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# PROTEIN CHROMATOGRAPHY WITH PYRIDINE- AND ALKYL-THIOETHER-BASED AGAROSE ADSORBENTS

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## SUMMARY

In an attempt to identify the part of the ligand of 3-(2-pyridylthiol)-2-hydroxypropylagarose that is responsible for the specific adsorption of immunoglobulins and $\alpha_2$-macroglobulin from serum, nine agarose derivatives were prepared: (I) 3-(N-2-iminopyridyl)-2-hydroxypropyl-, (II) 3-(4-pyridylthio)-2-hydroxypropyl-, (III) 3-(2-pyridylthio-N-oxide)-2-hydroxypropyl-, (IV) 3-(2-pyridylthio)-2-hydroxypropyl-, (V) 3-(ethylthio)-2-hydroxypropyl-, (VI) 3-(*n*-butylthio)-2-hydroxypropyl-, (VII) 3-(2-aminoethylthio)-2-hydroxypropyl-, (VIII) 3-(2-hydroxyethylthiol)-2-hydroxypropyl- and (IX) 3-(N-2-pyridyl-2-one)-2-hydroxypropylagarose. The selective adsorption of the above serum proteins to these derivatives was analysed by chromatography. The electron distributions were calculated for three of the investigated pyridine derivatives in order to establish whether there is any relationship between the electron distribution in the molecule and the absorption properties of the pyridine derivatives. By optimizing the preparation methods for the different derivatives, the possible side-reactions were minimized and checked. The adsorbed serum proteins were determined by the Ouchterlony technique and electrophoresis. The concentration of human serum albumin in the different fractions was determined by conventional radioimmunological methods. These data make it possible to classify the adsorbents according to their selectivity and hydrophobic thiophilic behaviour.

## INTRODUCTION

Ligands of different types coupled to matrices have been investigated as potential adsorbents for chromatography, and we have attempted to identify the structural features responsible for the cooperative effects often observed between the ligand, the polymer matrix of the adsorbent and the protein[1–7]. In 1985 Porath *et al.*[8] reported a new type of adsorbent, the thiophilic gels, typified by T-gel, which exhibited a characteristic, group-specific adsorption of immunoglobulins, C3, C4 and $\alpha_2$-macroglobulin. This explorative work was based on the use of divinyl sulphone-activated agarose gel. The sulphone group, together with a thioether, seemed to be essential for the characteristic salt-promoted adsorption of proteins. However, on further investigation of the thiophilic adsorption, we discovered that epichlorohydrin-activated gels to which 2-mercaptopyridine had been coupled exhibit similar behaviour[9].

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In an attempt to correlate structure and adsorption properties we have undertaken a chromatographic study of a number of adsorbents with ligands differing with respect to the presence or absence of atoms or groups of atoms.

## EXPERIMENTAL

### *Chemicals*

Sepharose 6B, 2-aminoethanethiol, 1-ethanethiol and the human serum albumin–radioimmunological assay (HSA–RIA) kit for HSA detection were kindly supplied by Pharmacia (Uppsala, Sweden). Potassium sulphate, 1-butanethiol, 2-mercaptopyridine and 2-mercaptopyridine-1-oxide sodium salt were purchased from Fluka (Buchs, Switzerland), 2-aminopyridine and 4-mercaptopyridine from Aldrich (Steinheim, F.R.G., and Milwaukee, WI, U.S.A., respectively), sodium borohydride, ethylene glycol, 2-propanol, $NaH_2PO_4 \cdot H_2O$, hydrochloric acid and epichlorohydrin from Merck (Darmstadt, F.R.G.), Trisma base from Sigma (St. Louis, MO, U.S.A.), acetone and $NaHCO_3$ from May and Baker (Dagenham, U-K-), toluene from Riedel-de Haan (Seelze, F.R.G.), 2-mercaptoethanol from Carl Roth (Karlsruhe, F.R.G.) and ethanol (99.5%) from Svensk Sprit (Stockholm, Sweden). Normal human serum was obtained from the Blood Bank at the University Hospital (Uppsala, Sweden) and gradient polyacrylamide gels (PAA 4/30) for electrophoresis and PD 10 columns for desalting from Pharmacia. Antisera against human serum proteins were kindly supplied by Professor C. B. Laurell (Department of Clinical Chemistry, Malmö Hospital, Malmö, Sweden).

### *Preparation methods*

*Epoxy-activated agarose gel beads.* Thoroughly washed and suction-dried Sepharose 6B (50 g) was added to a stirred mixture of 25 ml of 4 *M* NaOH, 4.25 ml of 1-chloro-2,3-epoxypropane and 0.17 g of $NaBH_4$. After reaction for 30 min with stirring, 10 ml of 4 *M* NaOH were added to the suspension, followed by continuous addition of 20 ml of 1-chloro-2,3-epoxypropane over a period of 1 h. After a total reaction time of 15 h, the slurry was washed with deionized water on a sintered-glass filter until the filtrate was neutral.

*(I) 3-(N-2-Iminopyridyl)-2-hydroxypropylagarose (PyNH$_2$-gel).* Suction-dried epoxy-activated agarose (25 g), prewashed with 0.1 *M* sodium phosphate buffer (pH 7.0), was added with stirring to a mixture of 50 ml of 0.1 *M* sodium phosphate buffer and 10 g of aminopyridine at pH 7.0. After 24 h at room temperature with stirring, the gel was transferred to a glass filter and washed with deionized water, ethanol and again with deionized water. The gel was found to contain 1343 μmol N per gram of dried product.

*(II) 3-(4-Pyridylthio)-2-hydroxypropylagarose (Py-S-4-gel).* Suction-dried epoxy-activated agarose (25 g), prewashed with 0.1 *M* sodium phosphate buffer (pH 6.8), was added to the reaction vessel. While nitrogen was allowed to flow through the reaction vessel for 10 min, 1.5 g of $NaBH_4$ was successively added to 100 ml of 0.1 *M* sodium phosphate buffer (pH 7.5) containing 3 g of 4-mercaptopyridine. The pH of this solution was adjusted to 6.8 before addition to the gel in the reaction vessel. After a 3-h reaction time with stirring in a nitrogen atmosphere at room temperature, the gel was washed with deionized water, ethanol and again with deionized water. The

ligand concentration was found to be 759 and 724 μmol per gram of dried product, as calculated from the sulphur and nitrogen content, respectively.

*(III) 3-(2-Pyridylthio-N-oxide)-2-hydroxypropylagarose (Py-S-NO-gel).* The preparation was performed according to the same method as described for 3-(4-pyridylthio)-2-hydroxypropylagarose. The ligand concentration was found to be 768 and 750 μmol per gram of dried product, as calculated from the sulphur and nitrogen content, respectively.

*(IV) 3-(2-Pyridylthio)-2-hydroxypropylagarose (Py-S-2-gel).* The preparation was performed according to the same method as described for 3-(4-pyridylthio)-2-hydroxypropylagarose. The ligand concentration was found to be 825 and 793 μmol per gram of dried product, as calculated from the sulphur and nitrogen content, respectively.

*(V) 3-(Ethylthio)-2-hydroxypropylagarose (Et-S-gel).* Suction-dried epoxy-activated agarose (25 g), prewashed with 0.1 *M* sodium phosphate buffer (pH 8.5), was added to 40 ml of 0.1 *M* sodium phosphate buffer (pH 8.5), then 20 ml of ethanethiol were added to the reaction vessel with stirring, followed by 40 ml of ethanol. The pH of the solution was adjusted to 8.5. After a total reaction time of 24 h at room temperature with stirring, the gel was transferred to a glass filter and washed thoroughly with deionized water, ethanol and again with deionized water. The washing procedure was repeated four times in the course of 2 days with 1–2-liter portions of the respective washing solutions. The ligand concentration was found to be 797 μmol per gram of dried product, as calculated from the sulphur content.

*(VI) 3-(n-Butylthio)-2-hydroxypropylagarose (Bu-S-gel).* The preparation was performed according to the method described for 3-(ethylthio)-2-hydroxypropylagarose. The ligand concentration was found to be 771 μmol per gram of dried product, as calculated from the sulphur content.

*(VII) 3-(2-Aminoethylthio)-2-hydroxypropylagarose ($NH_2$-et-S-gel).* Suction-dried, epoxy-activated agarose (25 g), prewashed with 0.2 *M* $NaHCO_3$, was added to 75 ml of 0.2 *M* $NaHCO_3$ (pH 8.5), then 0.5 g $NaBH_4$ was added gradually to 25 ml of 0.2 *M* $NaHCO_3$ containing 1 g of aminoethanethiol. After adjusting the pH to 8.5, the aminoethanethiol solution was added to the stirred gel suspension. After a total reaction time of 15 h at room temperature, the slurry was washed with deionized water, ethanol and again with deionized water on a sintered-glass filter. The ligand concentration was found to be 832 and 922 μmol per gram of dried product, as calculated from the sulphur and nitrogen content, respectively.

*(VIII) 3-(2-Hydroxyethylthio)-2-hydroxypropylagarose (OH-et-S-gel).* Suction-dried, epoxy-activated agarose (25 g), prewashed with 0.1 *M* sodium phosphate buffer (pH 7.5), was added to 50 ml of 0.1 *M* sodium phosphate buffer (pH 7.5), then 20 ml of mercaptoethanol were added to the stirred gel suspension. After a total reaction time of 21 h at room temperature, the slurry was washed with deionized water, ethanol and again with deionized water. The ligand concentration was found to be 881 μmol per gram of dried product, as calculated from the sulphur content.

*(IX) 3-(N-Pyridyl-2-one)-2-hydroxypropylagarose (PyO-gel).* The gel was prepared according to ref. 9, except that the reaction time was 3 h. The ligand concentration was found to be 729 μmol per gram, as calculated from the nitrogen content.

*Analytical procedures*

Nitrogen and sulphur analyses were performed by Microkemi (Uppsala, Sweden). The effluent from the chromatographic column was monitored at 280 nm on an Ultrospec II Biochrom spectrophotometer (LKB, Bromma, Sweden). Electrophoretic analyses of the pooled and concentrated fractions were performed in slabs of 4–30% polyacrylamide gradient gel according to the manufacturer's manual (Pharmacia). Serum proteins were immunologically identified by the conventional Ouchterlony technique, using monospecific antibodies against $\alpha_2$-macroglobulin, orosomucoid, hemopexin, IgG, IgA, IgM, albumin, prealbumin, transferrin, $\alpha_1$-antitrypsin, ceruloplasmin, haptoglobin, chymotrypsin, C3 and C4. The concentration of human serum albumin in the pooled fractions was determined by conventional immunological methods, such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA). Chromatography was performed according to standard procedures by use of a fast procedure liquid chromatographic (FPLC) system from Pharmacia, equipped with a programmable LCC-500 control unit.

*Thiol and disulphide content in the prepared agarose derivatives*

*Disulphide determination.* To a glass test-tube, 0.1 g of freeze-dried product of the respective gel and 5 ml of 50 m*M* dithioerythritol (DTE) in 0.1 *M* Tris buffer (pH 7.5) were added. After a total reaction time of 180 min at room temperature, the gel was suction-dried on a glass filter and the absorbance of the eluate was measured at 343 or 324 nm against a blank solution, consisting of 50 m*M* DTE in 0.1 *M* Tris buffer (pH 7.5). The concentration and amount of mercaptopyridine released from the gel were calculated by use of molar absorptivity of $0.71 \cdot 10^4$ l mol$^{-1}$ for 2-mercaptopyridine[10] and $1.98 \cdot 10^4$ l mol$^{-1}$ cm$^{-1}$ for 4-mercaptopyridine[10].

*Thiol determination.* To a glass test tube, 0.1 g of the respective freeze-dried gel and 5 ml of 5 m*M* 2,2-dipyridyl disulphide in 0.1 *M* Tris buffer (pH 8.1) were added. After a total reaction time of 180 min at room temperature, the gel was suction-dried on a glass filter and the absorbance of the eluate was measured at 343 nm against a blank solution consisting of 5 m*M* 2,2-dipyridyl disulphide in 0.1 *M* Tris (buffer (pH 8.1).

*MO calculations*

The electron distribution in the investigated molecules was calculated at the Department of Quantum Chemistry, University of Uppsala, by use of an Alliant FX 80 computer at the Faculty of Sciences (University of Uppsala) and a program, SCF/MO SCF Sirius, developed by Jensen and Ågren[11,12]. *Ab initio* MO wave functions were derived for the three molecules, using standard minimal basis sets of contracted Gaussian orbitals (STO/4G). All computations were carried out using the parameters published for 2-hydroxypyridine in ref. 13, for 2-mercaptopyridine in ref. 14 and for 2-aminopyridine in ref. 15. The coordinates for the last two molecules were transformed to the orthorhombic system. The coordinates for the hydrogens in 2-mercaptopyridine were calculated by use of a bond length of 1.738 Å and the same *Z*-coordinate as the corresponding carbon.

*Chromatography*

A 10 × 1 cm I.D. column was packed with the test gel to obtain a bed volume

of 4.6–4.7 ml, while maintaining a flow-rate of 50 ml/cm$^2$ · h during the sedimentation. The column was equilibrated with 0.1 *M* Tris buffer (pH 7.5), containing 0.5 *M* $K_2SO_4$. Potassium sulphate was added to the sample to a final concentration of 0.5 *M*. A 500-$\mu$l sample (containing 68 mg protein/ml) of centrifuged human serum (a pool of fifteen different serum samples) was applied to the column. A pump (P-500; Pharmacia) maintained a flow-rate of 25 ml/cm$^2$ · h via an injection valve (V7; Pharmacia), equipped with a 500-$\mu$l sample loop. The chromatographic procedure was programmed by use of a LCC-500 control unit (Pharmacia). The experiments were performed at room temperature. Fractions of 5 ml were collected. The adsorbed material was eluted from the column in three successive desorption steps: the first with 0.1 *M* Tris (pH 7.5), the second with 40% (v/v) ethylene glycol in 0.1 *M* (pH 7.5) and the third with 30% (v/v) 2-propanol in 0.1 *M* Tris (pH 7.5).

## RESULTS

Nine different types of ligands have been studied in order to clarify the effect of one essential factor in the adsorption process, the ligand structure. In two earlier studies[9,16] we investigated 2-(N-pyridyl-2-one)-2-hydroxypropyl-, 3-(phenylthio)-2-hydroxypropyl-, 3-(phenyloxy)-2-hydroxypropyl- and 3-(2-pyridyldisulphanyl)-2-hydroxypropylagarose. The effect of the pyridine nucleus in combination with the exocyclic sulphur in gel preparation IV (see Fig. 1) was investigated by replacing the pyridine nucleus with alkylmercaptans of different length (gel preparations V and VI) and alkylmercaptans with hydroxyl or amino groups as functional groups (gel preparations VII and VIII). The role of the exocyclic sulphur in gel preparation IV was investigated by replacing sulphur with nitrogen (gel preparation I) or oxygen (gel preparation IX) (see Fig. 1). The cooperative effect between the exocyclic sulphur and nitrogen in pyridine was investigated by comparing the adsorption on gel preparations III and II with the adsorption on gel preparation IV. We shall now classify all the investigated structures according to their ability to absorb proteins, especially immunoglobulins, in the presence of 0.5 *M* $K_2SO_4$ and at a ligand concentration of about 800 $\mu$mol S per gram of dried product.

### *Ligand structure vs. protein adsorption*

Of nine ligands investigated, four can adsorb significant amounts of proteins, which can also be desorbed. All of the adsorbing ligands contain sulphur, but sulphur is effective as an adsorption promoter only when combined with either a fully aromatic ring, as in thiophenol, with a less aromatic ring, such as pyridine, or with an unsubstituted alkyl group with a chain length of at least two carbon atoms without hydroxyl or amino groups as functional groups (see Table I).

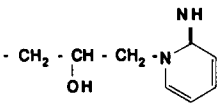
Simultaneous adsorption of immunoglobulins and a minor amount of albumin is possible only with gel preparations II and IV. When the pyridine nucleus is replaced by an alkyl chain, at least two carbon atoms long, the albumin will be adsorbed preferentially, and the amount of albumin adsorbed will increase from 0.28% of the total applied albumin to 11.5% for ethanethiol- and 18% for butanethiol-derivatized gels (see Table I and Fig. 2).

TABLE I

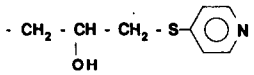
DISTRIBUTION OF PROTEINS IN THE CHROMATOGRAPHIC FRACTIONS ON GELS I–IX AFTER CHROMATOGRAPHY IN THE PRESENCE OF 0.05 *M* $K_2SO_4$–0.1 *M* TRIS (pH 7.5)

| *Fractions* | *Recovery (%) in $A_{280}$ units* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *I* | *II* | *III* | *IV* | *V* | *VI* | *VII* | *VIII* | *IX* |
| Non-adsorbed fractions | 99.4 | 70.0 | 92.2 | 53.0 | 32.8 | 78.8 | 102 | 103.0 | 95.5[a] |
| Desorbed by deleting $K_2SO_4$ | 0[b] | 16.4 | 0[c] | 35.9 | 48.4 | 12.8 | – | 0[c] | 1.4[a] |
| Desorbed with ethylene glycol | 0[c] | 1.0 | 0[c] | 1.0 | 11.8 | 0[c] | – | 0[c] | 0[c] |
| Desorbed with 2-propanol | 0[c] | 0[c] | – | – | 0[c] | 5.3 | – | 0[c] | 0[c] |
| % of the totally applied proteins still on the gel[a] | 1.1 | 3.7 | 2.4 | 3.7 | 0.4 | 1.3 | 0.4 | 0.4 | – |
| Total | 100.5 | 91.1 | 94.6 | 93.6 | 93.4 | 98.5 | 102.5 | 103.4 | 96.9 |

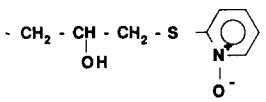
[a] Results obtained by amino acid analyses.
[b] The registered value has an absorbance $<0.5$.
[c] The registered values have absorbance $<0.1$.



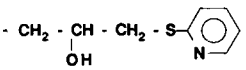
I. 3 - ( N - 2 - iminopyridyl ) - 2 - hydroxypropyl



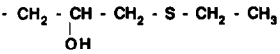
II. 3 - ( 4 - pyridylthio ) - 2 - hydroxypropyl



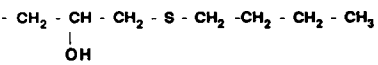
III: 3 - ( 2 - pyridylthio - N - oxide) - 2 - hydroxypropyl



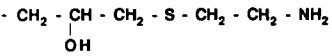
IV: 3 - ( 2 - pyridylthio ) - 2 - hydroxypropyl



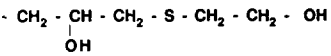
V: 3-(ethylthio ) - 2 - hydroxypropyl



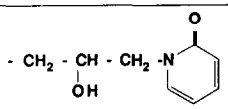
VI: 3 - ( n - butylthio ) - 2 - hydroxypropyl



VII: 3 - ( 2 - aminoetylthio ) - 2 - hydroxypropyl



VIII: 3- ( 2 - hydroxyetylthio ) - 2 - hydroxypropyl



IX: 3 - ( N - 2 - pyridyl - 2 - one ) - 2 - hydroxypropyl

Fig. 1. Structures of the ligands in the prepared agarose gels.

*Synthesis of mercaptopyridine gel derivatives*

In the synthesis of mercaptopyridine gel derivatives, several problems must be defined and solved. The mercaptopyridines tend to become oxidized, and the resulting product, 2,2′-dipyridyl disulphide or 4,4′-dipyridyl disulphide, can also react with

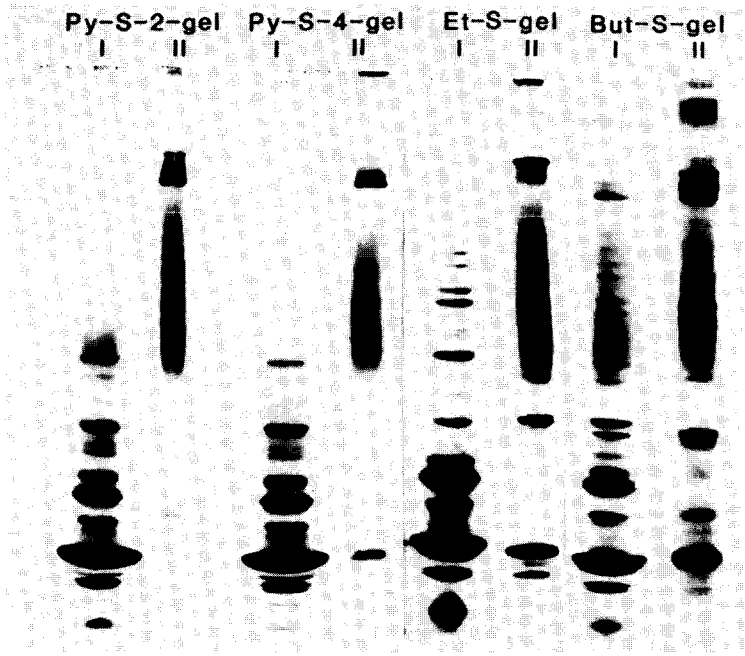



Fig. 2. Gel electropherogram of human serum that had passed the chromatographic bed of the adsorbent (I) and the material that was eluted deleting $K_2SO_4$ from the buffer (II).

oxirane to yield a ligand having a undesired structure (Fig. 3). Therefore, a thiol–disulphide exchange reaction should, if possible, be avoided, as should also the formation of a mixture of ligands, caused by simultaneous coupling to both nitrogen and sulphur. The first problem was minimized by using $NaBH_4$ and a nitrogen atmo-

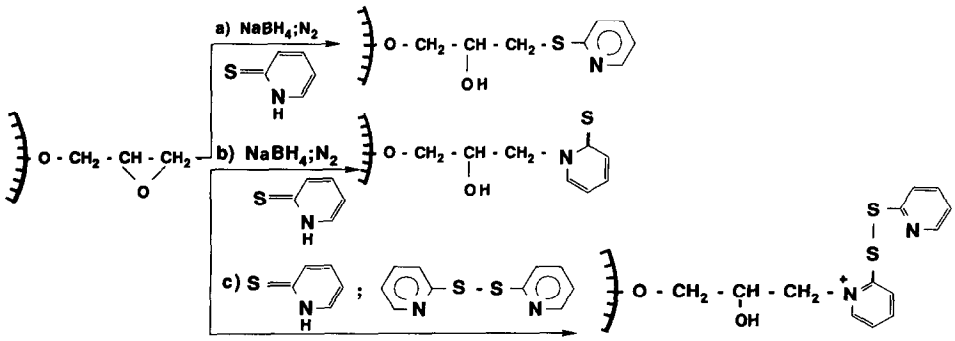



Fig. 3. Three possible pathways for the reaction of oxirane structures on the agarose with mercaptopyridine. (a) In the presence of $NaBH_4$ and in a nitrogen atmosphere the undesired disulphide structures are minimized. (b) The presence of two nueclophilic centres in the mercaptopyridine molecule can result in a coupling of the mercaptopyridine via the nitrogen atom instead of the exocyclic sulphur, as in reaction pathway (a). (c) In the absence of $NaBH_4$ and in air in the presence of 2,2-dipyridyl disulphide in the reaction mixture, a mixture of reaction products can result from either pathway (a) or (b) together with the undesired disulphide product.

sphere during the synthesis. With gel preparation II (Fig. 1), the protein remaining on the gel after desorption was reduced from 13.6 to 1.6%, and for gel preparation IV (Fig. 1) from 6.8 to 1.5%, after a final reduction of proteins on the gel with DTE, followed by amino acid analyses of these gels. The second problem has been investigated, and coupling will occur only via sulphur[17].

*Determination of the amount of thiol and disulphide groups on the agarose derivatives*

To complement the above-mentioned analyses, the thiol concentration in the prepared gels was also determined according to a modified version of the method of Grassetti and Murray[10]. The thiol content was found to be low for gel preparation IV (see Table II) but was about five times higher for gel preparation II. The possible presence of disulphides in gel preparations II and IV was investigated by reduction with DTE, followed by spectrophotometric detection of released 2-mercaptopyridine and 4-mercaptopyridine at 343 and 324 nm, respectively. Two gel preparations of 3-(2-pyridylthio)-2-hydroxypropyl- (gel preparation IV) and two of 3-(4-pyridylthio)-2-hydroxypropylagarose (gel preparation II) were compared. Preparations 2 and 4 were performed under nitrogen in the presence of $NaBH_4$ and show the lowest disulphide content. In order to minimize the disulphide content, it is therefore necessary both to include $NaBH_4$ and to exclude oxygen.

*Molecular orbital calculation of the electron distribution in the ligands calculated by an MO method*

The results (Fig. 4) obtained on calculation of the electron distribution show that the electron excess is of nearly the same magnitude in the three molecules and is concentrated on the exocyclic atom and the pyridine nitrogen. 2-Mercaptopyridine has a lower electron density in the ring (−0.40 electrons) compared with 2-hydroxypyridine (−0.03 electrons) and 2-aminopyridine (−0.05 electrons). These figures were obtained by summing the calculated electron density values contributed by the different atoms in the ring. These figures were subtracted from the sum of electron in the ring, where each carbon contributes six electrons, nitrogen seven electrons and hydrogen one electron.

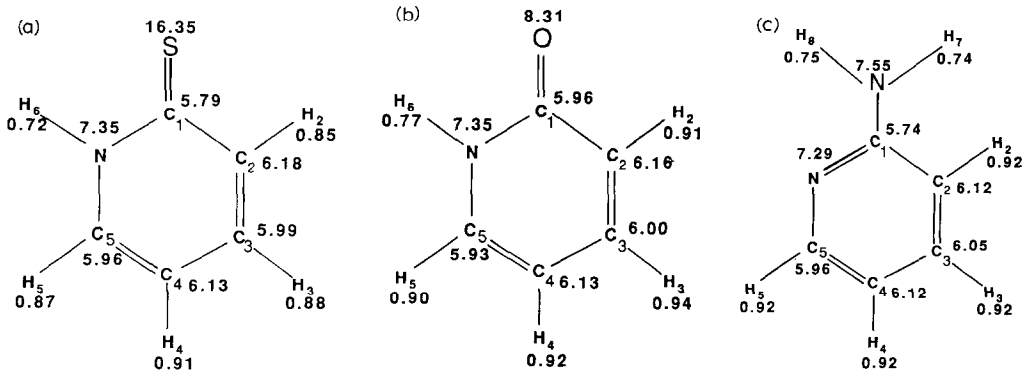
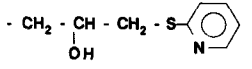
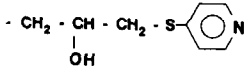



Fig. 4. Calculated electron densities (SCF/MO SCF Sirius) for (a) 2-mercaptopyridine, (b) 2-hydroxypyridine and (c) 2-aminopyridine. The value for each respective atom represents the total number of electrons.

TABLE II

CONTAMINATION OF THIOL[a]- AND DISULPHIDE[b]-CONTAINING LIGANDS IN GEL PREPARATIONS II AND IV

| *Main ligand and preparation* | *Total ligand concentration (μmol S/g dried product)* | *Preparation method* | *μmol thiol/g dried product* | *Thiol groups in total ligand (%)* | *μmol disulphide groups/g dried product* | *Disulphide groups in total ligand (%)* |
|---|---|---|---|---|---|---|
| Ligand:  | | | | | | |
| Prep. No.: | | | | | | |
| 1 | 909 | $NaBH_4$ | 1.9; 2.1[c] | 0.21; 0.23 | 4.78; 1.71[c] | 0.53; 0.19[c] |
| 2 | 653 | $NaBH_4$; $N_2$ atmosphere | 0.5 | 0.08 | 1.24 | 0.19 |
| Ligand  | | | | | | |
| Prep. No.: | | | | | | |
| 3 | 896 | $NaBH_4$ | 3.0 | 0.33 | 3.2; 3.7[c] | 0.36; 0.41[c] |
| 4 | 718 | $NaBH_4$; $N_2$ atmosphere | 3.3 | 0.45 | 0.58 | 0.08 |

[a] The number of thiol groups determined by reaction between the agarose derivatives and 2,2'-dipyridyl disulphide.
[b] The number of disulphide groups determined as released 2-mercaptopyridine and 4-mercaptopyridine when reduced with 5 m*M* DTE.
[c] Mean of two determinations.

TABLE III

PROTEINS ADSORBED ON Py-S-2-, Py-S-4-, Et-S- AND Bu-S-GELS

The serum sample was applied to the column in the presence of 0.5 *M* $K_2SO_4$–0.1 *M* Tris. After elution of the proteins not adsorbed on the gel, the column was desorbed by deleting $K_2SO_4$ from the buffer. These fractions were collected, and the amounts of IgG and HSA were determined. For Ouchterlony analysis, the pooled fractions were concentrated to an $A_{280}$ value of 1.0.

| *Gel type* | *Preparation No.* | *Amount of HSA (μg)* | *Identified components (major components in italics)* |
|---|---|---|---|
| Py-S-2- | IV | 65 | albumin, *IgG*, *$\alpha_2$-macroglobulin*, IgA, C3, C4, IgM |
| Py-S-4- | II | 42 | albumin, C3, C4, $\alpha_2$-macroglobulin, *IgG*, IgA |
| Et-S- | V | 2880 | *albumin*, C4, IgA, $\alpha_1$-antitrypsin, C4, *$\alpha_2$-macroglobulin*, *IgG*, chymotrypsin |
| Bu-S- | VI | 4550 | *$\alpha_2$-macroglobulin*, *IgG*, IgA, *albumin*, $\alpha_1$-antitrypsin, C3, C4 |

## DISCUSSION

In our first publication on the pyridine agarose gels[9], we pointed out that the ligands studied could be arranged in order of increasing hydrophobicity. The mercaptopyridine-derivatized gel was found to be weakly hydrophobic. A relationship seems to exist between the water solubility of the ligand-forming substances (their degree of hydrophilicity or hydrophobicity) and adsorption capacity of the type of protein being adsorbed. This is confirmed by the results obtained for the ligands studied here. The ligand affinity for proteins can be arranged in order of decreasing water solubility. 2-Mercaptopyridine and 4-mercaptopyridine are more hydrophilic than thiophenol. The thiophenol gel therefore adsorbs comparatively more albumin per ligand equivalent, and the electrophoretic pattern of the adsorbed proteins is different and more complex. Albumin adsorption on the thiophenol gel reflects the fact that the hydrophobicity of the thiophenol ligand is higher than that of the pyridylsulphido group.

When the pyridine nucleus in gel preparation IV is replaced by alkylthioethers, such as ethanethiol or butanethiol, the hydrophobicity of gel preparations V and VI increases, and consequently also the albumin adsorption (Table III). The introduction of functional groups, such as hydroxyl or amino, into the alkylthioethers (gel preparations VII and VIII) lowers the hydrophobicity of the ligands and consequently the capacity to adsorb proteins.

If mercaptopyridine in 3-(2-pyridylthio)-2-hydroxypropyl agarose (preparation IV) is replaced with aminopyridine (gel preparation I) or hydroxypyridine (gel preparation IX) or oxygen, the adsorption capacity is drastically reduced (see Table I).

In an attempt to investigate the factors responsible for the different adsorption capacities for the pyridine derivatives, the following parameters were investigated: (1) the sites of attachment of the pyridine nucleus to the matrix; (2) the hydrophobicity, and (3) the difference in electron distribution between the mercaptopyridine derivative and the distribution in 2-amino- and 2-hydroxypyridine derivatives.

The exact position of attachment of the pyridine derivatives (amino-, thio- and hydroxypyridines) to the epoxide groups on agarose has been determined. These results[17] show that amino- and hydroxypyridine (gel preparations I and IX, respectively) are attached only via the pyridine nitrogen, whereas mercaptopyridine is attached only via the sulphur (see Fig. 1). During the isolation of synthesized model substances of pyridine derivatives by flash chromatography, the relative hydrophobicities of the different pyridine derivatives could be compared, as the solvent used for elution of the different pyridine derivatives from the silica in flash chromatography reflects the hydrophobicity of the substances. The aminopyridine derivative could be eluted with methanol, whereas the mercapto- and hydroxypyridine derivatives could be eluted with diethyl ether. These results clearly show that there is no difference in hydrophobicity between the hydroxypyridine and mercaptopyridine derivatives.

The arylthioether gels treated here are only weakly hydrophobic in nature, as shown by their extremely low affinity for serum albumin. However, it is evident that the pyridine adsorbents possess affinity properties of a different kind (Table III). It is possible that they are electron donor to acceptor adsorbents[18]. Clearly, therefore, it is of interest to know the electron distribution over the ligands. The results of *ab initio* calculations have revealed a deficit of electrons on the pyridine-S-gels. It is highly unlikely that the water medium would convert the ligand properties to those of an electron donor. Admittedly, neglecting the influence of water in the calculation is a weakness in our attempt to formulate the basis for a theory, but that weakness is shared with application of quantum mechanics in other areas. As a parallel to our attempts to apply calculations of electron distributions in the gas phase to experimental results obtained in solution at room temperature, we can refer to results where the chemical shift value obtained from NMR studies in solution at room temperature shows a good correlation with the electron density calculated for the corresponding atom in the gas phase[19,20].

Unsubstituted pyridine nitrogen is a prerequisite for the characteristic protein affinity, as is also demonstrated by the properties of gel preparations I and IX.

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