# Affinity Chromatography using Agarose–Triazine Derivatives

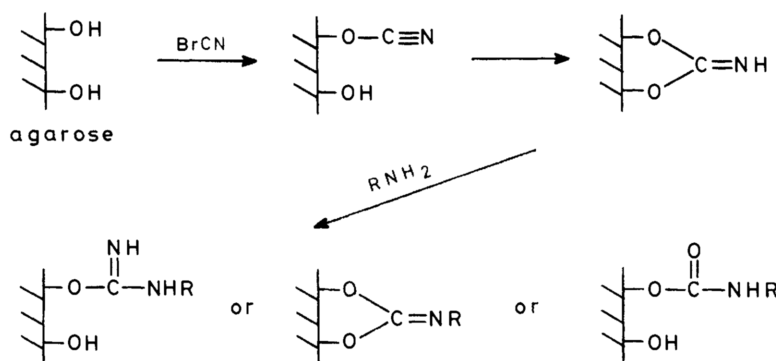
By **Thomas Lang, Colin J. Suckling,** and **Hamish C. S. Wood,*** Department of Pure and Applied Chemistry, University of Strathclyde, Cathedral Street, Glasgow G1 1XL

The preparation of derivatives of *s*-triazines and agarose and their application to the purification of chymotrypsin (E.C. 3.4.4.5), lactate dehydrogenase (E.C. 1.1.1.27), riboflavin synthase (E.C. 2.5.1.9), and dihydropteroate synthase (E.C. 2.5.1.15) is described. Methods for the preparation of both amino- and carboxy-activated agaroses are discussed. The stability of the chromatographic adsorbents prepared with *s*-triazines to hydrolysis and storage is shown by radioactivity measurements to be substantially greater than that of comparable adsorbents; *s*-triazinylagarose derivatives lose less than 1% of ligand in 2 months' storage and are fully active after 2 years' storage.

AFFINITY chromatography is now well established as a major method for the isolation and purification of biological macromolecules.[1] The specificity of this method compared with classical enzyme purification techniques makes it very attractive to chemists, because a purification procedure by affinity chromatography requires that a specific ligand for the target enzyme or macromolecule be attached to a porous, inert, chromatographic support. The support, or matrix, is commonly the polysaccharide agarose. A column composed of such a material will selectively bind the required enzyme to the ligand whilst unwanted protein is eluted. By changing the eluting conditions, the bound enzyme can be made to dissociate from the ligand and is eluted in pure form.

There are two main areas in which organic chemistry can make a contribution to affinity chromatography. First, the design of the ligand to which the biological macromolecule can bind may be studied,[2] and secondly, the chemistry of the process by which ligands are attached to the solid support requires investigation. This paper describes a new versatile method for the preparation of affinity columns using *s*-triazines as the link between support and ligand.

The most widely used method for attaching ligands to agarose employs cyanogen bromide to activate the carbohydrate.[3] It is thought that the active intermediate is an imidocarbonate which can subsequently react with amines such as bis-(3-aminopropyl)amine (Scheme 1). Despite several studies,[4] the chemistry of this reaction is not clear and consequently bioadsorbents for affinity columns prepared using cyanogen bromide are chemically ill defined. It is clear, however, that agarose does not behave in the simple way illustrated in Scheme 1 because it contains no vicinal diol. Also it has been shown that such columns have limited stability to storage due chiefly to hydrolysis of ligands bound in this way.[5] Although several supports other than agarose have been used in affinity chromatography,[6] agarose comes nearest to satisfying the conditions for an ideal support.[1] With the limitations of the cyanogen bromide technique in mind, we sought an alternative method of attaching ligands to agarose that would yield stable preparations of chemically defined structure.




SCHEME 1

We chose to adapt the chemistry of chloro-*s*-triazines, which have been used extensively in the preparation of fibre-reactive dyes[7] and which have also been widely

[1] C. R. Lowe and P. D. G. Dean, 'Affinity Chromatography,' Wiley–Interscience, New York, 1974.

[2] R. Wolfenden, *Accounts Chem. Research*, 1972, **5**, 10; B. R. Baker, 'The Design of Active Site Directed Irreversible Inhibitors,' Wiley, New York, 1967.

[3] P. Cuatrecasas, *J. Biol. Chem.*, 1970, **245**, 3059; S. C. March, I. Parikh, and P. Cuatrecasas, *Analyt. Biochem.*, 1974, **60**, 149.

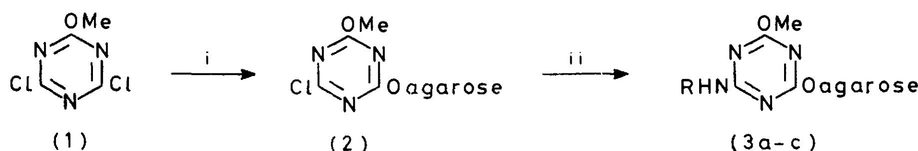
[4] R. Axen and S. Ernback, *European J. Biochem.*, 1971, **18**, 351; M. Hedayatullah, *Bull. Soc. chim. France*, 1968, **75**, 416; G. Bartling, H. Brown, L. Forrester, M. Koes, A. Mather, and R. Sasiw, *Biotechnol. Bioeng.*, 1972, **14**, 1039; L. Ahrgren, L. Kagedal, and S. Akerstrom, *Acta Chem. Scand.*, 1971, **25**, 2711; B. Stevenson, *F.E.B.S. Letters*, 1973, **29**, 167.

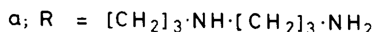
[5] G. I. Tesser, H-U. Fisch, and R. Schwyzer, (*a*) *F.E.B.S. Letters*, 1972, **23**, 56; (*b*) *Helv. Chim. Acta*, 1974, **57**, 1718.

[6] E. Steers, P. Cuatrecasas, and A. Pollard, *J. Biol. Chem.*, 1971, **246**, 196; P. Truffa-Bachi and L. Wofsy, *Proc. Nat. Acad. Sci., U.S.A.*, 1970, **66**, 685; M. K. Weibel, E. R. Doyle, A. E. Humphrey, and H. J. Bright, *Biochem. Bioeng. Symp.*, 1972, **36**, 29; W. Scouten, F. Torok, and W. Gitomer, *Biochim. Biophys. Acta*, 1973, **309**, 521.

[7] W. F. Beech, 'Fibre-Reactive Dyes,' Logos Press, London, 1970.

applied to the immobilisation of enzymes yielding very stable preparations.[8] Contrary to a report in a recent book,[1] halogenotriazines have not been used previously in the preparation of supports for affinity chromatography. Recently,[9] it has been shown that many



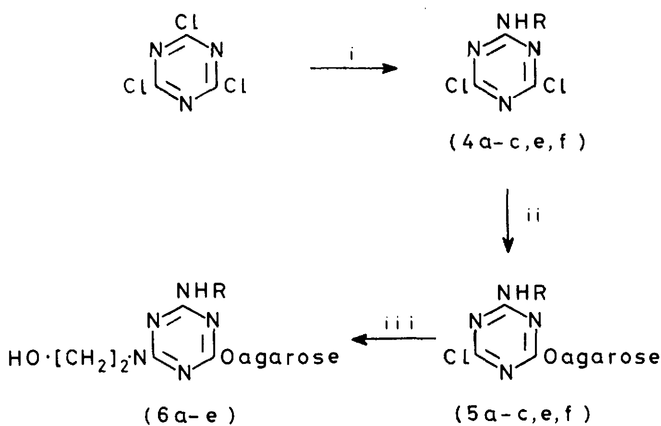
SCHEME 2 Reagents: i, $Na_2CO_3$, aq. dioxan, 25 °C, agarose; ii, $RNH_2$, pH 8.5, 25 °C

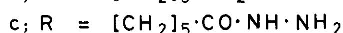
enzymes will bind to fibre-reactive dye derivatives of agarose. Although the ligand is non-specific, the purification of many enzymes, especially those involving phosphates, as substrates or coenzymes, has been achieved. Trichloro-*s*-triazine contains three labile chlorine atoms which can be replaced successively by nucleophiles. In order to prepare materials for affinity chromatography, one nucleophile must be agarose and another the ligand or a molecule, the linking arm, that joins agarose to the ligand. Accordingly our first preparations utilised dichloro(methoxy)-*s*-triazine (1); the reaction sequences are summarised in Scheme 2.

Thus an agarose (**3**) bearing either free amino or carboxylate groups can be prepared. It is not essential to replace one chlorine by an inert substituent such as methoxy before reaction with agarose; the triazine can be derivatised with a linking arm molecule prior to attachment to agarose [(4); Scheme **3**]. The remaining chlorine may be left intact during affinity chromatography or, advantageously, removed by reaction with 2-aminoethanol [see (6)]. Starting from the ethyl ester bearing triazinylagarose (5a), it is possible to prepare materials bearing acidic or basic end-groups. Mild alkaline hydrolysis at pH 10.5 yields a free carboxylate (5b) and hydrazinolysis of (5a)[10] affords an amino-group [in (5c)] to which a ligand may be coupled. Hydrazinolysis also caused partial removal of the remaining chlorine atom. Alternatively, coupling of 1,2-diaminoethane to (6b) using a carbodi-imide affords an agarose with a pendant basic group.

Routinely, the products of coupling reactions were assayed by elemental analysis for N and Cl on thoroughly washed and freeze-dried samples of the gels. Free primary amino groups were detected qualitatively by the trinitrobenzene colour test.[11] Carboxy end groups were determined quantitatively by esterification with *p*-nitrophenol, subsequent total hydrolysis of the ester, and estimation of *p*-nitrophenoxide concentration by u.v. spectroscopy. In general conversions took place in >80% yield and the concentration of coupled linking arms was about 5 μmol per ml of settled gel as shown by this method.

*Stability of Triazinylagaroses.*—The most thorough study of the stability of agarose derivatives activated





SCHEME 3 Reagents: i, $RNH_2$,HCl, $Et_3N$, $Me_2CO$, 4 °C; ii, $Na_2CO_3$, aq. dioxan, 25 °C, agarose; iii, aq. $H_2N{\cdot}[CH_2]_2{\cdot}OH$, pH 8.5, 25 °C

by the cyanogen bromide method involved an examination of the solvolytic cleavage of [$^{14}$C]alanine from a gel.[5b] It was found that solvolysis was more rapid at pH 9 than at pH 7 and that the half-life of the material was about 31 days at pH 8. In order to compare the

[8] J. F. Kennedy, *Adv. Carbohydrate Chem.*, 1974, **29**, 366.

[9] 'Blue Sepharose,' Pharmacia, Uppsala, 1976; P. Roschlau and B. Hess, *Z. physiol. Chem.*, 1972, **353**, 441; H-J. Bohme, G. Kopperschlager, J. Schulz, and E. Hofmann, *J. Chromatog.*, 1972, **69**, 209; R. L. Easterday and I. Easterday in 'Immobilised Biochemicals and Affinity Chromatography,' ed. R. B. Dunlap, Plenum, New York, 1974, p. 123; J. K. Baird, R. F. Sherwood, R. J. G. Carr, and A. Atkinson, *F.E.B.S. Letters*, 1976, **70**, 61.

[10] J. Davoll, *J. Chem. Soc.*, 1960, 5041.

[11] J. K. Inman and H. M. Dintzis, *Biochemistry*, 1969, **8**, 4074.

behaviour of triazinylagaroses with these results, we coupled [1-$^{14}$C]glycine methyl ester to trichloro-*s*-triazine, affording the aminodichlorotriazine (4f). The adduct was then coupled to agarose and radioactivity measurements showed that the yield of triazinylagarose (5f) was 95%. This product was stored at 4 °C in 0.01M-buffer solutions of pH 5.6, 7.0, and 9.6. Unlike the alanine-labelled cyanogen-bromide-treated agarose, the triazinylagarose lost less than 1% of its radioactivity in 30 days (Table) and there was no significant difference

Ligand released (%) from the triazinylagarose (5f) in buffer solutions

| Time (days) | pH of storage buffer 5.8 | 7.0 | 9.6 |
|---|---|---|---|
| 3 | 0.14 | 0.13 | 0.13 |
| 30 | 0.55 | 0.59 | 0.60 |
| 73 | 0.52 | 0.58 | 0.55 |

in release of ligand into the different buffer solutions. These results and the following examples of affinity chromatography show clearly the outstanding stability of triazinylagarose materials.

*Affinity Chromatography Experiments.*—Triazinylagarose derivatives have been successfully applied to affinity chromatography of four enzymes, α-chymotrypsin, lactate dehydrogenase (heart); riboflavin synthase, and dihydropteroate synthase.

*α-Chymotrypsin* (E.C. 3.4.4.5). D-Tryptophan methyl ester is a weak inhibitor of chymotrypsin ($K_i$ *ca.* $10^{-4}$ mol $l^{-1}$) and has been used widely as a ligand for affinity chromatography of this enzyme.[12] When this ligand was coupled to triazinylagarose (5b) by means of carbodi-imide, a bioadsorbent was obtained that retained pure α-chymotrypsin applied in 0.05M-Tris–HCl buffer. However only 70—80% of the applied protein was eluted when the buffer was changed to 0.1M-acetic acid. The residual adsorbtion of the enzyme on the column was traced to two sources. First, the third chlorine atom on the triazine was found to be reacting irreversibly with the enzyme; this effect was easily removed by substituting the chlorine with 2-aminoethanol [*cf.* (5b) ⟶ (6b)]. Secondly, it was discovered that agarose itself adsorbs proteins at pH <3; both α-chymotrypsin and bovine serum albumin were substantially adsorbed at pH 3 but were eluted when the pH was raised. Consequently optimal conditions for affinity chromatography of α-chymotrypsin using triazinylagarose derivatives requires the removal of all labile chlorine atoms from the triazine and elution with a dilute base such as 0.05M-ammonium hydroxide. Figure 1 illustrates the elution pattern obtained. After 20 weeks storage at 4 °C, a preparation based on triazinylagarose (6b) behaved identically with the freshly prepared bioadsorbent in affinity chromatography of α-chymotrypsin.

[12] P. Cuatrecasas, M. Wilchek, and C. B. Anfinsen, *Proc. Nat. Acad. Sci., U.S.A.*, 1968, **61**, 636.
[13] P. O'Carra and S. Barry, *F.E.B.S. Letters*, 1972, **21**, 281.

*Lactate dehydrogenase* (L-lactate: NAD oxidoreductase, E.C. 1.1.1.27). O'Carra and Barry[13] have investigated the affinity chromatography of lactate dehydrogenase using cyanogen-bromide-activated agarose with oxamate as inhibitor attached to the linking arm 1,6-diaminohexane. The enzyme was bound to the column only in

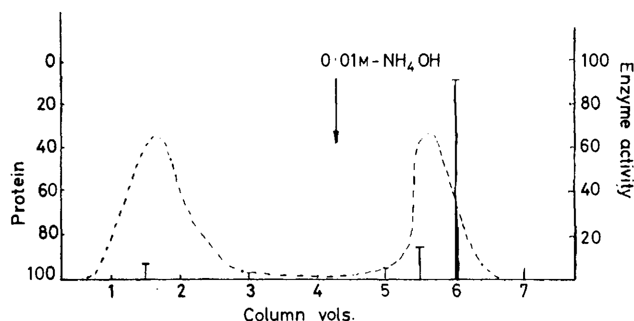


FIGURE 1 Affinity chromatography of α-chymotrypsin; (— — —) protein % transmittance at 280 nm; (⊤) enzyme activity $\Delta A$ at 400 nm min$^{-1}$ (4 ml samples)

the presence of the coenzyme NADH as would be expected from the compulsory ordered kinetics of lactate dehydrogenase and was eluted when the coenzyme was removed from the irrigant buffer. When oxamate was coupled to triazinylagarose (6c or d), in which the linking arms are hexanamides, affinity chromatography was not successful. Neither did the enzyme bind to a cyanogen-bromide-prepared column with bis-(3-aminopropyl)amine as linking arm. Only when the linking arm was 1,6-diaminohexane [(3b) or (6e)] could O'Carra's and Barry's results be reproduced, as is shown by Figure 2. This result emphasises the often neglected

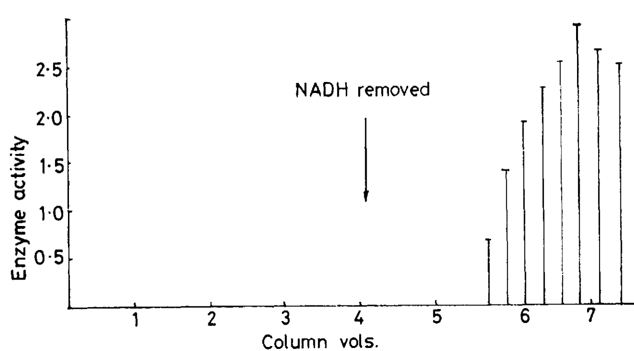


FIGURE 2 Affinity chromatography of lactate dehydrogenase; (⊤) enzyme activity $\Delta A$ at 340 nm min$^{-1}$ (1 ml samples)

importance of the nature of the linking arm in affinity chromatography. The triazinylagarose oxamate materials were still active in affinity chromatography after 22 weeks storage.

*Riboflavin synthase* (6,7-dimethyl-8-D-ribityl-lumazine: 6,7-dimethyl-8-D-ribityl-lumazine 2,3-butandiyltransferase, E.C. 2.5.1.9). It has been shown in these laboratories that 7-oxo-8-D-ribityl-lumazines are potent

inhibitors of yeast riboflavin synthase,[14] and the lumazine (7) has been attached through its carboxylate side chain to cyanogen-bromide-activated agarose. Such materials are effective in the purification of yeast riboflavin synthase by affinity chromatography. We have now coupled the lumazine (7) to triazinylagarose (3a) *via* a

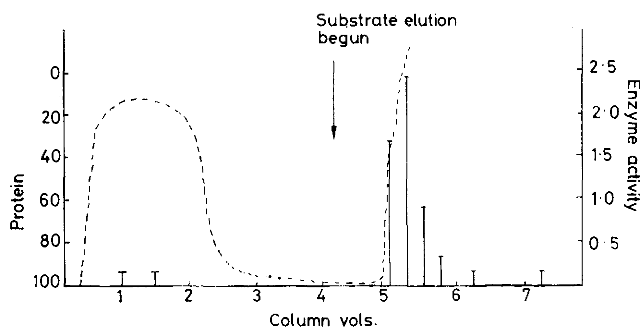
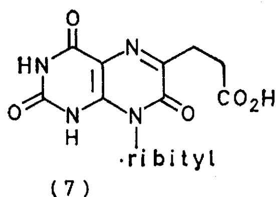
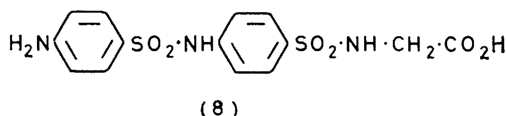



FIGURE 3 Affinity chromatography of riboflavin synthase (— — —) protein % transmittance at 280 nm; (⊤) enzyme activity $\Delta A$ at 470 nm min$^{-1}$ × 100 (2 ml samples)

mixed anhydride and have studied the affinity chromatography of this enzyme on the triazine derivative. The behaviour of the triazinylagarose did not differ from the reported properties of the cyanogen-bromide-prepared materials.[14] Crude enzyme was applied in phosphate buffer (0.2M; pH 6.9) containing $6 \times 10^{-3}$M-sodium hydrogen sulphite as reducing agent to protect the sensitive enzyme against oxidation. When all unbound protein had been eluted, the buffer was changed to 0.1M-phosphate (pH 9) containing $10^{-3}$M-cysteine hydrochloride and the enzyme's substrate, 6,7-dimethyl-8-D-ribityl-lumazine ($10^{-2}$M). The substrate competes for the enzyme with the ligand inhibitor and causes elution of active enzyme as shown in Figure 3. This bioadsorbent retained its full capacity for affinity chromatography after 2 years in water at 4 °C.




(7)



(8)

*Dihydropteroate synthase* (2-amino-7,8-dihydro-6-hydroxymethyl-4-pteridone diphosphate: *p*-aminobenzoic acid 2-amino-7,8-dihydro-4-pteridone-6-methenyl transferase, E.C. 2.5.1.15). Dihydropteroate synthase has been purified by affinity chromatography using cyanogen-bromide-derived materials by Sweeney in these laboratories.[15] He used the sulphonamide (8), which is a potent inhibitor of dihydropteroate synthase ($K_i$ *ca.* $10^{-7}$ mol l$^{-1}$) as ligand; this compound binds to the enzyme in competition with the substrate *p*-aminobenzoic acid. The sulphonamide (8) was coupled to the triazinylagarose (3a) and the conjugate was used by Sweeney in the successful purification of dihydropteroate synthase. Like lactate dehydrogenase, this enzyme has a compulsory binding order and it is necessary that the cosubstrate, 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine diphosphate (DHPP), is present in the

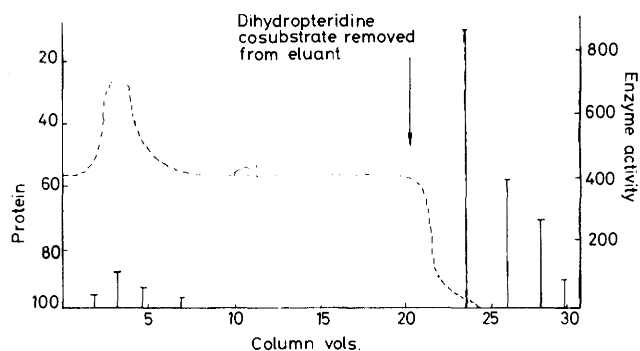



FIGURE 4 Affinity chromatography of dihydropteroate synthase; (— — —) protein % transmittance at 280 nm (includes absorption due to dihydropteridine cosubstrate); (⊤) enzyme activity (counts min$^{-1}$)

irrigating buffer for the enzyme to bind to the sulphonamide-bearing column. Removal of DHPP from the buffer causes the enzyme to be eluted (Figure 4).

## EXPERIMENTAL

*4-Chloro-6-methoxy-*s*-triazin-2-ylagarose* (2).—Dioxan-washed agarose (10 g) was added to a solution of 2,4-dichloro-6-methoxy-*s*-triazine [16] (0.45 g) in dioxan (20 ml) and the suspension was stirred for 15 min at room temperature. Water (20 ml) was added followed by 2M-sodium carbonate (2 ml). The temperature was increased to 25—30 °C and M-sodium hydroxide (4 ml) was added dropwise over 10 min. After a further 20 min stirring, the mixture was acidified to pH 6 with M-hydrochloric acid. The *triazin-2-ylagarose* (2) was filtered off and washed with dioxan (100 ml), 0.1M-sodium chloride (500 ml), and water (250 ml) (Found: %N/%Cl, 1.8; calc., 1.2).

*4-Aminoalkylamino-6-methoxy-*s*-triazin-2-ylagaroses* (3a *and* b).—4-Chloro-6-methoxy-*s*-triazin-2-ylagarose (2) (9 g) was suspended in a pH 8.5 solution of the diamine (0.01 mol) and concentrated hydrochloric acid in water (20 ml) which was then stirred for 24 h at room temperature. The triazin-2-ylagaroses (3a and b) were filtered off and washed with water (250 ml), 0.01M-hydrochloric acid–0.1M-sodium chloride (50 ml), water (50 ml), 0.01M-sodium

[14] R. J. Kulick, Ph.D. Thesis, University of Strathclyde, 1973; R. J. Kulick, H. C. S. Wood, R. Wrigglesworth, and C. D. Ginger, *J.C.S. Chem. Comm.*, 1975, 464.

[15] C. J. Suckling, J. R. Sweeney, and H. C. S. Wood, *J.C.S. Perkin I*, 1977, 439.

[16] J. R. Dudley, J. T. Thurston, F. C. Schaefer, D. Holm-Hanson, C. J. Hull, and P. Adams, *J. Amer. Chem. Soc.*, 1951, **73**, 2986.

hydroxide–0.1M-sodium chloride, 0.1M-sodium chloride (400 ml), and water (250 ml) [Found: %Cl (3a) 0.6, (3b) 0—0.6]; trinitrobenzene test positive for (3a and b).

*4-(5-Carboxypentylamino)-6-methoxy-s-triazinylagarose* (3c).—4-Chloro-6-methoxy-*s*-triazin-2-ylagarose (2) (5 g) was stirred in a pH 8.5 solution of 6-aminohexanoic acid (0.65 g) and M-sodium hydroxide in water (20 ml) for 24 h at room temperature. The triazin-2-ylagarose (3c) was filtered off and washed with water (200 ml), 0.01M-sodium hydroxide–0.1M-sodium chloride (50 ml), water (50 ml), 0.01M-hydrochloric acid–0.1M-sodium chloride (50 ml), 0.1M-sodium chloride (200 ml), and water (250 ml) (Found by *p*-nitrophenoxide analysis: 3.1 μmol carboxylate per ml settled gel).

*2,4-Dichloro-6-(5-ethoxycarbonylpentylamino)-s-triazine* (4a).—Ethyl 6-aminohexanoate hydrochloride (1.95 g) was added to a solution of 2,4,6-trichloro-*s*-triazine (1.84 g) in acetone (250 ml) at 4 °C. Triethylamine (2.0 g) in acetone (5 ml) was added to the stirred solution and the reaction was allowed to proceed at 0—4 °C for 16 h. The white precipitate was filtered off and the filtrate concentrated to 10 ml *in vacuo* below 40 °C. Addition of 50% aqueous acetone (100 ml), followed by evaporation of the acetone *in vacuo* below 40 °C, afforded the *ester* (1.97 g, 64%) as a white crystalline solid, m.p. 88—89° (Found: C, 43.2; H, 5.3; Cl, 22.9; N, 18.1. $C_{11}H_{16}Cl_2N_2O_4$ requires C, 43.0; H, 5.2; Cl, 23.1; N, 18.2%).

*4-Chloro-6-(5-ethoxycarbonylpentylamino)-s-triazin-2-ylagarose* (5a).—Dioxan-washed agarose (10 g) was stirred in a solution of 2,4-dichloro-6-(5-ethoxycarbonylpentylamino)-*s*-triazine (4a) (0.737 g) in dioxan (20 ml) for 15 min at room temperature. Water (20 ml) and 2M-sodium carbonate (2 ml) were added followed dropwise by M-sodium hydroxide (4 ml) over 10 min. The reaction was allowed to continue for a further 20 min and the solution was then brought to pH 6 with M-hydrochloric acid. The *triazin-2-ylagarose* (5a) was filtered off and washed with dioxan (100 ml), 0.1M-sodium chloride (500 ml), and water (250 ml) (Found: %N/%Cl, 1.58; calc., 1.58).

*4-Chloro-6-(5-carboxypentylamino)-s-triazin-2-ylagarose* (5b).—4-Chloro-6-(5-ethoxycarbonylpentylamino)-*s*-triazin-2-ylagarose (5a) (10 g) was stirred at 40 °C for 20 h in aqueous sodium hydrogen carbonate (0.025M) and sodium hydroxide (0.1M) (25 ml). The *triazinylararose* (5b) was filtered off and washed with water (250 ml), 0.01M-sodium hydroxide–0.1M-sodium chloride (50 ml), water (50 ml), 0.01M-hydrochloric acid–0.1M-sodium chloride (50 ml), 0.1M-sodium chloride (400 ml), and water (250 ml) (Found: %N/%Cl, 1.88; calc., 1.88; *p*-nitrophenoxide analysis: 4.7 μmol carboxylate per ml settled gel).

*Reactions of 6-(5-Ethoxycarbonylpentylamino)-s-triazin-2-ylagaroses* (5a) *and* (6a) *with Hydrazine Hydrate*.—The 6-(5-ethoxycarbonylpentylamino)-*s*-triazin-2-ylagarose (5a) or (6a) (10 g) was washed with dry methanol and then stirred in a solution of hydrazine hydrate (1.0 g) in methanol (20 ml) for 7 h at room temperature. The hydrazide (5c) or (6c) was filtered off and washed with methanol (200 ml), and water (250 ml) (Found: residual Cl, 0—0.7%).

*4-(5-Ethoxycarbonylpentylamino)-6-(2-hydroxyethylamino)-s-triazin-2-ylagarose* (6a).—4-Chloro-6-(5-ethoxycarbonylpentylamino)-*s*-triazin-2-ylagarose (5a) (10 g) was stirred in a pH 8.5 solution of 2-aminoethanol (0.06 g) in water (40 ml) for 20 h at room temperature. 2-Aminoethanol (0.06 g) was added and the reaction continued for 4 h; then more 2-aminoethanol (0.48 g) was added and the reaction continued for a further 20 h. The *triazinylagarose* (6a) was filtered off and washed with water (200 ml), 0.1M-sodium chloride (500 ml), and water (200 ml) (Found: residual Cl, 0.75%).

*4-(5-Carboxypentylamino)-6-(2-hydroxyethylamino)-s-triazin-2-ylagarose* (6b).—4-(5-Carboxypentylamino)-6-chloro-*s*-triazin-2-ylagarose (5b) (10 g) was stirred in a pH 8.5 solution of 2-aminoethanol (1.0 g) in water (40 ml) for 20 h at room temperature. The *triazinylagarose* (6b) was filtered off and washed with water (200 ml), 0.1M-sodium chloride (500 ml), and water (200 ml) (Found: residual Cl, 0.75%).

*Reaction of 4-(5-Carboxypentylamino)-6-(2-hydroxyethylamino)-s-triazin-2-ylagarose* (6b) *with 1,2-Diaminoethane*.—4-(5-Carboxypentylamino)-6-(2-hydroxyethylamino)-*s*-triazin-2-ylagarose (6b) (5 g) was stirred in a solution of 1,2-diaminoethane (0.06 g) in water (6 ml) adjusted to pH 4 with dilute hydrochloric acid. A solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride (0.2 g) in water (2 ml) was added and the mixture adjusted to pH 4.7 with 0.1M-sodium hydroxide. The reaction was allowed to continue for 20 h at room temperature. The *triazinylagarose* (6d) was filtered off and washed with water (200 ml), 0.1M-sodium chloride (500 ml), and water (200 ml) (Found: residual Cl, 0%).

*2,4-Dichloro-6-(methoxy[$^{14}$C]carbonylmethylamino)-s-triazine* (4f) (carried out by A. Shirra).—Glycine methyl ester hydrochloride (specific activity $2.28 \times 10^{10}$ counts $s^{-1}$ $mol^{-1}$) was coupled to trichlorotriazine by the method for the preparation of (4a). The product, recrystallised to constant activity from aqueous acetone, had specific activity $2.33 \times 10^{10}$ counts $s^{-1}$ $mol^{-1}$.

*4-Chloro-6-(methoxy[$^{14}$C]carbonylmethylamino)-s-triazin-2-ylagarose* (carried out by A. Shirra).—This material was prepared by the method for the preparation of (5a). A total of $4.16 \times 10^6$ counts $s^{-1}$ of starting material (4f) (42 mg) was used and the washings of the product contained a total of $0.17 \times 10^6$ counts $s^{-1}$. This is equivalent to a coupling yield of 96%.

The product was divided into three and a portion stored in each of three 0.01M-phosphate buffers (pH 5.8, 7.0, and 9.6) at 4 °C. Samples were removed at intervals and the radioactivity released was determined by scintillation counting using Bray's solution.[17]

*Coupling of D-Tryptophan Methyl Ester to the Triazinylagaroses* (5a) *and* (6a).—The triazin-2-ylagarose (5a) or (6a) (5 g) was washed with dioxan (6 × 10 ml) and suspended in 10 ml of this solvent. *N*-Hydroxysuccinimide (0.172 g) and *NN'*-dicyclohexylcarbodi-imide (0.31 g) were added to the stirred suspension to achieve a final concentration of 0.1M in each. The mixture was stirred gently for 70 min at room temperature and the *N*-hydroxysuccinimide ester was collected by filtration, washed with dioxan (6 × 10 ml) over a 10 min period, methanol (3 × 10 ml), and dioxan (3 × 10 ml), and then dried for 1 min under suction. This slightly moist ' activated ' agarose derivative was suspended in a solution of D-tryptophan methyl ester (150 mg) in 0.1M-sodium phosphate (pH 6.5; 20 ml) at 0 °C and the mixture was stirred gently for 6 h at 4°. The bioadsorbent was filtered off, washed with water (200 ml), 0.1M-sodium hydroxide (40 ml), water (50 ml), 0.1M-Tris buffer (pH 8)–1M-sodium chloride (20 ml), 0.1M-acetic acid (pH 3)–M-sodium chloride, 0.1M-Tris buffer

[17] R. Bray, *Analyt. Biochem.*, 1960, **26**, 279.

(pH 8)–M-sodium chloride, and finally equilibrated with 0.05M-Tris buffer (pH 8)–0.02M-calcium chloride.

*Coupling of Oxalic Acid to the Triazinylagarose* (6e).—A solution of potassium oxalate (1.22 g) and 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride (0.74 g) in water (5 ml) was stirred for 5 min at room temperature. The aminoalkyl-amino-hydroxyethylamino-triazinyl-agarose (6e) (5 g) was added and the suspension was stirred for 20 h at room temperature. The bioadsorbent was filtered off, washed with water (100 ml), 0.1M-sodium chloride (500 ml), and water (250 ml), and finally equilibrated with 0.02M-sodium phosphate (pH 6.8)–0.5M-sodium chloride–NADH (100μM).

*Coupling of* 6-*Carboxyethyl*-7-*oxo*-8-D-*ribityl-lumazine* (7) *to the Triazinylagarose* (3a).—Ethyl chlorocarbonate (0.14 g) was added to a stirred solution of 6-carboxyethyl-7-oxo-8-D-ribityl-lumazine (7) (0.5 g) and triethylamine (0.13 g) in dimethylformamide (20 ml) at 0 °C. After 30 min water (20 ml) was added followed by the 4-aminoalkylamino-6-methoxy-*s*-triazin-2-ylagarose derivative (3a) (5 g), and the suspension was allowed to warm to room temperature. The mixture was stirred at room temperature for 20 h and the bioadsorbent was filtered off, washed with water (100 ml), M-potassium dihydrogen phosphate (100 ml), water (100 ml), M-dipotassium hydrogen phosphate (100 ml), and water (250 ml), and finally equilibrated with 0.1M-sodium phosphate (pH 6.9).

*Affinity Chromatography*.—α-Chymotrypsin was examined by the method of Cuatrecasas [12] but eluting with 0.05M-$NH_4OH$. The enzyme was assayed using *p*-nitrophenyl acetate by spectrophotometric measurements at 400 nm.[18] Lactate dehydrogenase (heart muscle; Böhringer) was purified following O'Carra and Barry [13] and was assayed in pH 7.4 buffer at 25 °C by following the NADH absorption at 340 nm.[19] Riboflavin synthase was purified from bakers' yeast [20] and was assayed by observing riboflavin production at 470 nm.[14] Dihydropteroate synthase was purified by Sweeney [15] and assayed by means of [$^{14}$C]-*p*-aminobenzoic acid.

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