and B were calculated from the equations[20]

$$A = 1.82455.10^6 (DT)^{-3/2}, \qquad B = 50.2904.10^8 (DT)^{-1/2}$$

The dielectric constants and the densities of the pure solvents at various temperatures were taken from reported values[12]. The value of α was 3.99 Å. It was used the same value for all solvents and temperatures of this work. The parameter β is the «interaction coefficient». Combining equations (1a) and (2a) is obtained

$$E = E^* - 2k \log m + 2k \frac{A (m d_0)^{1/2}}{1 + \alpha B (m d_0)^{1/2}} - 2k\beta m \qquad (3)$$

A quantity $E^{*\prime}$ is defined by :

$$E^{*\prime} \equiv E + 2k \log m - 2k \frac{A' m^{1/2}}{1 + \alpha B' m^{1/2}} \qquad (3a)$$

and equation (3) becomes,

$$E^{*\prime} = E^* - 2k\beta m \qquad (4)$$

A plot of $E^{*\prime}$ against molality (m), yields a straight line of intercept E^* on the $m = 0$ axis, and of slop $2k\beta$, from which β can be calculated. Once E^* is known, $\gamma \pm$ can also be calculated from the equation (1a).

(b) Determination of the ion product of aliphatic alcohols. The ion product of an aliphatic alcohol is given by the equation

$$K = \alpha_{RO^-} \times \alpha_{ROH_2^+}$$

The galvanic cell used was of the type

$$Pt, H_{2(p\,atm)} \mid MOR_{(m_{RO^-})}, MCl_{(m_{Cl^-})} \mid AgCl \mid Ag$$

where (m_{RO^-}) and (m_{Cl^-}), are the molalities of the alcoxide and the chloride salt of Na or Li, respectively.

The «observed emf» is given by the equation

$$E = E^* - \frac{RT}{F} \ln K - \frac{RT}{F} \ln \frac{\alpha_{Cl^-}}{\alpha_{RO^-}} \tag{5}$$

which can be also written, as,

$$E = E^* - \frac{RT}{F} \ln K - \frac{RT}{F} \ln \frac{m_{Cl^-}}{m_{RO^-}} - \frac{RT}{F} \ln \frac{\gamma_{Cl^-}}{\gamma_{RO^-}} \tag{6}$$

A quantity $E^{*\prime\prime}$ is defined as

$$E^{*\prime\prime} \equiv \frac{E - E^*}{k} + \log \frac{m_{Cl^-}}{m_{RO^-}} \tag{6a}$$

thus equation (6) becomes

$$E^{*\prime\prime} = -\log K - \log \frac{\gamma_{Cl^-}}{\gamma_{RO^-}} \tag{7}$$

The term $\log \frac{\gamma_{Cl^-}}{\gamma_{RO^-}}$ is directly proportional to the ionic strength[13], in dilute solutions, and a plot of the $E^{*\prime\prime}$ against (I) should yield (–log K) by linear extrapolation to infinite dilution.

Experimental

Cell design and apparatus. The cell was made of Pyrex glass and was designed in a special way, so that a possible diffusion of the hydrogen gas from the hydrogen electrode to the silver-silver chloride electrode compartment, be prevented.

The electrodes were connected to the cell through ground joints, so that no contact with the air be possible. The glass cell, as well as, a presaturator bubbler for the incoming hydrogen, were immersed in a thermostat maintained at $t \pm 0.05\,^{\circ}C$. The cell contained about 55 cm^3 of the solution and the pressaturator 150 cm^3.

The emf measurements were carried out using a Cambridge Vernier type potensiometer, equipped with a Cambridge Standard cell and a mirror type galvanometer. The precision of the system was 0.001 mV.

All density measurements of the solutions were made by a thermostated digital densitometer of Anton Paar K.G. DMA 02C Type.

Electrodes. The hydrogen electrodes were prepared from a platinum foil, of total area 2 cm^3 and thickness 0.015 cm, which was platinized according to the Hills and Ives method [14]. They were washed in de-ionized water and stored in dilute hydrochloric acid solution. Before their use they were pre-soaked in an alcoholic-hydrochloric acid solution, of the same concentration to the one used. The hydrogen gas (of purity 99.999%), during a measurement, was presaturated by bubbling through a solution of the same concentration and of double volume, before passing into the electrode compartment.

An hydrogen flowing rate of one bubble per second, was maintained throughout each experiment.

The test of equilibrium was a constant emf value for one hour. It was observed that the time required for obtaining a stable emf value, was increasing with the number of measurements; that could be attributed to the electrode becoming inert. Thus a cleaning and replatinizing of the electrode surface became necessary before any new experiment.

The silver-silver chloride electrodes were of the «silver mirror» type [15,16]. They were prepared from a platinum foil of a total area 2 cm^3 and thickness 0.015 cm, on which silver was deposited by the Rochelle salt mirror process [17]. The silver coating was anodized in dilute hydrochloric acid solution for 2-3 min, at a current density of 1.0 $mA.cm^{-2}$. A conversion of 15-25% of Ag to AgCl was achieved. This type of electrode has been used to an excellent effect [18], as it is free from aging effects, very well reproduced (better than 0.05 mV) [15,16]. It is recommended for use in media of low dielectric constants. The present work consists the first use of the mirror-type electrode in non-aqueous media.

In these experiments two electrodes were used, chosen from a group which showed the lowest bias potentials against an aged one. Storage was achieved by transfer to an alcoholic-hydrochloric acid solution.

Concerning the reproducibility and the long life of the electrode, the following precautions were taken :

(i) The electrodes were stored in a dark place and the experiments were carried out in a dark room, as the light could influence the percentage of the silver on the surface.

(ii) The solutions were deoxygenated before their use, by bubbling N_2 gas through them, because the air dissolved could cause a slow oxidation of the silver, in the acidic solutions.

(iii) A diffusion of the hydrogen gas from the hydrogen electrode to the silver-silver chloride compartment, was hindered by a special design of the measuring cell, as the hydrogen could cause reduction of the silver in the AgCl.

The two electrodes used were frequently checked and were proved to be of an extremely good reliability, up to the end of the present work.

Thus, provided that the precautions mentioned are taken, the use of this type of electrode in low-dielectric media, is confirmed. The bias potential was very low ($\pm$ 0.02 mV), and the reproducibility extremely good, as it didn't need a repreparation throughout all series of experiments in this work.

Chemicals

The starting alcohols were of pA grade. They were subject to purification by fractional distillation in an all-glass system. A Na-Hg amalgam was introduced into the distillate, so that the last traces of water be moved.

The hydrochloric acid solutions were prepared by bubbling HCl gas, directly into the alcohol in the glass vessel. Anhydrous hydrogen chloride was prepared in an all glass apparatus, by introducing concentrated sulphuric acid on sodium chloride, and dehydrated by passing through a series of glass containers with $CaCl_2$, H_2SO_4 and P_2O_5, in order to be fully dehydrated. An amount of about 50 cm^3 absolute alcohol was introduced from the distillation apparatus in the glass vessel, which was connected to the last part of the hydrogen chloride production apparatus. The gas produced was permitted to bubble in the alcohol for some minutes. Afterwards the vessel was closed and put in the thermostat. This way any contact of the solution with the air was avoided. The hydrochloric acid solutions were prepared just before their use.

The alcoxide solutions were prepared by direct addition of the metal in the absolute alcohol (Na in ethanol and Li in the other alcohols). The process took place in a glove box, in a nitrogen atmosphere. The stoke solutions were diluted in conceivable molalities and the title was determined titrimetrically. The alcoxide solutions were prepared at the same time of the experiment.

Salt, alcoholic solutions (NaCl in ethanol and LiCl in the other alcohols).

Stoke solutions were prepared by dilution of p.a. grade salt in the alcohol. The title was determined gravimetrically. The solutions were rejected every second day and new ones were prepared.

Procedure

(a) The hydrochloric acid-abs. alcohol solution was prepared directly in the glass vessel as previously described, put in the thermostat and connected to the hydrogen gas bubblers. After some minutes of the hydrogen flow, the electrodes were introduced into the measuring cell and, emf measurements were taken every 2-3 min. Equilibrium was reached when the emf values were stable within 0.00003 volt for 60 min.

Afterwards the title of the solution was estimated by titration with a NaOH standard solution; no difference was found with the titration done before the measurement. The solutions were of random concentrations so that systematic errors to be avoided.

(b) A mixture of alcoxide and salt solution, of total ionic strength not exceeding 0.13 m, consisted the solution to be measured, for the second part of the experiments, which were carried out as previously described. Equilibrium was reached within 60 min, when the emf was stable at 0.00003 volt.

The measurement lasted 120 min. All experiments were carried out in a dark room.

Results and Discussion

(a) The results of the emf measurements of the cell $Pt,H_2 \mid HCl_{(m)},ROH \mid AgCl \mid Ag$ are given in Table II. A typical graphical representation of the function $E^{*\prime}$, as identified by eq.(3a), against the molality (m) of HCl, ROH solutions, is shown in Fig. 1.

This plot should result in a straight line of zero slope, provided correct value of the parameter α is chosen. As it is observed in Fig. 1 the plot gave a straight line of a very small slope, for an α equal to 3.99 Å, over the whole concentration range.

The value 3.99 Å of α, is proved to be correct for HCl-ROH solutions [10], but no measurable differences in the standard potentials were obtained by using, in the present calculations, a different α value which made the curve horizontal, thus confirming that the small magnitude of the slope did not decrease the accuracy of the extrapolation. The curves are drawn according to the least squares' method and, the obtained standard (molal) emfs are summarized in Table III.

The standard emfs, plotted against the absolute temperature (T), are shown in Fig. 2. In the curve of EtOH in this Fig., a value at 10 °C is placed (—0.0313 v) taken from an unpublished work of the author, which fits very well in the graph. From this representation the thermal coefficient $\frac{(\partial E^*)}{(\partial T)}$ of the standard emf is obtained, which appears to exceed a stable value for all alcohols within $\pm$ 0.0004 volt K^{-1}.

To compare the E* values in EtOH appearing in this work with those in references, an interpolated value at 25^0 is obtained from Fig. 2, which is —0.067 volt.

This is more negative than the ones of Harned and Fleysher [6], and Lucasse [7], and less negative than those of Taniguchi and Janz [10], Woolcock and Hartley [8], Schaal and Teze [3], LeBas and Day [4]. The E* in PrOH and i-PrOH at 20° deviate from those of Schaal and Teze [3], being more negative about 0.005 volt.

(b) The results of the emf measurements of the cell $Pt,H_2 \mid m_{RO^-}, m_{Cl^-}, ROH \mid AgCl \mid Ag$ are given in Table IV, together with the ionic strength ($I = m_{RO^-} + m_{Cl^-}$) of the solution.

A typical graphical representation of the function $E^{*\prime\prime}$ as identified by eq.(6a), against I, is given in Fig. 3. The extrapolated values (pK), as well as the K values calculated from them, are summarized in Table V. Comparing the K of EtOH at 20^0 with the one of Schaal and Teze [3], a descrepancy is noticed (pK = 18.95) whereas a fairly good agreement is observed for K of PrOH and i-PrOH at the same temperature (pK = 19.33 and 20.73 respectively).

TABLE II : EMF readings of the cell: $Pt,H_{2(p\ atm)} \mid HCl_{(m)},ROH \mid AgCl \mid Ag$, at various molalities, at 15,20,30 °C.

	EtOH		PrOH		i-PrOH		BuOH		i-BuOH	
t (°C)	m (mol kg^{-1})	E (volt)	m (mol kg^{-1})	E (volt)	m (mol kg^{-1})	E (volt)	m (mol kg^{-1})	E (volt)	m (mol kg^{-1})	E (volt)
	0.0013	0.28970	0.0006	0.29010	0.0039	0.20502	0.0043	0.18063	0.0143	0.13630
	0.0025	0.26690	0.0012	0.25680	0.0214	0.14700	0.0330	0.11650	0.0266	0.12102
15	0.0104	0.20311	0.0062	0.19349	0.0223	0.14698	0.0375	0.11200	0.0290	0.11487
	0.0152	0.18990	0.0271	0.13802	0.0450	0.12608	0.0590	0.10003	0.0452	0.10200
	0.0248	0.17193	0.0476	0.12153	0.0765	0.11282	0.0811	0.08780	0.0509	0.09700
	0.0259	0.17030	0.0886	0.10002	0.1030	0.10383			0.0733	0.08801
	0.0012	0.29248	0.0062	0.18400	0.0065	0.17828	0.0075	0.15950	0.0143	0.13301
	0.0068	0.22350	0.0130	0.15688	0.0159	0.15150	0.0186	0.13220	0.0200	0.11781
20	0.0088	0.20852	0.0270	0.13350	0.0196	0.14403	0.0265	0.11813	0.0247	0.11520
	0.0119	0.19502	0.0471	0.11110	0.0208	0.14227	0.0650	0.09347	0.0290	0.11200
	0.0213	0.17590	0.0509	0.10772	0.0454	0.11600	0.0920	0.08303	0.0541	0.09111
			0.0610	0.10128	0.0765	0.10400				
	0.0018	0.26170	0.0031	0.19980	0.0063	0.17277	0.0137	0.13703	0.0010	0.20700
	0.0029	0.23980	0.0107	0.15302	0.0079	0.16650	0.0156	0.12748	0.0055	0.15279
30	0.0032	0.23420	0.0200	0.12750	0.0082	0.16420	0.0336	0.10600	0.0204	0.11331
	0.0134	0.17161	0.0295	0.11902	0.0210	0.13349	0.0530	0.09400	0.0291	0.10690
	0.0212	0.15550	0.0722	0.08500	0.0298	0.12401	0.0598	0.09002	0.0542	0.08602
	0.0232	0.15321			0.0764	0.09790				

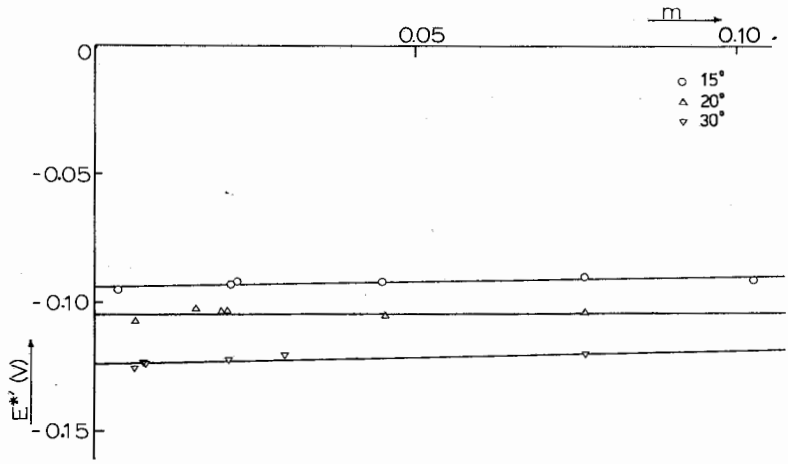

FIG. 1 : *Graphical representation of the E*' against molality, for HCl-abs.i-PrOH solutions, and evaluation of the standard emf $E_{(m)}$ of the silver-silver chloride electrode in i-PrOH solutions, at 15,20,30 °C.*

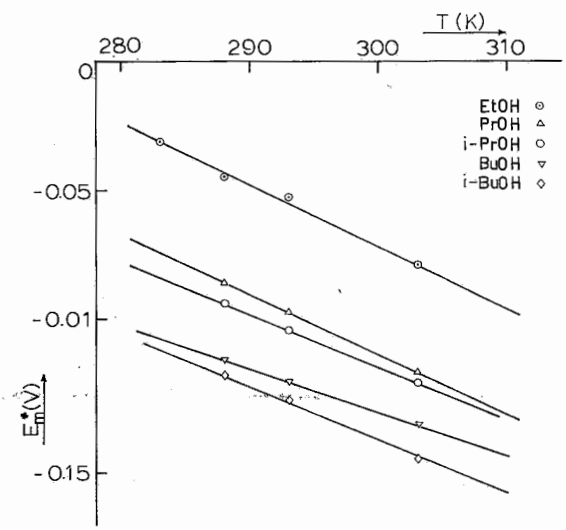

FIG. 2 : *Standard electrode potentials of the silver-silver chloride electrode, in various alcoholic solutions, plotted as a function of temperature.*

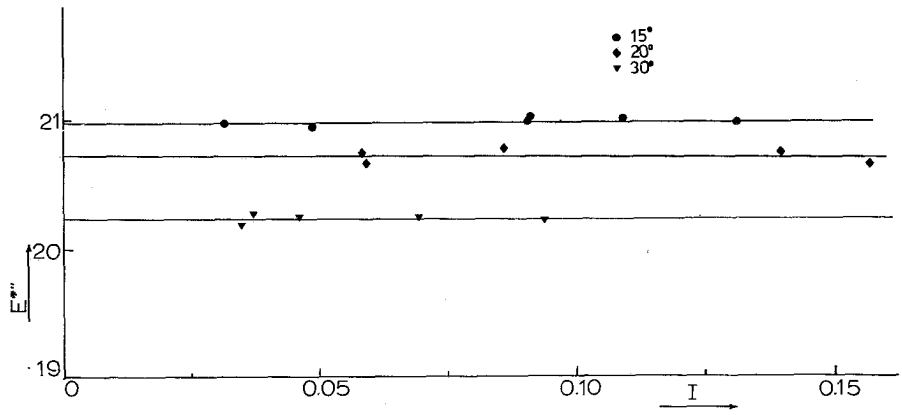

FIG. 3 : *Graphical representation of the E*′ against the ionic strength of a LiCl, i-PrOLi, abs. i-PrOH solution, and evaluation at the I = 0 axis of the pK of i-PrOH at 15,20,30 °C.*

TABLE III : Standard EMF (E^*_m) in molality scale of the silver-silver chloride electrode in HCl-alcoholic solutions, and its thermal coefficient

t°	EtOH	PrOH	i-PrOH	BuOH	i-BuOH
15	-0.04496	-0.08630	-0.09432	-0.11590	-0.12172
20	-0.05250	-0.09715	-0.10484	-0.12498	-0.13159
30	-0.07938	-0.12117	-0.12459	-0.14153	-0.15526
$\frac{(\partial E^*)}{(\partial T)}$ (voltK^{-1})	-0.00244	-0.00239	-0.00210	-0.00173	-0.00210

In Fig. 4, the pK values are plotted against the absolute temperature, and it is observed that, as was expected, the pK decreases with the temperature.

At constant temperature, a gradual drop of the K values (an increase in pK), is observed, as the chain of the molecule gets longer, with the exception of i-PrOH, which deviates towards the smallest value.

The order they follow is, $K_{EtOH} > K_{PrOH} > K_{BuOH} > K_{i\text{-}BuOH} > K_{i\text{-}PrOH}$ This could be explained considering two factors which mainly affect the acidity of the alcohols, making them less acidic than water:

The inductive effect, which alkyl groups, replacing an H atom of the molecule, display, and the magnitude of the dielectric constant of the molecule.

TABLE IV : EMF readings of the cell: $Pt,H_{2(p\ atm)} \mid MOR_{(m_{RO^-})}, MCl_{(m_{Cl^-})}, ROH \mid AgCl \mid Ag$, at various ionic strengths ($I = m_{RO^-} + m_{Cl^-}$) at 15,20 and30 °C.

	EtOH		PrOH		i-PrOH		BuOH		i-BuOH	
t (°C)	I (mol kg^{-1})	E (V)	I (mol kg^{-1})	E (V)	I (mol kg^{-1})	E (V)	I (mol kg^{-1})	E (V)	I (mol kg^{-1})	E (V)
	0.0449	1.02280	0.0219	1.01352	0.0315	1.08002	0.0299	1.01188	0.0189	0.99511
	0.0489	1.02517	0.0238	1.02000	0.0484	1.05590	0.04376	1.01600	0.0320	1.00610
	0.0611	1.03191	0.0426	1.02501	0.0909	1.00510	0.0453	1.02311	0.0322	0.99000
	0.0785	0.04921	0.0472	1.03000	0.1091	1.04403	0.0747	1.01802	0.0402	0.96521
	0.1237	1.03900	0.0597	0.99200	0.1313	0.98600	0.0794	0.98900	0.0435	0.98300
	0.0417	1.04332	0.0289	1.00301	0.0583	1.06517	0.0194	1.01608	0.0279	0.96778
	0.0449	1.03478	0.0356	1.02100	0.0592	1.03500	0.0274	1.02812	0.0318	0.98101
	0.0727	1.04550	0.0557	1.00298	0.0861	1.01602	0.0350	1.01930	0.0404	0.96002
	0.0856	1.05878	0.0841	0.94701	0.1397	1.02928	0.0380	1.02180	0.0435	0.98100
	0.1052	1.06220	0.0998	0.99900	0.1572	1.00200	0.0536	0.98100	0.0547	0.98579
	0.0209	1.04440	0.0225	1.01701	0.0349	1.03511	0.0220	1.03108	0.0166	1.02000
	0.0279	1.01448	0.0258	1.03400	0.0370	1.06008	0.0294	1.02780	0.0223	1.02309
	0.0398	1.05700	0.0290	0.99401	0.0458	1.03300	0.0343	1.01000	0.0320	1.00110
	0.0548	1.03400	0.0527	1.03308	0.0693	1.04101	0.0421	1.02212	0.0419	0.99000
	0.1232	1.07088	0.1062	0.96903	0.0938	0.99019	0.0983	0.98210	0.0698	0.98278

TABLE V : Ion product (K) of EtOH, PrOH, i-PrOH, BuOH, i-BuOH at 15,20 αvδ 30 °C.

	EtOH		PrOH		i-PrOH		BuOH		i-BuOH	
t (°C)	pK	K	pK	K	pK	K	pK	K	pK	K
15	18.27	$5.37\ 10^{-19}$	19.54	$2.88\ 10^{-20}$	20.97	$1.07\ 10^{-21}$	19.98	$1.05\ 10^{-20}$	20.48	$3.31\ 10^{-21}$
20	18.15	$7.08\ 10^{-19}$	19.40	$3.98\ 10^{-20}$	20.74	$1.82\ 10^{-21}$	19.87	$1.34\ 10^{-20}$	20.26	$5.50\ 10^{-21}$
30	18.02	$9.54\ 10^{-19}$	19.22	$6.02\ 10^{-20}$	20.26	$5.50\ 10^{-21}$	19.52	$3.02\ 10^{-20}$	19.86	$1.38\ 10^{-21}$

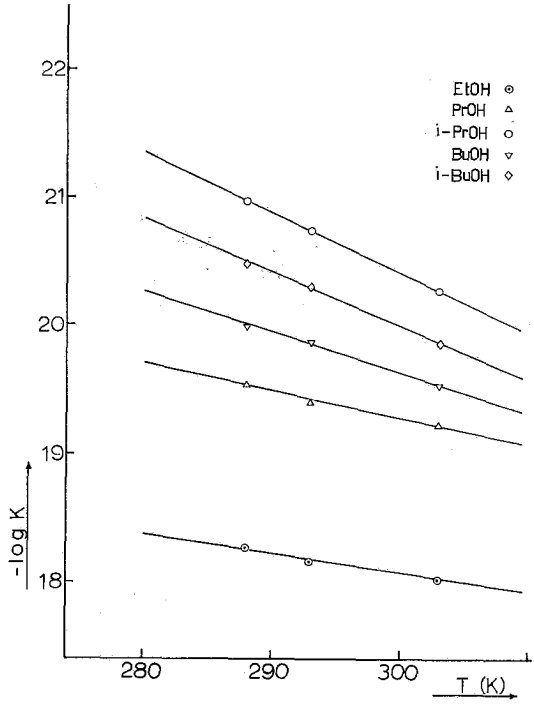

FIG. 4 : *pK values of EtOH, PrOH, i-PrOH, BuOH and i-BuOH as a function of absolute temperature.*

The alkyl groups are poorer electron attractors than the hydrogen atoms, and display an «electron donating», positive, inductive effect. This effect increases the electron availability of the O atom (of the OH^-), thus decreasing the acidic character of the molecule. The relative magnitude of the inductive effect of alkyl groups is normally found to follow the order,

$\begin{matrix}Me\\Me\end{matrix}\!\!>\!\!CH\!\rightarrow\!\!> MeCH\!\rightarrow\!\!> CH_3\!\rightarrow$ which is also the order of increase of the electron availability of the O atom; hence this is the order of decrease of the acidic character of the molecule [19].

The K of i-PrOH being smaller than these of BuOH and i-BuOH, although the chain is shorter, is well explained by the fact, that the i-PrOH molecule contains two methyl groups substituted on the α-carbon atom. The inductive effect is in that case stonger (the methyls are closer to the O atom), than the one existing in the longer molecules of BuOH and i-BuOH. The dielectric constants of these alcohols are decreasing in the same order with the K.

A lower dielectric constant means greater energy to separate charges. So this factor together with the inductive effect could explain the order of K obtained in this work.

Περίληψη

«Μετρήσεις ΗΕΔ σέ μή ὑδατικά διαλύματα ἀλειφατικῶν ἀλκοολῶν. Προσδιορισμός ἰοντικοῦ γινομένου».

Ἀπό μετρήσεις ΗΕΔ τοῦ στοιχείου $Pt,H_2 \mid HCl_{(m)},ROH \mid AgCl \mid Ag$ ὑπολογίζεται τό κανονικό δυναμικό (E*) τοῦ ἠλεκτροδίου ἀργύρου-χλωριούχου ἀργύρου, σέ μή ὑδατικά διαλύματα EtOH, PrOH, i-PrOH, BuOH, i-BuOH στούς 15,20 καί 30 °C.

Χρησιμοποιεῖται γιά πρώτη φορά ὁ τύπος τοῦ ἠλεκτροδίου «κατόπτρου» ἀργύρου-χλωριούχου ἀργύρου, πού ἀποδεικνύεται ἐξαιρετικά σταθερό καί ἀναπαραγωγίσιμο σέ ὅλη τήν διάρκεια τῶν πειραμάτων.

Ὑπολογίζεται ἐπίσης ὁ θερμικός συντελεστής τοῦ κανονικοῦ δυναμικοῦ γιά κάθε ἕνα διαλύτη.

Ἀπό τίς τιμές αὐτές καί ἀπό μετρήσεις ΗΕΔ τοῦ στοιχείου

$$Pt,H_2 \mid MOR_{(m_{RO^-})}, MCl_{(m_{Cl^-})}ROH \mid AgCl \mid Ag$$

ὑπολογίζονται τά ἰοντικά γινόμενα τῶν παραπάνω ἀλειφατικῶν ἀλκοολῶν γιά τίς ἴδιες θερμοκρασίες.

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WITTIG REACTIONS OF α-DICARBONYL COMPOUNDS WITH CARBALKOXYALKYLIDENETRIPHENYL-PHOSPHORANES. SYNTHESIS AND STUDY OF (Z)- AND (E)-ISOMERIC ESTERS OF SOME 4-OXO-α,β-UNSATURATED ACIDS.

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Summary

Wittig reactions of benzil *(1a)*, 1-phenyl-1,2-propanedione *(1b)* and phenylglyoxal *(1c)* with carbomethoxy- and carbethoxy-methylenetriphenylphosphoranes *(2a, 2b)* as well as with α-carbomethoxyethylidenetriphenylphosphorane *(2c)* have been studied. Although two equivalents of the ylid were used in all reactions the dicarbonyl compounds *1a-c* underwent alkene formation at only one carbonyl group and gave mixtures of the possible stereo- and peri-isomeric esters of the corresponding 4-oxo-α,β-unsaturated acids. The proposed structures for the new compounds were confirmed by their spectral data (^{1}H-NMR, IR, MS) and were further supported by the transformation of some of them to five-membered methoxy- or hydroxylactones.

Key words : Wittig reactions, esters of 4-oxo-α,β-unsaturated acids, five-membered lactones.

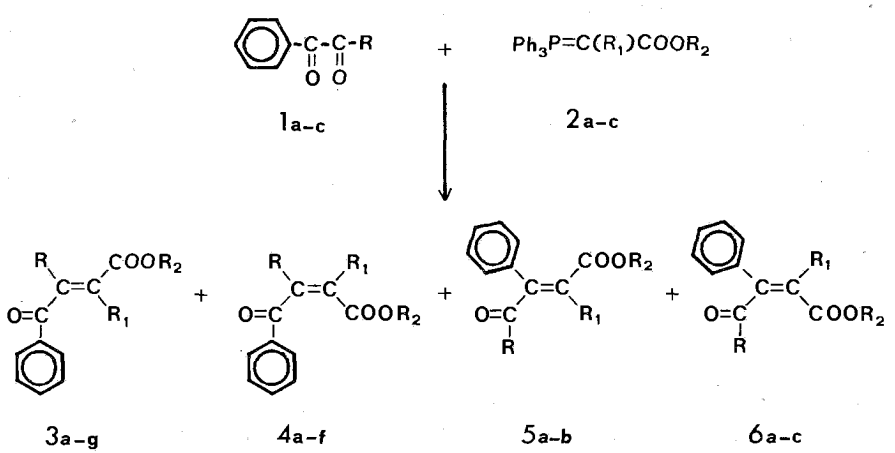
Introduction

There are a number of reports in the literature concerning Wittig reactions of α-dicarbonyl compounds and mainly of symmetric α-diketones with mono- and bis-phosphonium ylids. The reactions with bis-ylids generally result to the formation of unsaturated cyclic compounds, through a normal bis-Wittig reaction [1-3] but an unusual reaction of benzil has also been observed [4]. The less reactive mono-ylids usually react only with one carbonyl group giving α,β-unsaturated carbonyl derivatives. Although two configurational isomers may be produced, the formation of only one isomer has been reported in most of these reactions and in some cases a Michael addition of a second ylid molecule to the originally prepared carbonyl derivative occured [5]. In contrast to the extensive literature on Wittig reactions of several symmetric α-dicarbonyl compounds, reactions with non-symmetric α-diketones have not so far been reported. In connection with our previous work on bis-Wittig

reactions of some α- and γ-dicarbonyl compounds[6] we wish now to report reactions of compounds *1a-c* with two equivalents of ylids *2a-c*. In some similar reactions benzil *(1a)* gave α,β-unsaturated ketones[7,8] and phenylglyoxal *(1c)* led to unilateral condensation of only the aldehydic carbonyl group[9,10]. Taylor[11] reported recently that dimethyl- and diethylglyoxal give with cyanomethylenetriphenylphosphorane bis-Wittig reactions in contrast to *1a* which converts under the same conditions only one carbonyl group to olefin. The reactions studied in the present work generally resulted through a Wittig condensation of one carbonyl group of compounds *1a-c* to the formation of mixtures of the possible isomeric esters of the corresponding 4-oxo-α,β-unsaturated acids. The structure of the new compounds prepared was studied on the basis of their spectral and analytical data and also by cyclization of some of them to 3-butenolactone derivatives.

Results and Discussion

The Wittig reactions between the α-dicarbonyl compounds *1a-c* and the ylids *2a-c* are given in Scheme 1, while the analytical and spectral data of esters prepared are listed in Tables I, II.



1a: R = C_6H_5
1b: R = CH_3
1c: R = H

2a: R_1 = H, R_2 = CH_3
2b: R_1 = H, R_2 = C_2H_5
2c: R_1 = R_2 = CH_3

3a, 4a: R = C_6H_5, R_1 = H, R_2 = CH_3
3b, 4b: R = C_6H_5, R_1 = H, R_2 = C_2H_5
3c, 4c: R = C_6H_5, R_1 = R_2 = CH_3
3d, 4d, 5a, 6a: R = CH_3, R_1 = H, R_2 = CH_3
3e, 4e, 5b, 6b: R = CH_3, R_1 = H, R_2 = C_2H_5
3f, 6c: R = R_1 = R_2 = CH_3
3g, 4f: R = H, R_1 = R_2 = CH_3

Scheme 1

TABLE I : Analytical data of compounds *3a-g, 4a-f, 5a-b, 6a-c.*

Reaction N°	Carbonyl compound	Ylid	Product Yield [%][a]	M.p. or b.p. [°C] (recryst. solvent) (lit.m.p. or b.p.)	Molecular formula	Calculated/Found C %	H %
I	1a	2a	3a (17)	70-71 (petr.ether)	$C_{17}H_{14}O_3$ (266.28)	76.67 76.52	5.30 5.27
			4a (65)	91-92 (petr.ether) (91-92)[17]	$C_{17}H_{14}O_3$ (266.28)	76.67 76.43	5.30 5.24
II	1a	2b	3b (61)	212-215/4.5 torr	$C_{18}H_{16}O_3$ (280.31)	77.12 77.50	5.75 6.01
			4b (28)	77-79 (ether/petr. ether) (83-84)[14]	$C_{18}H_{16}O_3$ (280.31)	77.12 76.93	5.75 5.72
III	1a	2c	3c (3)	96-97 (petr.ether)	$C_{18}H_{16}O_3$ (280.31)	77.12 77.46	5.75 5.68
			4c (2)	76-77 (petr.ether)	$C_{18}H_{16}O_3$ (280.31)	77.12 77.08	5.75 5.76
IV	1b	2a	3d (30)	oil (159-160/16 torr)[21]	$C_{12}H_{12}O_3$ (204.22)	70.57 70.25	5.92 5.65
			4d (5)	——— [b] (50-52)[23]	$C_{12}H_{12}O_3$ (204.22)	——— [b]	
			5a (36)	oil (GLC)[c]	$C_{12}H_{12}O_3$ (204.22)	70.57 70.20	5.92 5.71
			6a (25)	——— [b] (yellow oil)[24]	$C_{12}H_{12}O_3$ (204.22)	——— [b]	
V	1b	2b	3e (20)	155-156/5 torr	$C_{13}H_{14}O_3$ (218.24)	71.54 71.17	6.47 6.40
			4e (5)	——— [b]	$C_{13}H_{14}O_3$ (218.24)	——— [b]	
			5b (30)	154-155/4.5 torr	$C_{13}H_{14}O_3$ (218.24)	71.54 71.78	6.47 6.64
			6b (21)	154-155/4.5 torr	$C_{13}H_{14}O_3$ (218.24)	71.54 71.83	6.47 6.63
VI	1b	2c	3f (3)	oil (GLC)[c]	$C_{13}H_{14}O_3$ (218.24)	71.54 71.26	6.47 6.31
			6c (19)	145-146 (ether/ chloroform)	$C_{13}H_{14}O_3$ (218.24)	71.54 71.42	6.47 6.36
VII	1c	2c	3g (65)	152-153/5 torr (176/23 torr)[21]	$C_{12}H_{12}O_3$ (204.22)	70.57 70.73	5.92 5.90
			4f (4)	78-80 (petr.ether)	$C_{12}H_{12}O_3$ (204.22)	70.57 70.23	5.92 5.68

a : Based on the carbonyl compound used. b : Not isolated from the reaction mixture c : See experimental.

TABLE II : IR and ^{1}H-NMR spectral data of compounds *3a-g, 4a-f, 5a-b, 6a-c*[a].

Compound	IR (CCl_4) $\nu_{C=O}$ [cm^{-1}]	^{1}H-NMR (CCl_4) δ [ppm]
3a	1730, 1673	3.54(s, 3H); 6.16(s, 1H); 7.20-7.57(m, 8H); 7.77-7.98(m, 2H)
4a	1723, 1678	3.55(s, 3H); 6.41(s, 1H); 7.18-7.58(m, 8H); 7.77-7.98(m, 2H)
3b	1723, 1674	1.11(t, 3H, J=7 Hz); 4.07(q, 2H, J=7 Hz); 6.17(s, 1H); 7.20-7.58 (m, 8H); 7.78-8.03(m, 2H)
4b	1720, 1680	1.08(t, 3H, J=7 Hz); 4.01(q, 2H, J=7 Hz); 6.40(s, 1H); 7.20-7.58 (m, 8H); 7.80-8.02(m, 2H)
3c	1727, 1675	2.02(s, 3H); 3.74(s, 3H); 7.30-7.68(m, 6H); 7.88-8.08(m, 4H)
4c	1722, 1673	2.02(s, 3H); 3.47(s, 3H); 7.20-7.52(m, 8H); 7.78-7.98(m, 2H)
3d	1726, 1666	2.38(d, 3H, J=1.5 Hz); 3.73(s, 3H); 6.09(q, 1H, J=1.5 Hz); 7.38-7.62(m, 3H); 7.70-7.90(m, 2H)
4d	———[b]	2.08(d, 3H, J=1.5 Hz); 3.47(s, 3H); 5.95(q, 1H, J=1.5 Hz)[c]
5a	1730, 1704	2.18(s, 3H); 3.55(s, 3H); 6.64(s, 1H); 7.05-7.47(m, 5H)
6a	———[b]	2.33(s, 3H); 3.73(s, 3H); 6.08(s, 1H)[c]
3e	1725, 1665	1.30(t, 3H, J=7 Hz); 2.38(d, 3H, J=1.5 Hz); 4.20(q, 2H, J=7 Hz); 6.09(q, 1H, J=1.5 Hz); 7.22-7.60(m, 3H); 7.67-7.93(m, 2H)
4e	———[b]	0.98(t, 3H, J=7 Hz); 2.08(d, 3H, J=1.5 Hz); 3.94(q, 2H, J=7 Hz); 5.94(q, 1H, J=1.5 Hz)[c]
5b	1725, 1705	1.04(t, 3H, J=7 Hz); 2.20(s, 3H); 3.98(q, 2H, J=7 Hz); 6.65(s, 1H); 7.05-7.50(m, 5H)
6b	1722, 1687	1.30(t, 3H, J=7 Hz); 2.32(s, 3H); 4.20(q, 2H, J=7 Hz); 6.06(s, 1H); 7.22-7.62(m, 5H)
3f	1724, 1676	1.72(q, 3H, J=1.5 Hz); 2.19(q, 3H, J=1.5 Hz); 3.78(s, 3H); 7.28-7.65(m, 3H); 7.82-8.02(m, 2H)
6c	1724, 1709	1.83(s, 3H); 2.13(s, 3H); 3.76(s, 3H); 7.13-7.57(m, 5H)
3g	1723, 1666	2.18(d, 3H, J=1.5 Hz); 3.83(s, 3H); 7.35-7.63(m, 3H); 7.70(q, 1H, J=1.5 Hz); 7.87-8.08(m, 2H)
4f	1747, 1722	1.98(d, 3H, J=1.5 Hz); 3.78(s, 3H); 7.23-7.68(m, 3H); 8.05-8.25(m, 2H); 8.40(q, 1H, J=1.5 Hz)

a : IR spectral data have been reported for compound *3d*[21], *3g*[21], *4d*[24,25], *4e*[24], *6a*[24]; ^{1}H-NMR spectral data have been reported for compounds *3d*[21,23], *3e*[26], *3g*[21,23], *4d*[23], *4e*[24,26], *6a*[24].

b : The compound has not been isolated from the reaction mixture.

c : The absorptions of the aromatic protons were masked by those of the other components of the mixture.

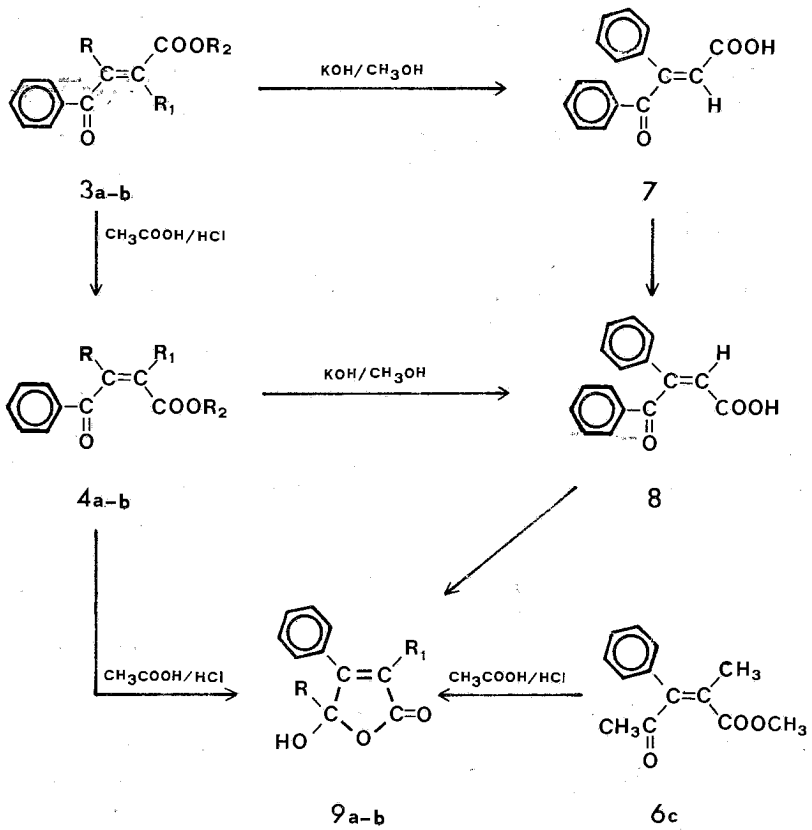
It is obvious that the reactions of α-diketones *1a*, *1b* with the carbalkoxymethylenetriphenylphosphoranes *2a*, *2b* (reactions I, II, IV, V, Table I) gave all the possible stereo- and peri-isomers in high total yields (76-96%), while the reaction between *1a* and the α-carbomethoxyethylidenetriphenylphosphorane *2c* (reaction III) resulted again to the formation of both possible isomers, but in low total yield. In the reaction of *1b* with *2c* (reaction VI) only the (E)-benzoyl and the (Z)-acetyl products were detected and isolated from the reaction mixture in a moderate total yield (22 %). α-Ketoaldehyde *1c* gave with ylid *2c* through a unilateral Wittig reaction of only the aldehydic carbonyl group the benzoyl derivatives *3g*, *4f* (reaction VII) in high total yield, in agreement with the higher reactivity of the aldehydic carbonyl group in ketoaldehydes [12]. In a similar reaction of *1c* with *2b* only the preparation of a (Z)-benzoyl derivative was reported [10,13]. It was also reported [11] that the reaction of *1a* with cyanomethylenetriphenylphosphorane gave a mixture of both isomers in 77% yield, analysed without separation. In general, structural assignments of compounds isolated from the reaction mixtures were made by means of elemental analyses, spectral data and (wherever reasonably feasible) comparison with literature data.

Compounds *3d*, *3e*, *3g*, *4a*, *4d*, *4e* and *6a* are known and their data are in good agreement with those given in the literature. The preparation of an ethyl 3,4-diphenyl-2-buten-4-on-1-oate and its hydrolysis in 5% KOH in methanol to the corresponding acid (m.p. 152-154 ^{0}C) has also been reported [14] but without configurational assignment and spectral data. The reported m.p. 83-84 ^{0}C for this ester differs from the data found for both isomers *3b*, *4b* prepared in the present work and it was difficult to identify it with one of them. Although a variety of isolation and purification methods were employed for the separation of the mixtures produced from the reactions IV and V we failed to isolate and purify compounds *4d*, *4e* and *6a*. These known compounds were identified by their ^{1}H-NMR spectra.

All the isolated compounds gave correct elemental analysis. The recorded mass spectra gave the expected molecular ion (M^+) and furthermore all the benzoyl derivatives gave the ion m/e 105 (C_6H_5-$C\equiv\overset{+}{O}$) and the acetyl derivatives the ion m/e 43 ($CH_3C\equiv\overset{+}{O}$), usually as a base peak. The benzoyl derivatives exhibited in the IR spectra two strong absorptions in the range 1730-1720 and 1680-1665 cm^{-1} for their ester and keto carbonyl group respectively, while the acetyl derivatives showed these two bands in the range 1730-1722 and 1709-1687 cm^{-1}, with the exception of the benzoyl derivative *4f*. An inspection of the ^{1}H-NMR spectra of the esters prepared reveals that the chemical shifts of the groups common in some of the esters depend largely not only upon their configuration but also upon the substituents and mainly the phenyl groups present. The α-olefinic proton of all compounds prepared from ylids *2a*, *2b* resonates at characteristic δ values and its absorption proved useful for the configurational assignments of some

of the new compounds as well as for the distinction between the benzoyl and acetyl derivatives prepared from reactions IV, V. This proton exhibited in the ^{1}H-NMR spectra of the acetyl derivatives a singlet signal while in those of the benzoyl derivatives a quartet, because of the β-methyl protons. Similarly the β-olefinic proton of compounds *3g*, *4f* showed a quartet but at a lower field than that of the α-olefinic protons.

The new compounds *3a*, *4f*, *5a*, *5b* and *6b* were fully characterized by their analytical and spectral data which are in good agreement with the proposed structures and with those of similar compounds given in the literature. The «E» and «Z» configuration of *3b* and *4b* was assigned by comparing their ^{1}H-NMR spectra with those of compounds *3a*, *4a*. For a further configurational comparison of the reported [14] ethyl 3,4-diphenyl-2-buten-4-on-1-oate with the esters *3a*, *3b*, *4a* and *4b* we tried to hydrolyse them to the corresponding acids. Hydrolysis of *3a* and *3b* in 5% KOH in methanol gave in both cases at first the known [15] (E)-β-phenyl-β-benzoyl-acrylic acid *(7)*, m.p. 120-123 °C, in 45% yield, which was isomerised during the efforts for puri-



3a-b 7 4a-b 8 9a-b 6c

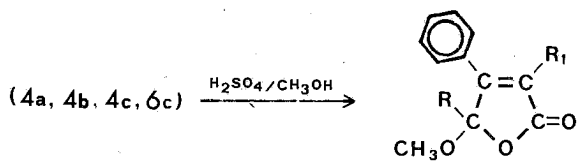
9a: R = C_6H_5, R_1 = H
9b: R = R_1 = CH_3

Scheme 2

fication by recrystallisation to the corresponding known [14] (Z)-isomer *8*, m.p. 150-153 °C. Hydrolysis of compounds *4a* and *4b* under the same conditions resulted directly to the acid *8* in 90% yield. Compound *8* was converted by recrystallization from chloroform to a solid m.p. 141-142 °C, for which the structure of the 4,5-diphenyl-5-hydroxy-3-butenolactone *(9a)* was assigned. Then we tried to hydrolyse the esters *3b*, *4a*, *4b* by refluxing them in hydrochloric-acetic acid (1:9) solution. The treatment of *3b* under these conditions resulted originally to isomerisation to *4b* and further by complete hydrolysis to *9a*. The reaction was monitored by ^{1}H-NMR spectroscopy. Compound *4a* was directly hydrolysed also to *9a*. Although the structure of cis-β-benzoyl-β-phenyl-acrylic acid has been proposed for the solid m.p. 141-142 °C [16,17] we consider that the cyclic form *9a* is in good agreement with the recorded spectral data. The compound under question exhibited a strong band in the infrared at 1759 cm^{-1}, diagnostic of the five-membered hydroxy-lactone structure [18,19]. Another strong evidence in favour of the cyclic structure is the fact that in contrast to all the benzoyl derivatives, which show in the ^{1}H-NMR spectra a two-proton multiplet at δ 7.80-8.00 ppm for the o-aromatic protons of the benzoyl group, compound *9a* exhibited one multiplet at δ 7.20-7.80 ppm (10 H) for all the protons of both phenyl groups. Earlier investigations demonstrated the occurence of (E)-(Z)-isomerization and of a ring-chain tautomerism in 4-oxo-α,β-unsaturated acids [18,19] and it was also reported that the cis-acids in solution are cyclic [20]. From these results it is obvious that the previously reported ethyl ester m.p. 83-84 °C is identical to compound *4b*.

More evidence were necessary for the configurational assignment of compounds *3c*, *4c*, *3f* and *6c*, since there are no general informative correlations between the spectral data of the esters and their configuration. In the ^{1}H-NMR spectra of the isomers *3c*, *4c* only the methyl protons of their methoxy-groups resonate in different fields. This difference is not informative, because in the spectra of the similar isomers *3a* - *4a* and *3b* - *4b* the protons of the alkoxy-groups in each pair of (Z)-(E)-isomers show the same chemical shift. On the other hand the chemical shift of the methoxy-protons of compound *3f* is very similar to that of the (E)-isomer *3d*, while the shift of its β-methyl protons is closer to that of the (Z)-isomer *4d*. A similar relationship between the chemical shifts of methoxy and acetyl protons of compound *6c* and the (E)-, (Z)-isomers *5a-6a* was also observed in their ^{1}H-NMR spectra. Additional evidence for the configurational assignment of compounds in question was provided by refluxing them with methanol-sulfuric acid solution. El-Ghandour et al. [21] reported the transformation of cis-α-methyl-β-acetyl-acrylic and cis-α-methyl-β-acetyl-2-butenoic acid methyl esters to 3,5-dimethyl- and 3,4,5-trimethyl-5-methoxy-3-butenolactones respectively by treatment with methanol-sulfuric acid solution. We tried this experiment to fully characterised compounds *3b*, *3g*, *4a*, *4b* and found that only the (Z)-isomers *4a*, *4b* were partially transformed to the known [17] 4,5-diphenyl-5-methoxy-3-butenolactone *(10a)*. In both cases an equilibrium between the cyclic product

10a and the methyl ester *4a* was established even after prolonged refluxing. In the experiments with the (E)-isomers, the ethyl ester *3b* was only trans-esterified to the methyl ester *3a* while the methyl ester *3g* was recovered unaltered. Then we tried the same experiment with compounds *3c*, *4c*, *3f* and *6c* and found that only compounds *4c* and *6c* were transformed to the corresponding lactones *10b* and *10c* in 40% and 47% yield respectively, while compounds *3c* and *3f* maintained the acyclic form and were recovered. The cyclisation of compounds *4c* and *6c* was completed much more quickly



10a-c

10a: R = C_6H_5, R_1 = H
10b: R = C_6H_5, R_1 = CH_3
10c: R = R_1 = CH_3

Scheme 3

than that of compounds *4a*, *4b* and in these cases no starting esters were recovered. The results of these experiments support the (Z)-configuration for compounds *4c* and *6c* and the (E)-configuration for compounds *3c* and *3f*. Hydrolysis of *6c* under acidic conditions gave also the hydroxy-lactone *9b* (Scheme 2). All the cyclic products *9a-b* and *10a-c* gave the expected molecular ion (M^+) in their mass spectra and correct elemental analyses; their spectral data (Table III) are in good agreement with the five-membered lactone structure.

From the data presented we can conclude that although two equivalents of ylid were used in all reactions, compounds *1a-c* underwent alkene formation at only one carbonyl group. Table I shows that the benzoyl and acetyl-(E)-isomers *3* and *5* were prepared generally in higher yield than the corresponding (Z)-isomers *4* and *6* respectively, with the exception of isomers *3a-4a*. In the case of diketone *1b* the acetyl derivatives *5* and *6* were prepared in a significant higher total yield than the benzoyl derivatives *3* and *4*.

Experimental

M.p's are given without correction and were determined with a Kofler hot-stage apparatus. ^{1}H-NMR spectra were obtained with a Varian A-60A spectrometer with tetramethylsilane as internal standard. The mass spectra were obtained with a Hitachi Perkin-Elmer RMU-6L mass spectrometer; the

TABLE III : Analytical and spectral data of compounds *9a-b* and *10a-c*

Compound	M.p.[°C] (recryst. solvent) (lit. m.p.)	Molecular formula	Calculated/ Found C %	H %	IR[a,b] $\nu_{C=O}$ [cm^{-1}]	^{1}H-NMR[b,c] δ [ppm]
9a	141-142 ($CHCl_3$) (141-142)[17]	$C_{16}H_{12}O_3$ (252.26)	76.18 75.99	4.80 4.87	1759[a]	6.45(s, 1H); 7.20-7.80 (m, 10H)[c]
9b	146-147 ($CHCl_3$)	$C_{12}H_{12}O_3$ (204.22)	70.57 70.60	5.92 6.01	1758[a]	1.65(s, 3H); 1.98(s, 3H); 7.20-7.73(m, 5H)[c]
10a	98-100 (Ether/ petr. ether) (103-104)[17]	$C_{17}H_{14}O_3$ (266.28)	76.67 76.46	5.30 5.26	1770[b]	3.37(s, 3H); 6.55(s, 1H); 7.17-7.68(m, 10H)[b]
10b	oil	$C_{18}H_{16}O_3$ (280.31)	77.12 76.88	5.75 5.79	1775[b]	2.08(s, 3H); 2.38(s, 3H); 7.05-7.55(m, 10H)[c]
10c	oil	$C_{13}H_{14}O_3$ (218.24)	71.54 71.44	6.47 6.53	1768[b]	1.56(s, 3H); 2.08(s, 3H); 3.24(s, 3H); 7.35-7.67(m,5H)[b]

a : In $CHCl_3$ solution
b : In CCl_4 solution
c : In $CDCl_3$ solution

ionization energy was maintained at 70 eV. Earlier reported procedures were used for the preparation of the ylids *2a-c*[22].

Wittig reactions. General procedure

The reactions between the compounds *1a-c* and *2a-c* were carried out according to the following general procedure. A solution of dicarbonyl compound *1* (0.01 mole) and ylid *2* (0.02 mole) in dry methylene chloride (100 ml) was boiled under reflux for 15 h. Then the solvent was evaporated under reduced pressure and the residue was roughly separated by column chromatography on silica gel eluting with chloroform. Fractions of 20 ml were collected and the content of each fraction was checked by TLC and ^{1}H-NMR spectroscopy. The (E)-benzoyl derivatives *3*, formed in all the reactions studied, were generally moved through the column faster than the other isomers of the reaction mixtures and eluted before them with the exception of compound *3g* (reaction VII, Table I) which was eluted after the other isomer *4f*. A satisfactory separation between the isomers *3a-4a*, *3b-4b*, *3c-4c*, *3f-6c* and *3g-4f*, prepared from the reactions I, II, III, VI and VII respectively was achieved in this way. Similarly compounds *3d* and *3e* were also partially separated from the produced mixtures of reactions IV and V respectively, while the other products of these reactions were further separated by GLC (column 20% OV-1. 60/80 Chrom, 4 ft) at 169° and 173 °C respectively. In the first case compound *5a* was eluted first as an individual fraction followed

by the mixture of *3d*, *4d*, *6a* and in the second case compounds *5b* and *6b* were separated as the first and last fraction, while a mixture of *3e* and *4e* was eluted between them. Compounds *3f* and *6c* were also purified by GLC on the same column at 161 ^{0} and 163 ^{0}C respectively. The products were further purified by methods given in Table I.

Hydrolyses of compounds 3a, 3b, 4a, 4b, 6c

A. Hydrolysis with methanolic 5 % potassium hydroxide

Compound *3b* (52 mg) was dissolved in 5% potassium hydroxide in methanol (3ml) and the mixture was boiled under reflux for 5 min. The solution was then cooled, acidified with dilute hydrochloric acid and extracted with chloroform. The organic layer was dried with anhydrous sodium sulfate, concentrated under reduced pressure and the residue was separated by preparative TLC on silica gel, eluting with methanol-chloroform (5:95) to give 20 mg of (E)-β-phenyl-β-benzoyl-acrylic acid *(7)*, m.p. 119-122 ^{0}C (lit.[15] m.p. 120-123 ^{0}C); yield 45%. Recrystallisation of *7* from chloroform-petroleum ether gave the (Z)-isomer *8*, m.p. 150-153 ^{0}C (lit.[14] m.p. 152-154 ^{0}C). Further recrystallisation of *8* from chloroform-petroleum ether gave 4,5-diphenyl-5-hydroxy-3-butenolactone *(9a)*, m.p. 141-142 ^{0}C.

Compound *3a* was also hydrolysed like compound *3b* to give generally the same results.

Compound *4b* (35 mg) was hydrolysed like compound *3b* to give directly the acid *8* (29 mg, 93% yield) and by recrystallisation of *8* compound *9a* (18 mg).

Similarly compound *4a* (31 mg) was hydrolysed to *8* (25 mg) which was then transformed by recrystallisation to *9a* (17 mg).

B. Hydrolysis with hydrochloric-acetic acid (1:9) mixture

Compound *4a* (100 mg) was dissolved in a mixture of hydrochloric- acetic acid (1:9) (3 ml) and was refluxed for 8 h. Then the solution was concentrated under reduced pressure, water (2 ml) was added and the mixture was extracted with chloroform. The organic layer was washed with water, dried (Na_2SO_4) and concentrated to give crystals of *9a* (75 mg, 80% yield) m.p. 141-142 ^{0}C. The reaction was monitored by ^{1}H-NMR spectroscopy and it was found that the gradual transformation of *4a* to *9a* was completed after 8 h of refluxing.

Treatment of compound *3b* under the same conditions resulted originally to the isomerisation of *3b* to *4b* and finally to the preparation again of compound *9a* in 78% yield. The reaction was monitored by ^{1}H-NMR spectroscopy.

Similarly compound *6c* (100 mg) was dissolved in the above acidic mixture (3 ml), boiled under reflux for 3 h and then worked up as in the case of *4a* to give by crystallisation from chloroform 3,5-dimethyl-4-phenyl-5-hydroxy-3-butenolactone *(9b)* (42 mg, 45% yield) m.p. 146-147 ^{0}C.

Preparation of 5-methoxy-3-butenolactones 10a-c

A solution of compound *4a* (100 mg) in 1.5% sulfuric acid-methanol mixture (4 ml) was boiled under reflux for 2 h. The solvent was evaporated under reduced pressure, the residue was diluted with water (2 ml) and the mixture was extracted with chloroform. The recorded ^{1}H-NMR spectrum of the dried (Na_2SO_4) and concentrated organic layer showed the partial transformation of *4a* to 4,5-diphenyl-5-methoxy-3-butenolactone *(10a)*. The treatment with the acidic mixture was repeated as far as the progressive transformation of *4a* to *10a* was continued. After 6 h of refluxing an equilibrium between the two isomers *4a-10a* was established. The mixture was then separated by preparative TLC on silica, eluting with ether-petroleum ether (1:1) to give from the faster moving band compound *10a* (38 mg, 38% yield), m.p. 98-100 ^{0}C (lit[17] m.p. 103-104 ^{0}C). The starting ester *4a* (44 mg) was recovered from the next band.

The same experiment was also carried out with compounds *4b*, *4c*, *3b*, *3c*, *3f* and *6c*. Compound *4b* (110 mg) underwent after 6 h of refluxing partial cyclization to *10a* (40 mg, 38% yield) and trans-esterification to *4a* (51 mg).

Compound *4c* (50 mg) was transformed after 2 h of refluxing to 3-methyl-4,5-diphenyl-5-methoxy-3-butenolactone *(10b)* (20 mg, oil, 40% yield), separated from the reaction mixture by preparative TLC, as compound *10a*. No starting ester *4c* was recovered.

Compound *6c* gave after 1.5 h of refluxing 3,5-dimethyl-4-phenyl-5-methoxy-3-butenolactone *(10c)* in 47% yield (oil), separated also by preparative TLC as above. No starting material *6c* was recovered.

The ethyl ester *3b* was only trans-esterified to methyl ester *3a*, even though it was refluxed for 26 h.

Compounds *3c* and *3f* remained under the same conditions unchanged.

Περίληψη

Ἀντιδράσεις Wittig α-δικαρβονυλικῶν ἐνώσεων μέ καρβαλκοξυαλκυλιδενοτριφαινυλοφωσφοράνια. Σύνθεση καί μελέτη (Z)- καί (E)-ἰσομερῶν ἐστέρων μερικῶν 4-οξο-α,β-ακορέστων ὀξέων.

Στήν ἐργασία αὐτή μελετήθηκαν ἀντιδράσεις Wittig τῶν α-δικαρβονυλικῶν ἐνώσεων *1a-c* μέ τά ὑλίδια τοῦ φωσφόρου *2a-c*. Ἄν καί σ' ὅλες τίς περιπτώσεις χρησιμοποιήθηκε τό ὑλίδιο σέ διπλάσια μοριακή ποσότητα ἀπό τήν δικαρβονυλική ἕνωση, τά προϊόντα πού σχηματίσθηκαν ἦταν μίγματα τῶν ἐστέρων τῶν ἀντιστοίχων δυνατῶν ἰσομερῶν 4-οξο-α,β-ακορέστων ὀξέων καί προῆλθαν ἀπό ἀντίδραση Wittig τοῦ ἑνός καρβονυλίου τῶν ἐνώσεων *1a-c* (Σχῆμα 1, Πίνακας Ι). Σέ ἀνάλογες ἀντιδράσεις πού ἀναφέρονται στή βιβλιογραφία συνήθως λαμβάνεται ἕνα μόνο ἀπό τά δυνατά ἰσομερή προϊόντα. Ἰδιαίτερο ἐνδιαφέρον παρουσίασαν οἱ ἀντιδράσεις τῆς μή συμμετρικῆς α-δικετόνης *1b* ἡ ὁποία ἔδωσε στερεοχημικά ἰσομερῆ ἁπλῆς ἀντιδράσεως Wittig καί τῶν δύο καρβονυλικῶν της ὁμάδων. Ἄν καί ἡ δομή πού προτείνεται γιά τίς νέες ἐνώσεις *3a, 4f, 5a, 5b* καί *6c* συμφωνεῖ

μέ τά φασματοσκοπικά τους δεδομένα (Πίνακας II) καθώς καί μέ τά δεδομένα τῶν γνωστῶν στή βιβλιογραφία ἐνώσεων *3d, 3e, 3g, 4a, 4d, 4e* καί *6a*, γιά τήν εὕρεση τῆς στερεοχημικῆς δομῆς τῶν ὑπολοίπων νέων ἐνώσεων καθώς καί γιά τήν παραπέρα διευκρίνηση τῆς δομῆς τῶν *3b, 4b*, ἔγινε δοκιμή γιά τήν κυκλοποίησή τους πρός τίς ἀντίστοιχες 5-ὑδροξυ- (Σχῆμα 2) ἤ 5-μεθοξυ-λακτόνες (Σχῆμα 3) *9a-b* καί *10a-c*. Ἡ μελέτη ἔδειξε ὅτι μόνο τά (Z)-ἰσομερή κυκλοποιοῦνται καί ἔτσι βρέθηκε ἡ δομή πού προτείνεται γιά τίς ἐνώσεις *3b, 3c, 3f, 4b, 4c,* καί *6c*.

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A TWO-STAGE POLYCONDENSATION OF 2,5-FURANDICARBONYL DICHLORIDE WITH N^1,N^5-DIPHENYL-1,2,4,5-TETRAAMINOBENZENE, 3

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Summary

The reaction variables (time, concentration, temperature, reactant mole ratio, solvent) for the low-temperature solution polymerization of 2,5-furandicarbonyl dichloride with N^1,N^5-diphenyl-1,2,4,5-tetraaminobenzene were studied.

The yielded by the optimum conditions poly[furylene-(2,4-diphenylamino)amide] was converted by thermal polycyclodehydration to the corresponding new poly [furylene-(1,7-diphenyl)benzodiimidazole].

The prepared polymers were studied by measuring inherent viscosity, molecular weight, UV and IR spectra, thermal analysis and the results are discussed.

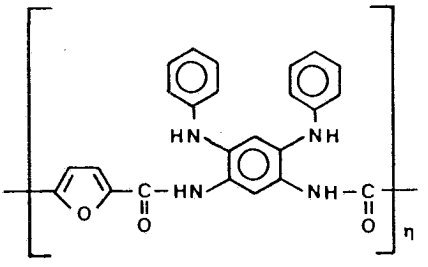
Key words : Solution polymerization, Poly[furylene-(2,4-diphenylamino)amide], Poly[furylene-(1,7-diphenyl)benzodiimidazole], or Poly(imidazo[4,5-f]benzimidazole-2,6-diyl-2,5-furanediyl).

Abbreviations : NMP = N-Methyl-2-pyrrolidone; DMA = N,N-Dimethylacetamide; DMF = N,N-Dimethylformamide; DPTAB = N^1,N^5-diphenyl-1,2,4,5-tetraaminobenzene; FDC = 2,5-furandicarbonyl dichloride; IMC = initial monomer concentration; DSC = Differential Scanning Calorimetry; TGA = Thermogravimetric Analysis.

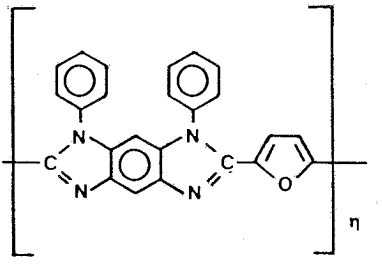
Introduction

2,5-Furandicarboxylic acid derivatives which are prepared from the available raw-material 2-furoic acid have already been used with tetrafunctional aromatic compounds to obtain heat resistant polymers, as well as in some cases, their precursors polyamides. Thus, poly(furylene)-benzimidazoles[1,2], -benzoxazoles[3], -benzothiazoles[4], -benzoxazinones[5,6], and -benzodioxazinones[6] have been reported. Also in two recent papers[7,8], the poly (furylene)-benzodiimidazole[7] and its di(α- or β-)naphthyl derivatives[8] were obtained by a two-stage polycondensation process.

In this work the unknown poly[furylene-(2,4-diphenylamino)amide] (I) was prepared by a low-temperature solution polymerization of 2,5-furandicarbonyl dichloride with N^1,N^5-diphenyl-1,2,4,5-tetraaminobenzene. Also, the variables of the above reaction were studied to obtain this polyamide in a highest molecular weight. In a second step, this polyamide I was converted, by thermal cyclodehydration, to the corresponding new, thermally stable, poly[furylene-(1,7-diphenyl)benzodiimidazole] (II).



(I)



(II)

The polyamide I and the polybenzodiimidazole II were studied and compared with the corresponding polymers of terephthalic and isophthalic acid[9,10] which were analogously prepared, as well as with the corresponding unsubstituted[7] and di(α- or β-)naphthyl substituted[8] polymers of furandicarboxylic acid.

Experimental

Materials : The 2,5-Furandicarbonyl dichloride was prepared as reported in previous work[6,7]. The N^1,N^5-diphenyl-1,2,4,5-tetraaminobenzene was prepared according to the literature[11,12,13] from m-dichlorobenzene and recrystallized twice from chloroform; m.p. 213.5-215 ^{0}C (in sealed tube) (Lit.[13] 210-211 ^{0}C). The N-Methyl-2-pyrrolidone (NMP), (Fluka) was purified by fractional distillation at reduced pressure (b.p. 82-84 ^{0}C, 10 mm Hg) over calcium hydride and stored over activated 3A molecular sieves. Its water content, determined by the Karl-Fischer method, was found 0.004%. The N,N-Dimethylacetamide (DMA), (Merck) was stirred with fresh barium oxide for three days, fractionally distilled at reduced pressure (b.p. 55-58 ^{0}C/11 mm Hg), and stored over activated 3A molecular sieves. The water content determined by the Karl-Fischer method, was found 0,019%. The N,N-Dimethylformamide (DMF), (Fluka) was dried over $MgSO_4$, filtered, fractionally distilled at reduced pressure (b.p. 39-40 ^{0}C/10 mm Hg), and stored over activated 3A molecular sieves. The water content, determined by the Karl-Fischer method, was found 0,020%.

Polymers

Poly[furylene-(2,4-diphenylamino)amide]: According to IUPAC nomenclature, it is a poly[2,5-furylene-carbonylimino(2,4-diphenylamino-1,5-phenylene)-iminocarbonyl] (I).

All the low-temperature solution polymerizations were carried out in a four necked polymerization flask equipped with stirrer, thermometer, nitrogen inlet and outlet tube, connected with a calcium chloride tube. The apparatus was immersed in a refrigerated thermostatic bath and previously heated for the removal of any moisture.

Solutions of N^1,N^5-diphenyl-1,2,4,5-tetraaminobenzene in dry solvent (NMP, DMA or DMF) were obtained with stirring under a nitrogen stream at -10 ^{0}C and the solid 2,5-furandicarbonyl dichloride was added in portions in a period of 0.5 h under vigorous stirring. Then the temperature was kept constant at -10, 0, 25, or 50 ^{0}C for a studied period of reaction time under continuous stirring and nitrogen stream. The viscous polymer solution was poured into distilled water. The precipitated polymer was collected by suction filtration, washed with water, then with methanol and dried in vacuum (3 mm Hg) at 60 ^{0}C.

Thus, from 2.90 g (10 mmole) of N^1,N^5-diphenyl-1,2,4,5-tetraaminobenzene in 19.5 ml of NMP and 1.97 g (10.2 mmole) of 2,5-furandicarbonyl dichloride (overall reactants 25% w/v) and for a reaction time of 1 h at 25 ^{0}C (optimum conditions), 3.98 g of polyamide I were isolated (yield 97%).

Anal. Calcd. for $(C_{24}H_{18}N_4O_3)_n$: C, 70,24; H, 4.42; N, 13,65%
Found : C, 70,09; H, 4.49; N, 13,55%

The IR spectrum (KBr) showed absorptions at 3300 cm^{-1} (NH), 1650 and 1530 cm^{-1} (NH-CO).

Poly[furylene-(1,7-diphenyl)benzodiimidazole]: According to IUPAC nomenclature, it is a poly[(1,7-diphenylbenzo(1,2-d:4,5d′)diimidazole-2′,6′-diyl)-2′,5′-furylene] (II).

It was prepared by thermal polycyclodehydration of I at 290-300 ^{0}C for 6 h in vacuum (0.01 mm Hg) and then it was pulverized and reheated under the same conditions for more 6 h.

Anal. Calcd. for $(C_{24}H_{14}N_4O)_n$: C, 76.99; H, 3.77; N, 14.96%
Found : C, 76.05; H, 4.03; N, 15.00%

The IR spectrum (KBr) showed the disappearance of absorptions at 3300 cm^{-1} (NH), 1530 and 1660 cm^{-1} (NH-CO) and the appearance of absorption maxima at 1600 (C=N), 1500, 1450 and 1380 cm^{-1}, which are characteristic of benzimidazole ring [14].

Analytical Methods

Melting points were taken on a Kofler melting point or a Buchi (dr. Tottoli) apparatus and are uncorrected. Elemental analysis was performed with a Perkin-Elmer model No 240 analyzer and infrared spectra were recorded on a Perkin-Elmer model 281-B spectrophotometer using the KBr pellet technique. Ultraviolet spectrum of polybenzodiimidazole was obtained from its dilute solution in conc. sulfuric acid using a Pye-Unicam SP-8000 spectrophotometer. The inherent viscosities were measured with Ubbelohde viscometers at 25 ^{0}C. The number average molecular weight ($\bar{M}_n$) of the polyamide was determined by a Knauer membrane osmometer using Sartorius regenerated cellulose membrane and DMF as solvent. The water content of the solvents was determined with a Baird and Tatlock Karl-Fischer apparatus using a solution equivalent to 2.5 mg H_2O/ml. The thermal properties of the polymers were studied on a Perkin-Elmer DSC-2 Differential Scanning Calorimeter ($\Delta T = 20\,^0$C/min) under nitrogen atmosphere and on a Perkin-Elmer TGS-2 Thermobalance ($\Delta T = 5\,^0$C/min) for dry samples approximately 5 mg.

Results and Discussion

The optimum polymerization conditions for the low-temperature solution polymerization of N^1,N^5-diphenyl-1,2,4,5-tetraaminobenzene with 2,5-furandicarbonyl dichloride were determined by measuring the variation in the extent of polymerization (viscosity) with the reaction variables such as reaction time (T), initial monomer concentration (total percentage weight of monomers in the solution), temperature of reaction (t), reactant mole ratio (FDC/DPTAB) and solvent.

In all polymerizations of this work an initial temperature of -10 ^{0}C during the addition of the dichloride was choosen, in order to prevent hydrolysis of dichloride whereas in lower temperatures precipitation of the tetraamine was observed.

According to the data of Table I the maximum molecular weight (inherent viscosity) was obtained at the reaction time of one hour (after the addition of the dichloride) and further increase of the reaction temperature had no pratical effect in extent of polymerization.

Four analogous polymerizations with different initial monomer concentrations (Table II) and reaction temperature of 0 ^{0}C (at -10 ^{0}C no homogeneous system was obtained for the high initial monomer concentrations), showed that the highest value of inherent viscosity was obtained for an initial monomer concentration of 25% (w/v) and an additional reaction time of one h at 50 ^{0}C insignificantly increased the extent of polymerization.

The reduction of n_{inh} observed when a 30% initial monomer concentration was used, should be attributed to insufficient stirring caused by the high viscosity of the reaction solution.

TABLE I : Effect of reaction time (T) in the extent of polymerization in DMA (IMC = 10%, t = -10 ^{0}C, FDC/DPTAB = 1.02).

T (min)	n_{inh}(dl/g)
15	0.176
30	0.182
60	0.192
120	0.192
140*	0.193
180**	0.193

* The last 20 min the temperature raised at 50 ^{0}C.
** The last 40 min the temperature raised at 50 ^{0}C to 100 ^{0}C.

TABLE II : Effect of initial monomer concentration (IMC) on the extent of polymerization in DMA (FDC/DPTAB = 1.02, (a) = reaction time 1 hour at 0 ^{0}C, (b) = additional reaction time 1 hour at 50 ^{0}C).

C %	(a) n_{inh}(dl/g)	(b) n_{inh}(dl/g)
10	0.172	0.187
20	0.201	0.216
25	0.221	0.228
30	0.188	0.192

The study of the effect of the reaction temperature on the extent of polymerization showed that the temperatures of 25 ^{0}C or 50 ^{0}C gave the best result (Table III).

TABLE III : Effect of reaction temperature on the extent of polymerization in DMA (IMC = 25%, T = 1 hour, FDC/DPTAB = 1.02).

Reaction temperature	n_{inh}(dl/g)
-10	0.201
0	0.221
25	0.228
50	0.228

The results of the effect of the mole ratio reactants on the extent of polymerization showed that a slight excess of dichloride (FDC/DPTAB=1.02) was beneficial, propably because it counteracted losses from hydrolysis. On the contrary a higher excess of dichloride reduced the extent of polymerization because of the unequivalence of the reactants (Table IV).

TABLE IV : Effect of reactant mole ratio on the extent of polymerization in DMA (IMC = 25%, T = 1 hour, t = 25 ^{0}C).

FDC/DPTAB mole	n_{inh}(dl/g)
1.00	0.192
1.02	0.228
1.04	0.199

Among the polar solvents studied, NMP was the most effective (Table V). The low value of n_{inh} resulted when DMF was used, was explained by its rapid and irreversible reaction with acid chlorides which reported in literature [15,16].

TABLE V : Effect of solvent on the extent of polymerization in (IMC = 25%, T = 1 hour, t = 25 ^{0}C FDC/DPTAB = 1.02).

Solvent	n_{inh} (dl/g)
DMA	0.228
DMF	0.055
NMP	0.290

The poly[furylene-(2,4-diphenylamino)amide], prepared by the found optimum conditions (IMC = 25%, T = 1 hour, t = 25 ^{0}C, FDC/DPTAB = = 1.02, solvent NMP), was soluble in polar aprotic solvents such as NMP, DMA, DMF, dimethylsulfoxide and hexamethyl-phosphoramide. It showed an inherent viscosity 0.29 dl/g in DMF (C = 0.5%, 25 ^{0}C) and 0.22 dl/g in a mixture of tetrachloroethane-phenol(3:1) (C = 0.5%, 25 ^{0}C). Transparent films were obtained from its DMF solutions. The number average molecular weight of this polyamide determined osmometrically was found to be 18,600. The prepared polyamide I showed lower inherent viscosity than the corresponding polyamides of terephthalic and isophthalic acid which were obtained by analogous about conditions in DMA [9,10].

The DSC and TGA thermograms in nitrogen (50-480 ^{0}C) showed that the polycyclodehydration of the polyamide to the polybenzodiimidazole II started at 240 ^{0}C with a maximum rate at about 300 ^{0}C, as it was observed for

the corresponding unsubstituted poly[furylene-(2,4-diamino)amide][7] and poly [furylene-2,4-di(α- or β-naphthylamino)amides][8]. The appearence of a second endotherm peak at 388 ^{0}C in the DSC-curve for the polyamide was proved by TGA to due to the completion of the cyclodehydration resulting to a total percentage weight loss in agreement with that calculated for its conversion to the corresponding polybenzodiimidazole.

The poly(furylene)benzodiimidazole II was insoluble in polar solvents, slightly soluble in conc. sulfuric (n_{inh} = 0.13, C = 0.2%, 25 ^{0}C) and formic acid (n_{inh} = 0.20, C = 0.2%, 25 ^{0}C) and soluble in a mixture of tetrachloroethane-phenol (3:1) (n_{inh} = 0.33, C = 0.5%, 25 ^{0}C). It is remarkable that tenfold higher values of the inherent viscosity for the corresponding and analogously prepared poly(1,7-diphenyl)benzodiimidazoles of terephthalic and isophthalic acid have been reported[9].

The UV spectrum of II measured in conc. sulfuric acid solution (C = 5 × 10^{-4} g/100 ml) showed a strong band at 416 nm ($E^{1\%}_{1cm}$ = 864) and a shoulder at 235 nm.

The thermal stability of polybenzodiimidazole II was studied from its TGA thermograms (Fig. 1) determining various criteria of thermostability[17,18] (Table VI). The activation energy of decomposition in nitrogen was determined by the multiple heating rate procedure[18] as reported in a previous paper[1].

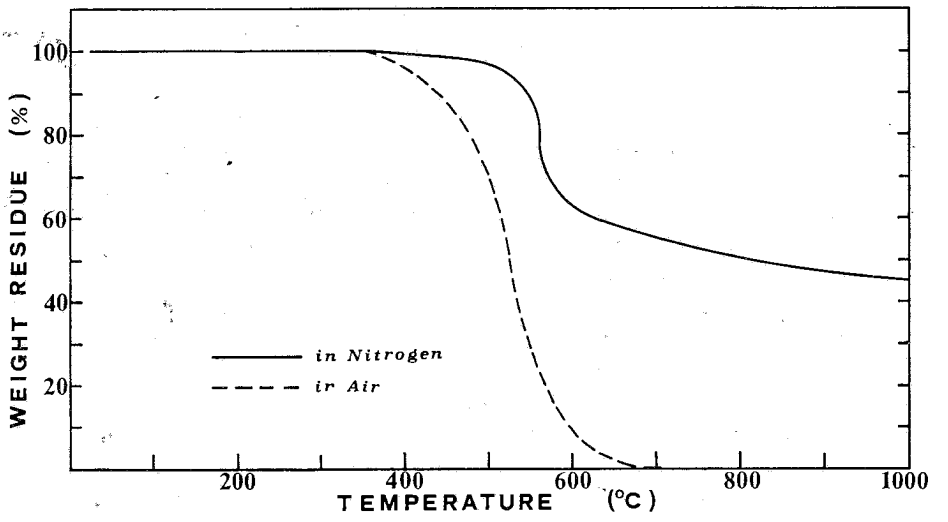


FIG. 1 : *Thermogravimetric curves of polybenzodiimidazole II.*

Comparison of thermal stability of the polybenzodiimidazole II with that of the corresponding unsubstituted poly(furylene)-benzodiimidazole[7] as well as, that of the corresponding phenyl substituted and unsubstituted polybenzodiimidazoles of the terephthalic and isophthalic acid[9,13], showed that

TABLE VI : Data of thermal degradation of poly[furylene-(1,7-diphenyl)benzodiimidazole] (II).

Decomp. Temp. of 5% weight loss		Half-volatilization point[a]	Integral procedural decomposition temperature[17,19]	% of total weight loss at 900 °C[a]	Activation energy[a]
in air °C	in nitrogen °C	°C	°C[a]		Kcal × mole^{-1}
405	510	578	590	55	40

[a] Determined in nitrogen.

the presence of phenyl substituents reduced the thermal stability in nitrogen but less than the presence of α- or β-naphthyl substituents[8]. On the contrary the presence of the phenyl substituents very slightly improved the thermostability in air and less than the presence of α- or β-naphthyl substituents.

Περίληψη

Πολυσυμπύκνωση τοῦ διχλωριδίου τοῦ 2,5-φουρανοδικαρβονικοῦ ὀξέος μέ τό N^1,N^5-διφαινυλο-1,2,4,5-τετρααμινοβενζόλιο σέ δύο στάδια.

Στήν ἐργασία αὐτή μελετοῦνται ἀρχικά οἱ παράμετροι τῆς ἀντιδράσεως πολυμερισμοῦ, σέ διάλυμα καί χαμηλή θερμοκρασία, (χρόνος, συγκέντρωση, θερμοκρασία, μοριακή σχέση τῶν ἀντιδρώντων μονομερῶν καί διαλύτης) τοῦ διχλωριδίου τοῦ 2,5-φουρανοδικαρβονικοῦ ὀξέος μέ τό N^1,N^5-διφαινυλο-1,2,4,5-τετρααμινοβενζόλιο.

Τό νέο πολυ[φουρυλενο-(2,4-διφαινυλαμινο)αμίδιο] πού παρασκευάστηκε μέ τίς βέλτιστες συνθῆκες παρουσίασε μέσο μοριακό βάρος 18.600 (ὡσμωμετρικά) καί ἔδωσε ἀπό διάλυμά του σέ DMF διαφανή εὔκαμπτη μεμβράνη. Αὐτό μέ θερμική κυκλοσυμπύκνωση μετατράπηκε στό νέο ἀντίστοιχο πολυ[φουρυλενο(1,7-διφαινυλο)βενζοδιιμιδαζόλιο], ἀδιάλυτο σέ πολικούς διαλύτες, μέ καλή θερμική ἀντοχή (405 °C στόν ἀέρα, 510 °C στό ἄζωτο) ὅπως ἔδειξε ἡ θερμοσταθμική ἀνάλυση.

Τά δύο πολυμερή πού παρασκευάστηκαν μελετήθηκαν μέ στοιχειακή ἀνάλυση, μέτρηση τοῦ λογαριθμικοῦ ἰξώδους (n_{inh}), λήψη φασμάτων IR καί UV, καί μέ διαφορική θερμιδομετρία σαρώσεως DSC.

Ἡ σύγκριση τῆς θερμικῆς σταθερότητας τοῦ πολυβενζοδιμιδαζολίου αὐτοῦ μέ αὐτήν τοῦ ἀντίστοιχου μή ὑποκαταστημένου ἔδειξε ὅτι ἡ παρουσία τῶν φαινυλο- ὑποκαταστατῶν μείωσε τή θερμική σταθερότητα στό ἄζωτο ἐνῶ αὔξησε αὐτήν στόν ἀέρα.

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ELECTROLYTIC FLOTATION IN INDUSTRIAL EFFLUENT TREATMENT

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Summary

A detailed study of electrolytic flotation and its application to the processing of industrial effluents was carried out to determine the feasibility of the process. The current density, the conductivity of the solution, the depth of the tank and the initial concentration were taken as operating variables. Electrodes of different configuration and material were also tried. Preliminary information on flotation characteristics obtained from batch tests have been tested in a continuous unit. The kinetics and the scale-up of the process were then studied.

Key words : Parameters, bubbles, flocculation, electrodes, continuous-flow, hydrodynamics, membrane, operation.

Introduction

Electroflotation relies on electrolysis of the liquid between electrodes with the resultant liberation of hydrogen and oxygen gases as extremely fine bubbles, which rise to the surface acting as the bubble «blanket» (1). Electroflotation competes, in a broad sense, with air flotation as a means of achieving or accelerating the physical separation of phases, whether these are emulsions or suspensions. It has, therefore, a wide range of applications in the field of industrial and mixed effluent treatment (2), (3), in addition to its original field of minerals beneficiation. It is generally envisaged as a system involving a tank in which two electrodes placed horizontally at the bottom create, during passage of d.c., a stream of bubbles which achieve the flotation process (Fig. 1).

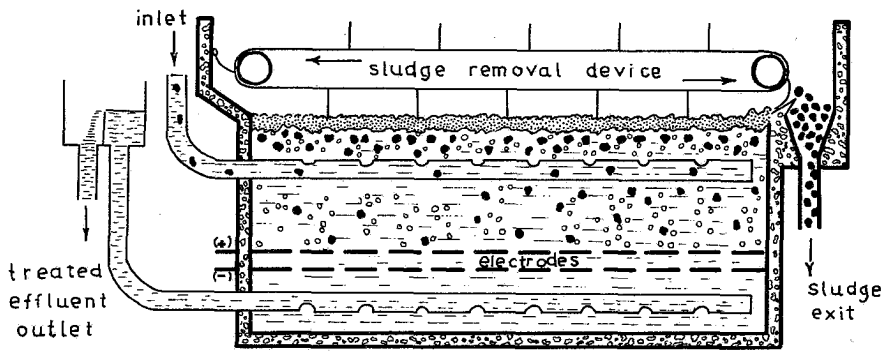

FIG. 1 : *Schematic representation of an electrolytic flotation unit.*

Results and Discussion

Type of Effluents Treated

Two laboratory prepared dispersions have been tried in the main study, in order to test the fundamental aspects of the process. The first, being an emulsion paint dilution, was chosen as a suitable suspension to determine the feasibility of electrolytic flotation as a solid/liquid separation method. The second was an emulsified oil-water dispersion and was studied to examine the effectiveness of the process as a liquid/liquid separation technique.

The process was then tried effectivelly on a industrial waste, from a power station, consisting of a mixture of oil, pulverized fly ash and an aqueous solution. A series of experiments on milk dilutions were also tried giving promising results; protein recovery could be a profitable aspect of waste treatment.

An organic «haze» was also tested. The problem arises during the coalescence of phases in copper solvent extraction. The removal of the organic entainment from the advanced electrolyte presents a valuable product recovery, keeping in mind both the production of pure copper electrodes and the solvent economy.

Finally, an oil-water sludge sample taken from a refinery's interceptor was treated successfully by electroflotation. The process is expected to cover the field of breaking and separating the emulsions as the common oil-water gravity separators are not designed for these purposes (4).

Processing Variables

The current density applied to the horizontal pair of electrodes was in the range of 100-300 A/m^2. The voltage was usually in the range of 5-20 V,

depending on the effluent conductivity; while the interelectrode gap was around 3 mm. On certain cases a common electrolyte (once, seawater) was added to increase the conductivity of the solution. This can make an appreciable saving in power consumption.

Increasing the current density results in a great deal more gas being produced by the electrolytic cell. A large proportion of the gases may be ineffective, but the net effect was to reduce the time required for solids removal (Fig. 2). However, a higher current density brings an increase in

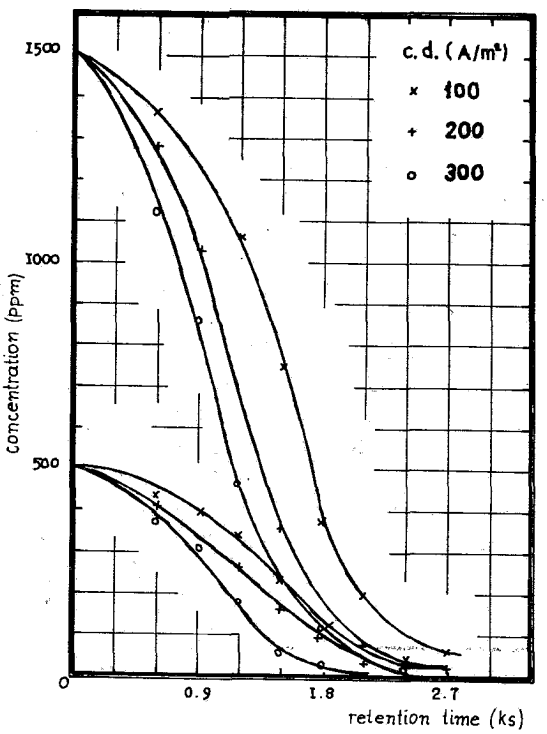

FIG. 2 : *The change of concentration of paint with time. The effect of current density.*

running costs. It was observed that the retention time of the process was increasing with feed concentration and with decreasing power input. Tank depths up to 1.5 m were tried successfully. On one occasion a solids concentration reduction of 99.95 % was observed.

The gas produced by electrolysis was the working tool in the flotation process. It was calculated that the hydrogen flux was 173 mm^3/s for the current density of 100 A/m^2. Assuming that the bubbles have a spherical shape, with a diameter of 0.05 mm, the number of bubbles evolved was around $4 \times 10^6/s$, at the above current. Observations of the gas bubbles evolved flowing upwards gave the impression of laminar flow with no disturbance or mixing of the liquid.

A phenomenon has been observed where air bubbles – generated by dispersed-air flotation – were, in some occasions, only mixing the liquid and no phase separation was produced. However, flotation rate was found in other cases as being somehow independent of bubble size, and more dependent on gas volumetric flow.

A very stable and relatively dry froth layer was formed during the electroflotation experiments, which consisted of gas bubbles and solids, oil, or both depending on the experiment. Even when the current was switched off, there was essentially no tendency for the solids to return to the solution. The thickness of the layer was a function of current density, being greater at higher densities. If thickening is the important function of the process then a low current density should be used. The common method of removal of this surface layer in flotation processes is by a large number of slow moving flights combined with a speed controlled conveyor drive. It is then usually disposed at approved areas, or burnt as it mainly contains organic matter, or even further processed for by-products recovery, on certain cases.

Flocculation

Chemical additives have been found to increase the flotation rate. Clarification time and effluent quality were shown to depend strongly upon pH. This is a known effect from minerals flotation; there is a definite relation between zeta potential, the ionic strength of the solution and flotation recovery.

Flocculation is one of the dominant factors in dissolved-air flotation. In difficult cases, such as stabilized emulsions or where stringent effluent discharge conditions are applicable, it is thought necessary to have a well designed flocculator. It seems, at first, that flocculation increases the running costs, however, the decrease in the retention time more than makes up for this increase (5).

In electrolytic flotation the need for charge neutralizers and coagulating agents may be reduced to a minimum, and in some applications will not be needed at all, due to the surface charges of the gas bubbles. The addition of flocculants succeeded indeed in shortening the retention time. However, the use of them should depend on the optimization of the process, keeping in mind their cost.

Electrolytic Bubbles

Gas bubble size is one of the parameters that affect flotation design. The electrolytically produced bubbles, being of two separate gases, presented some difficulty when, after measured from the projected photographs, plotted on a probability paper. Gas bubbles evolved at electrodes under different conditions may have different sizes.

Most of the bubbles measured were in the range 20-90 μm. Those bubbles around the upper limit were believed to be the outcome of coalescence. At 100 A/m^2 the mean diameter was 50 μm. The statistical mean in the empirical distributions decreased with respect to bubble size as the applied current density was increased. The number of bubbles increases with increasing current density.

Separated Electrodes

Another consideration was if the chemical differences of the gases had any effect on the process. This was accomplished by the construction of another cell having the two horizontal electrodes separated by a cation exchange membrane. In the meantime, any different effect introduced by the anode and cathode separately was investigated (Fig. 3).

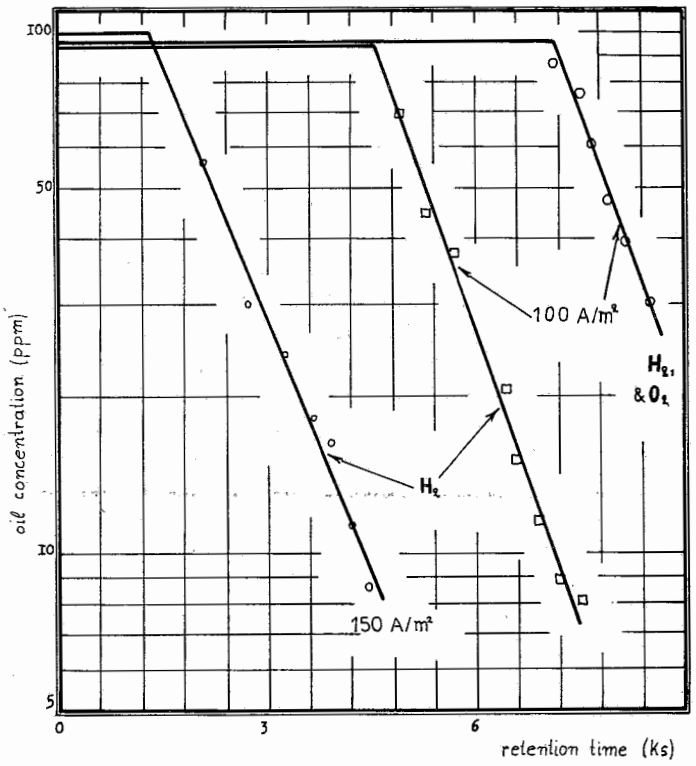

FIG. 3 : *Experiments with the conventional electrolytic cell and with separated anode. Verification of the first-order equation.*

The specific role of the chemical effect of various gases during flotation has been studied in early works applied on minerals research. Now coming to electrolysis, one of the fundamental theoretical aspects concerns the mechanism of gas evolution. In fact, despite the considerable literature on the subject, there is no universal agreement as to the precise mechanism, which indeed may vary with the electrode surface and other operating conditions.

By using atomic hydrogen and atomic oxygen, it was established that their chemical action on the suspended particles surfaces change the physicochemical properties and either assist or suppress flotation. A gas removing medium was introduced in the lower part of the cell under the membrane, to carry away the bubbles generated by the separated electrode (4).

It is understood that by separating the cathodic and anodic spaces of the electrolytic cell in the flotation chamber, the whole design becomes more complex. However, the advantage of operating with either electrodes can be applied, when moving from the laboratory scale to an industrial plant, by using the effect of the difference in the surface areas of the working electrodes.

Continuous Flow Experiments

Batch flotation tests were used to obtain data to evaluate a continuous flow flotation unit, to foresee the way it should work and predict the expected result. The basic information desired from the batch tests included answers to questions including the following :

1) Will the material being considered float?
2) How rapidly will it float?
3) What kind of electrodes does it need?
4) What degree of effluent clarification can be expected?
5) What will be the rate of flotation?
6) How do changes in current density and feed concentration influence these answers?

The answers to these questions are important when one is considering the design of a flotation equipment for a particular application. Attention should be paid on the sensitivity of the process with respect to each application.

As the flow of gas bubbles is necessarily rising, there are three choices for the liquid entering the tank, among the following possibilities: 1) horizontal, 2) ascending, 3) descending (6). In the horizontal flow system it was found difficult to avoid parasitic currents of recirculation due to density differences of the bubbles. The cocurrent system was said to be less useful because of the difficulty presented at the superior part to choose between the treated liquid and the impurities. An homogeneous descending system provides countercurrent flow between the gas bubbles and the solid particles, and was said to give a more favourable movement from the point of concentration gradients and probability of encounter. The counterflow circulation of the liquid was found to give a high efficiency «filtration» effect. This was maximum when

the liquid passed through the electrodes to reach the lower part of the tank. Since the downward liquid velocity is less than the upward velocity of the bubbles, it was suggested that the treated water was almost entirely clarified.

Generally, the batchwise tests can be used to gain preliminary information on the flotation characteristics, while continuous flow experiments may give the final design data. However, it has been reported that the results obtained from prototype flotation units do not always agree with the results predicted from the data obtained from batch tests (7).

Detention time and feed concentration were found to affect the exit effluent concentration, for the specific application. Detention times were recorded in the range of 3.3 ks for easily floatable matter and with feed concentrations around 1,500 ppm, giving a concentration reduction of an order of 90 %. The process was shown to be affected by high throughputs. With difficult emulsions, the optimum detention time was approximately twice the above mentioned, succeeding concentration reductions of around 80 % this time, with feed concentrations as low as 60-100 ppm of oil.

One of the factors on which greatly depends the performance of the electrolytic flotation unit is the inlet design. A proper inlet could maximize the adhesion efficiency by reducing turbulence. Any hydrodynamic streams would contribute to detachment of bubbles from the suspended particles. It was realised that the process is not intended to replace completely existing forms of waste water treatment, but rather to provide a facility which will considerably reduce the polluting load of effluents by simple and economical means.

Flotation Hydrodynamics

The term «hydraulic efficiency» has been introduced in sanitary engineering to describe the detention time distribution of the fluid and the flow regime in the system. The measurement and analysis of the hydrodynamics is usually of great importance in evaluating the system performance.

This investigation was undertaken as the efficiency was expected to be a function of the residence time distribution. The stimulus-response technique was used in this experimentation. The tracer input signal was an electrolyte (acid) and had the form of a step function. Assuming that the flow regime was composed of various flow types, a theoretical mixed model (8) was applied and the experimental data were fitted accordingly.

It was concluded that no by-pass flow existed, while there was a deadwater region during the cocurrent mode. The flow regime was dominantly backmix flow. Attention should be paid at the settling of suspended mater as a result, usually, of turbulence around the inlet. This is an antagonistic process when the particles are heavier than water.

Operation and Design Aspects

Before a viable electrolytic flotation process could be developed it is necessary to find electrode materials of adequate mechanical and electrical properties with an extended lifetime. The basic requirement for the electrodes is to evolve very fine bubbles by electrolysis of the influent fluid. To accomplish this in practice a number of criteria must be satisfied :

1) no electrode corrosion,
2) avoidance of scaling,
3) capability of operating at high current densities, and
4) should not produce objectionable gaseous products.

In this study the cell has been constructed from stainless steel expanded mesh. The corrosion of the anode is indeed a disadvantage of the process, particularly in high anion concentration in the effluent. The use of lead dioxide covered titanium was reported as a reasonable solution; this was found from accelerated life tests (9). Electrolytic cells of different material and construction have been tested also here, and among them a set of bipolar electrodes (4).

A comparison of running expenses between flotation systems showed (Gardner, (2)) that electrolytic flotation requires significantly less power on units of 5 m^2 area or less, i.e. units able to accept flows in the range up to 13.9 m^3/ks. The advantage decreases with increase in the size of cell. Calculations from the present data on power consumption gave approximately 3.6 MJ/m^3 –depending always on the conductivity of the effluent– which means that the power costs of the process were 0.75 pence per m^3 of effluent.

This treatment could still be considered as an «art» and more research was needed on the basic theory of flotation performance and design. The beginning was done with the bubbles measurement and the residence time considerations described above. The hydrodynamics study proved an earlier assumption that the vessel resembles a perfectly mixed reactor.

The principles of reactor design and scale-up are generally well established in chemical technology (10). This knowledge would not only benefit operation and prediction, but also flotation plant design would be placed on a surer foundation, reducing the excessive reliance on costly trial and error development of new and improved units.

An account of flotation machine development, together with the general principles governing the operation of these machines, was given (11); the main effort has been to find the most suitable impeller design. Process design theory developed for foam separation was based on its resemblance, in a way, with distillation with entrainment (12). While, the batch flux analysis, known from the sedimentation of suspensions, was applied in dissolved-air flotation (13). It seems that there are still fruitful areas for investigation.

Scaling-up experiments have been carried out – with surface area ratio of 0.017 : 0.36 m^2 (Fig. 4). The conclusion was that the rate of flotation was decreased, which means longer detention times. The same result was obtai-

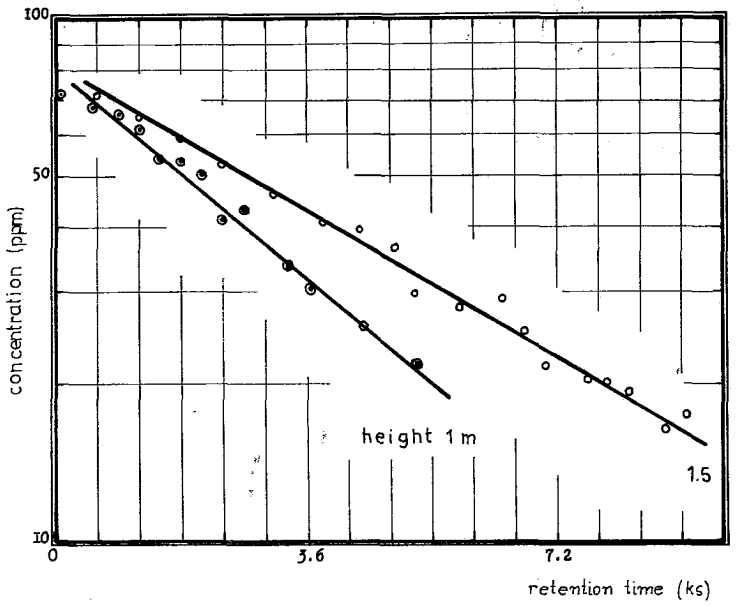

FIG. 4 : *Pilot plant electrolytic flotation tests with the emulsified oil dispersion.*

ned when the effect of liquid height was investigated. Two reasons were given: the wall effects in the small vessel, and the internal reflux of suspended matter from the disturbed froth.

The knowledge of the rate at which suspended matter will be floated in a unit is very important; also, an operator should be able to identify the variables that control the rate. This understanding of the process can lead to improvement to flotation design.

A macroscopic method was followed in the kinetics study. It consisted of the analysis of variations in suspended matter concentration as a function of time. The conditions governing the entire period of the batch process were not constant. It was divided in stages and each was examined separately. The actual flotation stage was found to follow a first-order equation.

Flotation rates were found to be greater than settling rates under the same conditions. This suggests a difference in the surface area requirement of the two processes. Also, better effluent quality and shorter retention times were involved in the former.

The flotation coefficient was calculated as a function of time, and from that the flotation rate, which was observed to reach a maximum. The effects of initial concentrations and current density were studied (Fig. 5). These flotation rate maxima when plotted as a function of initial cell concentration, in the range tested, were found to increase at first until a constant maximum value is reached.

It seemed that there was an optimum concentration, where the coefficient

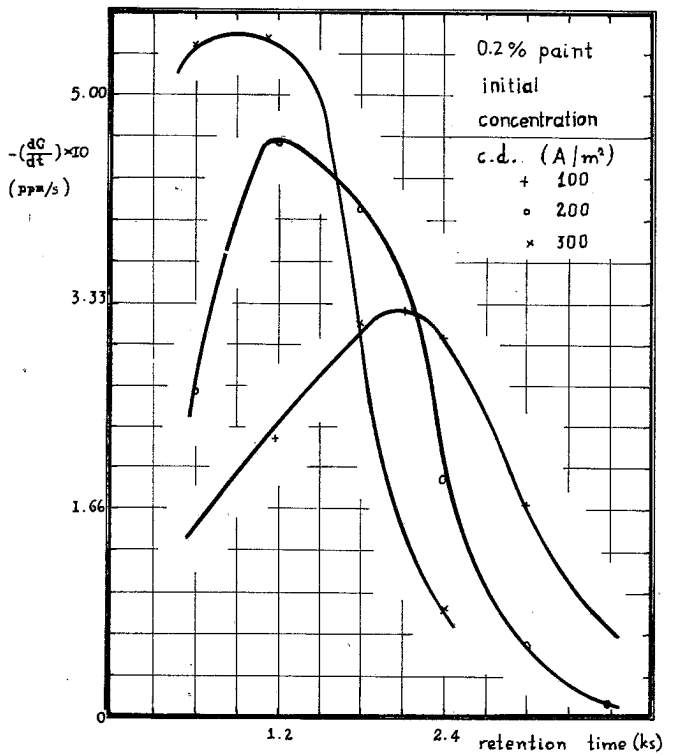

FIG. 5 : *Variation of flotation rate with time at different power inputs.*

reached a maximum. There was also evidence that there was a certain limit in suspended solids concentration, over which the process could not operate. The generated bubbles were merely mixing the liquid.

General Discussion

When an effluent is brought between two electrodes, of which one is the positive anode and the other is the negative cathode, and energy is applied to the electrodes, an electric field is built up between them through the use of the conductivity of the suspension. Without the addition of chemicals a preliminary coagulation occurs within the suspension, which seeks to group the negative and positive particles together. Furthermore, with the decomposition of water into its constituents, in the diffusion layer of the anode comes free atomic oxygen, which is carried by convection into the suspension and it immediately combines with organic and inorganic matter resulting in its oxidation. In a similar manner, there is also a transformation brought about by the electrically excited hydrogen resulting in the reduction of contained matter.

The four basic electrokinetic phenomena depend upon the same phenomenon of selective ion distribution on interfaces, and are involved in the interface theory of flotation. It has been suggested that if it is desired to remove all suspended matter from a liquid, advantages should result from using a continuous stream of extremely fine bubbles, possessing potentials in the positive and negative sense. These electrolytically produced micro-gas bubbles possess very high surface charge densities, and because of the random movement in the solution, they will find particles and globules with an opposite charge. Thus, one may envision that the electrokinetic forces which cause colloids and emulsions to be dispersed may be neutralized by charges on the gas bubbles, which will then be available to collect around all suspended matter and float it to the surface.

The electric field gradient between the electrodes aids the flocculation of suspended matter. Further, when an electric current is applied to an effluent the oxidation-reduction potential changes depending on the type of electron treatment. Also, nascent hydrogen and oxygen are highly active and as they attack the particle surface, a change of the flotation characteristics of this particle can be effected. Any or more of the above effects could be operating during electroflotation, and they have been described with the general term «electrochemical effect» of the process. There should be a differentiation between that and the application of gas bubbles in flotation as the working medium for the transport process.

Concluding Remarks

Reviewing the process it should be noticed that :

1. The electrode grids can be arranged to provide good coverage of the whole surface area of the flotation tank, so uniform mixing between the effluent and the gas bubbles is achieved.

2. A large amount of very small bubbles is formed with minimum turbulence, with result that clarification can be effected with effluents that previously would not have been considered suitable for treatment by flotation.

3. The electric field gradient between the electrodes aids flocculation of suspended matter.

4. Gas production, residence time and the other operating conditions can be checked quickly and are easily controlled.

5. No high pressure pumps, pressurized vessels, or other complex machinery is needed, and the moving parts are minimal.

6. The equipment is reliable and safe in operation since only low voltage is used.

7. The relative quantities of gases produced are a function of current density and salinity of the solution. A simple blower system would eliminate any hazard from the predominant escaping gas (hydrogen).

8. Electrolytic flotation can be used in cases where air could be difficult to dissolve in a particular effluent.

These are some of the reasons that make the process of electroflotation attractive.

Περίληψη

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Στά τελευταῖα χρόνια ἡ ἰδέα τῆς χρησιμοποίησης τῶν ἀνερχόμενων ἀέριων φυσαλίδων γιά τό διαχωρισμό ὑλικοῦ (ὑγροῦ ἤ στερεοῦ) σέ διασπορά, ἀπό ἕνα αἰώρημα, ἔχει ἐφαρμοσθεῖ μέ καλά ἀποτελέσματα στόν καθαρισμό τῶν ὑγρῶν ἀποβλήτων. Ἡ ἠλεκτρόλυση μπορεῖ νά θεωρηθεῖ σάν μιά ἀπό τίς μεθόδους πού παράγουν μικρές φυσαλίδες, ὑδρογόνου καί ὀξυγόνου, οἱ ὁποῖες ἔχουν τήν ἱκανότητα νά προκαλέσουν διαχωρισμό φάσεων. Ἔτσι, ἡ ἠλεκτρολυτική ἐπίπλευση εἶναι μιά τεχνική γιά τήν ἀπομάκρυνση αἰωρούμενων σωματιδίων (καί σταγονιδίων) μέ ἐπίπλευση καί χρησιμοποιεῖ τίς φυσαλίδες πού σχηματίζονται σάν ἀποτέλεσμα τῆς ἠλεκτρόλυσης τοῦ ὑδάτινου μέρους τοῦ ὑγροῦ. Τό μηχάνημα ἀποτελεῖται ἀπό δύο τμήματα οὐσιαστικά, τό ἕνα εἶναι ἡ μονάδα ἐπιπλεύσεως καί περιλαμβάνει τό ἀπαιτούμενο σύστημα ἠλεκτροδίων, καί τό ἄλλο εἶναι ἕνας μετασχηματιστής-ἀνορθωτής πού παρέχει συνεχές ρεῦμα χαμηλῆς τάσεως στήν κυψελίδα.

Ἡ διεργασία ἐφαρμόσθηκε σέ πολλούς τύπους ἀποβλήτων μέ ἐπιτυχία. Ἐξετάστηκαν πρῶτα οἱ μεταβλητές τοῦ συστήματος, τό εἶδος τῶν ἠλεκτροδίων, τό μέγεθος τῶν φυσαλίδων, καί ἡ χρήση τῆς κροκίδωσης. Τά ἀποτελέσματα ἀπό τά πειράματα καί οἱ πρῶτες πληροφορίες χρησιμοποιήθηκαν μετά σέ λειτουργία συνεχοῦς ροῆς. Ὁ χρόνος παραμονῆς καί ἡ συγκέντρωση τροφοδοσίας φάνηκαν νά ἐπιδροῦν στή συγκέντρωση ἐξόδου τῶν ἀπονέρων. Δοκιμάσθηκε ἀκόμα ὁ διαχωρισμός τῶν δύο ἠλεκτροδίων στήν ἠλεκτρολυτική κυψελίδα μέ κατιονική μεμβράνη. Ἐπίσης, μελετήθηκαν ἡ κινητική τοῦ συστήματος, προβλήματα μεγέθυνσης καί θέματα λειτουργίας καί σχεδιασμοῦ. Γενικά, ἡ ἠλεκτρολυτική ἐπίπλευση προσφέρει μιά πρακτική διεργασία καθαρισμοῦ πού παρουσιάζει ὁρισμένα πλεονεκτήματα σέ σχέση μέ τίς συνηθισμένες τεχνικές ἐπίπλευσης.

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OXIDATIVE CYCLISATION OF SOME 1,3- AND 1,4-DIOXIMES WITH PHENYLIODINE (III) BIS TRIFLUOROACETATE

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Summary

1,3-Dioximes with an active methylene group are oxidised by the title reagent into 4-oxo-4-H-pyrazole 1,2-dioxides, while substituted isoxazoles are simultaneously formed. 2-Unsaturated 1,4-dioximes are oxidised into a mixture of pyridazine 1,2-dioxides and 3a,6a-dihydroisoxazolo[4,5-d] isoxazoles.

Key Words : 4-oxo-4-H-pyrazole 1,2-dioxides, Isoxazoles, Pyridazine 1,2-dioxides, 3a,6a-dihydroisoxazolo(4,5-d)isoxazoles.

Introduction

Oximes are known to undergo easily oxidation with a variety of oxidising agents to a variety of products[1,2]. With the exception of 1,2-dioximes which are cyclised to furoxanes[1,3], no other dioximes had been examined at the time the present work begun. We report here our results with 1,3- and 1,4-dioximes, using as oxidant phenyliodine(III)bis trifluoroacetate[4], abbreviated PIB. It should be noted that oxidation of several monoximes of both aldehydes and ketones have also been studied with PIB[5], the results being similar to analogous oxidations with lead tetraacetate[2].

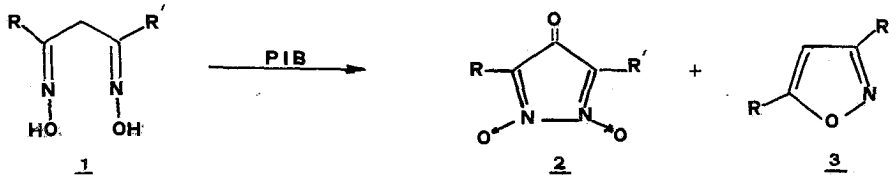
Results and Discussion

1,3-Dioximes. The dioximes oxidised were known compounds of unknown configuration. They were oxidised at 0^0C in suitable solvents with equimolecular amounts of PIB. The results for five 1,3-dioximes are presented in Table I. It can be seen that flexible dioximes having an unsubstituted active methylene group (1) give two products, i.e. 4-oxo-2H-pyrazole 1,2-dioxides (2) and 3,5-disubstituted isoxazoles (3), according to Scheme 1.

TABLE I. : Oxidation Products from Dioximes *1*

Oxime	R	R′	Isoxazole *3* M.p.°C(Lit. m.p.), Yield	Pyrazole dioxide *2* M.P.°C(Lit. m.p.), Yield
1a	Me	Me	oil, 33%	108-110(109-110[c]) 20%
1b	Me	Ph	41-42(42-43[a]), 60%	162-164(163-164[c]) 23%
1c	Ph	Ph	139-141(141[b]), 44%	189-190(191-192[c]) 8%
1d	$-(CH_2)_3$	-	-	-

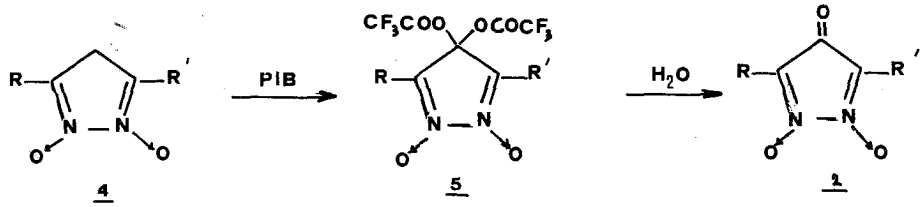
[a]L. Claisen, *Chem. Ber.*, **40**, 3903 (1907).
[b]H. Goldschmidt, *Chem. Ber.*, **28**, 2540 (1895).
[c]Ref. 6.



Scheme 1

4-Oxo-4H-pyrazole dioxides have been previously prepared from oximes of 2-unsaturated ketones upon treatment with $NaNO_2$[6,7] and also by oxidation of 1,3-dioximes with NaOBr[8]; in that case the initially formed 4,4-dibromo-4H-pyrazole 1,2-dioxides were subsequently hydrolysed into *2*. The identification of 2 as reaction products was effected by comparison of their physical and spectroscopic properties with those of authentic samples or with values from the literature.

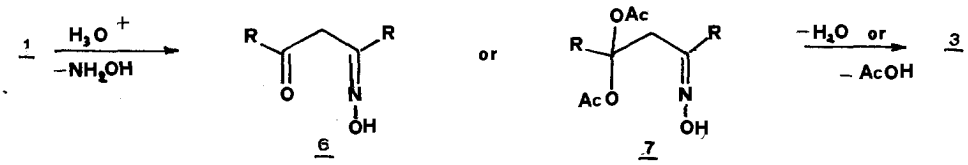
A characteristic spectroscopic feature in their mass spectra is the low abundance $[M\text{-}16]^+$, (M-oxygen), and $[M\text{-}28]^+$, (M-carbonyl), fragment ions; the major fragment ions correspond to nitrile oxides, $[RCNO]^+$. These features were later corroborated in a publication[9] dealing exclusively with the mass spectra of *2*. Concerning the mechanism of the reaction, several possibilities exist leading to the 4H-pyrazole 1,2-dioxide *4*, e.g. the dioxime may first be transformed into an iminoxy radical, by abstraction of H·, $RC(=NOH)CH_2C(NO\cdot)R$, or give an iodine (III) derivative, $RC[=N\text{-}OI(Ph)OCOCF_3]CH_2C(=NOH)R$, or a gem-nitroso-trifluoroacetate $RC(NO)(OCOCF_3)CH_2C(=NOH)R$. Such intermediates are known to be formed from simple oximes[1,2,5]; by eventual release of iodobenzene and trifluoroacetic acid they may be converted into *4*, which reacts with more PIB and gives the bis trifluoroacetoxy derivative *5* and this is finally hydrolysed, probably during work-up (Scheme 2).



Scheme 2

Reactions with PIB where an active methylene group is transformed into trifluoroacetate of a gem-diol have been observed in some instances, e.g. in the oxidation of fluorene to fluorenone[5]. The propensity of gem-trifluoroacetates to hydrolyse into ketones is well documented. Air-oxidation of *4* to *2* probably occurs also to a considerable extent. Thus, by carrying out the reactions by bubbling through atmospheric air yields of *2* were increased up to 30%, while under nitrogen yields were only marginally smaller (1-3%).

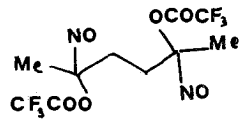
The isoxazoles *3* are formed by an acid-catalysed ring-closure of the dioximes from the trifluoroacetic acid released upon the reduction of PIB. An independent experiment showed that the dioxime of 1,3-diphenyl 1,3-propanedione is converted into *3* by 3% trifluoroacetic acid in chloroform. A similar transformation has been observed when *6* was treated with formic acid[10]. There is no doubt that in these cases a partial hydrolysis (or acidolysis) of *1* gives the monoxime *6* (or the acylal *7*), which cyclise to *3* by dehydration (or removal of acid), Scheme 3 :



Scheme 3

The dioxime of 1,3-cyclohexanedione was not cyclised, because the two oximino groups are too far away to interact. The only isolated product was the parent diketone. Similarly the carbonyl compound is only isolated from the dioxime of 4-ethyl-3,5-heptanedione. Presumably the ketones result from gem-nitrosotrifluoroacetates, which hydrolyse upon work-up or decompose thermally, like other similar compounds[11].

1,4-Dioximes. The only reaction studied was that between the dioxime of 2,5-hexanedione and PIB. No cyclisation occured and the only product formed was the bis-gem-nitrosotrifluoroacetate *8*.

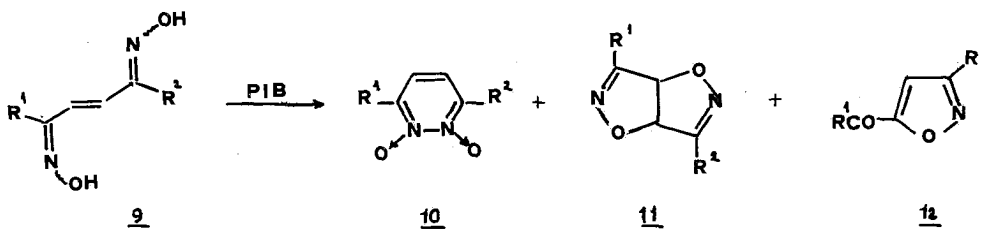


As with simple oximes[5] the compound could not be obtained pure but its formation was deduced by its u.v. spectrum (λ_{max} at 640nm) and its i.r. spectrum (ν_{CO} at 1795 cm^{-1}, lack of OH absorption).

Attempts to obtain Diels-Alder adducts from *8* or other simple gem-nitrosotrifluoroacetates and anthracene failed, although nitrosocompounds with α- substituents of strong electron-withdrawing character behave as dienophiles[12].

2-Unsaturated 1,4-dioximes. These dioximes, from trans-diketones but of unknown configuration, *9*, are new compounds[5] and they have been reported[13] to cyclise with PIB to pyridazine 1,2-dioxides *10*, a class of compounds with few known representatives.

However Ohsawa et al.[14] showed that oxidation of the dioxime of 1,4-diphenyl-2-butene-1,4-dione *9a*, gives mainly 3a,6a-dihydroisoxazolo [5,4-d] isoxazole *11a*, along with small amounts of the isomeric pyridazine 1,2-dioxide *10a* and some other minor products (Scheme 4). Furthermore, the reported 3,6-diphenyl-pyridazine 1,2-dioxide[13] was proved to be actually 3,6-diphenyl-3a,6a-dihydroisoxazolo[4,5-d]isoxazole.



9 10 11 12

Scheme 4

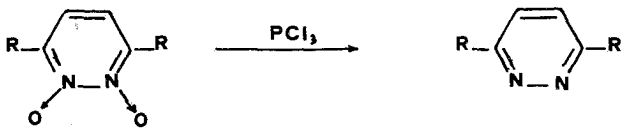
The above finding has led to a reexamination of the reactions between 2-unsaturated 1,4-dioximes and PIB. It was found that the dioximes of 1,2,4-triphenyl-2-butene-1,4-dione and of 2-hexene-1,4-dione give exclusively *10*, as already reported[13], while all other dioximes, (with the exception of that of 1-p-nitrophenyl-4-phenyl-2-butene-1,4-dione, which gives only *11*) give both *10* and *11*, but those assigned as *10* are actually *11*. Pyridazine 1,2-dioxides are eluted from the chromatographic column after the dihydroisoxazolo-isoxazoles with fairly polar solvent mixtures and they are isolated in small yields, 2-7%. Sometimes a third product may also be eluted, before *10*, i.e. isoxazole *12*. The results obtained appear in Table II.

The distinction between *10* and *11* is based on both chemical and spectroscopic evidence. Pyridazine 1,2-dioxides are deoxygenated with PCl_3 to the known pyridazines *13*, while dihydroisoxazolo-isoxazoles remain unchanged under these conditions (Scheme 5).

TABLE II : Pyridazine *1,2*-Dioxides[a] *10* from the Oxidation of Dioximes *9*

Oxime	R^1	R^2	Yield (%)	m.p.[b](dec.) (solvent)	Molecular formula	I.R.(nujol) νN-O(cm^{-1})	U.V.(C_2H_5OH) λ_{max}(nm)	^{1}H-N.M.R.($CDCl_3$) δ (ppm)	M.S. (70eV) m/e (relative intensity)
9a	C_6H_5	C_6H_5	3	258-260° (ethanol)	$C_{16}H_{12}N_2O_2$ (264.3)	1320	278	7.60-8.00 (m)	264(20)M^+, 248(11), 234(11), 119(100)
9b	C_6H_5	p-BrC_6H_4	7	269-270° (methanol)	$C_{16}H_{11}BrN_2O_2$ (343.2)	1330	289	7.65-8.04 [d](m)	344, 342(16)M^+, 329, 327(23), 312, 310(21) 103(100)
9c	p-BrC_6H_4	p-BrC_6H_4	2	305-307° (—)[e]	$C_{16}H_{10}Br_2N_2O_2$ (422.1)	1320	289	—[c]	M^+(7, 13, 7), (M-0) (3) (M-20) (2), 225, 223(100)
9d	C_6H_5	p-$MeOC_6H_4$	6	248-249° ($CHCl_3$-hexane)	$C_{17}H_{14}N_2O_3$ (293.3)	1330	290	3.58(s), 6.97(d) 7.50-7.94 (m)	294(12)M^+, 278(30), 262(63), 132(100)
9e	C_6H_5	β-naphthyl	3	248-250° ($CHCl_3$-hexane)	$C_{20}H_{14}N_2O_2$ (314.3)	1330	286	7.5 (m) 7.80-8.15 (m)	314(14)M^+, 298(13), 282(11), 153(100)
9f	C_6H_5	p-ClC_6H_4	4.5	258-260° (methanol)	$C_{16}H_{11}ClN_2O_2$ (298.7)	1330	288	7.60-8.15 (m)	300(6), 298(19)M^+, (M-0) (6, 20), (M-20) (18, 50) 179(100)
9g	p-ClC_6H_4	p-ClC_6H_4	6.5	300-301° (dioxane)	$C_{16}H_{10}Cl_2N_2O_2$ (333.1)	1320	291	—[c]	M^+(2,6,10), (M-O)(2,6,8), (M-20)(5,11,18), 179(100)

[a] All compounds gave satisfactory elemental analyses (C ± 0.36%, H ± 0.34%, N ± 0.52%) except 10g.
[b] Uncorrected.
[c] Reported m.p. 258° (ref. 14).
[d] In 80% $CDCl_3$ + 20% CF_3COOH.
[e] Insoluble in common solvents.



10 13

Scheme 5

Several attempts to oxidise *11* (with MnO_2 and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone) to isoxazolo-isoxazoles were unsuccessful. Between the two classes of compounds, *10* have invariably some 50 °C higher melting point than *11*, with decomposition.

The u.v. spectra of *10* and *11* differ in that λ_{max} of *10* and *11* appear at 290 nm and 260 nm, respectively. The i.r. spectra of *10* and *11* have no significant differences. The n.m.r. signals of protons on C-4 and C-5 of the pyridazine ring in *10* appear at 7.5δ, while the protons on C-3a and C-6a of the isoxazoline rings in *11* resonate at higher field, 6.5 δ. Finally the mass spectra of *10* have both $[M\text{-}O]^+$ ions, which are absent in *11*.

The 3-arylo-5-aroylo-isoxazoles were (Table II) *12a* (m.p. 64 °) and *12f* (m.p. 149 °), in yields of 2% and 3%. They showed the expected molecular ion and fragmentation pattern in their mass spectra, but no microanalyses have been performed.

Experimental

NMR (60 MHz, Me_4Si internal standard) and mass spectra (70 eV) were recorded on Varian A-60A and Hitachi-Perkin-Elmer RMU-6L spectrometers, respectively. IR spectra were recorded on a Perkin-Elmer 257 spectrophotometer. Melting points have been obtained on a Kofler hot stage apparatus and they are uncorrected.

All starting materials were known compounds, with the exception of the 2-unsaturated, 1,4-dioximes which were prepared by standard methods from the respective trans-ketones. Their physical and spectral data as well as elemental analyses may be found in reference 5.

General procedure for the reactions of 1,3-dioximes. 4.1 mmol of PIB were added into a solution of 4 mmol of the dioxime in chloroform or acetone (20 ml) at 0 °C. After a few hours all dioxime was consumed. The solution was evaporated to dryness and the residue chromatographed in a silica gel column, using as eluent hexane-chloroform. The order of elution was iodobenzene, isoxazole and pyrazole dioxide.

General procedure for the reactions of 1,4-dioximes. The experimental conditions were identical to those of reference 13. The order of elution during column chromatography was iodobenzene, isoxazolo-isoxazole, (isoxazole), and pyridazine dioxide.

General procedure for the deoxygenation of pyridazine 1,2-dioxides. A sample of *10* was refluxed with an excess of PCl_3 in $CHCl_3$ for 2 h. Water was added to the reaction mixture, the aqueous layer was alkaline and the pyridazine was extracted with $CHCl_3$. After drying and removal of the solvent, the residue was recrystallised from ethanol. Yields ranged between 50% and 90%. The following pyridazines *13* were obtained: *13a*, m.p. 220-221° (lit.[15] 221-222°), *13b*, m.p. 240° (new compound), *13c*, m.p. 281-284° (lit.[16] 285°), *13d*, m.p. 198-200° (lit.[17] 198-199°), *13e* m.p. 206-208° (lit.[17] 206-208°), *13f*, m.p. 230-231° (lit.[17] 231-232°), *13g*, m.p. 260-264° (lit.[16] 264°).

Περίληψη

'Οξειδωτική κυκλοποίηση 1,3- καί 1,4-διοξιμῶν μέ διτριφθορακετοξυ-ιωδοβενζόλιο

Διοξίμες 1,3-δικαρβονυλικῶν ἐνώσεων πού ἔχουν ἐνεργή μεθυλενική ὁμάδα κατά τήν ἐπίδραση διτριφθορακετοξυ-ιωδοβενζολίου ὀξειδώνονται σέ 4-ὀξο-4H-πυραζολο-1,2-διοξείδια, ἐνῶ παράλληλα σχηματίζονται καί 3,5-διυποκατεστημένα ἰσοξαζόλια.

Διοξίμες ἀκορέστων 1,4-δικαρβονυλικῶν ἐνώσεων ὀξειδώνονται σέ μίγμα 1,2-διοξειδίων τῆς πυριδαζίνης καί διυδρο-ισοξαζολο-ισοξαζολίων.

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