PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Large-Scale Purification of Enzymes [and Discussion]

C. J. Bruton, A. R. Thomson and C. R. Lowe

Phil. Trans. R. Soc. Lond. B 1983 **300**, 249-261
doi: 10.1098/rstb.1983.0003

Phil. Trans. R. Soc. Lond. B **300**, 249–261 (1983)
Printed in Great Britain

Large-scale purification of enzymes

By C. J. Bruton

Department of Biochemistry, Imperial College of Science and Technology, London, SW7 2AZ, U.K.

Many standard procedures for the purification of proteins in the laboratory do not readily lend themselves to scaling up, whereas, on the other hand, some techniques relatively unsatisfactory in the laboratory are much more effective on a large scale. When producing gram or kilogram quantities of enzymes for use over an extended period, the storage properties and general tractability of the purified products become increasingly important. Hence enzymes from thermophilic sources frequently have advantages over those from mesophiles. The possible economic advantages of simultaneous large-scale multi-enzyme isolation over separate individual enzyme purifications are evaluated.

Batchwise adsorption and elution from ion-exchange celluloses frequently replace traditional precipitation techniques in the early stages of a large-scale purification. Dialysis is replaced by concentration, dilution and reconcentration with the use of hollow-fibre ultrafiltration equipment. Antiphonally direct scaling-up of column chromatographic procedures is usually possible. Modifications to column geometry to maximize flow rates are often desirable but purification factors and recoveries comparable with those obtained on the laboratory scale can be achieved relatively easily.

Classical affinity chromatographic techniques have not proved so amenable to large-scale work, mainly because of the enormous expense and rather short life of the matrices. However, the quasi-affinity chromatography afforded by the triazine dye conjugates has proved of great benefit. The materials are cheap to prepare. The coupling procedures are both simple and rapid and do not involve the use of noxious chemicals such as cyanogen bromide. Moreover the triazine linkage is more stable under a variety of conditions than the isourea formed in cyanogen bromide coupling. Considerable further exploitation of these versatile matrices is expected.

Introduction

In this paper I shall be discussing mainly enzyme purification on the gram scale: that is large by the standards of most laboratory biochemists and molecular biologists, but not perhaps by the standards of the industrialists or commercial producers. However, it is generally in the transition from the laboratory (milligram) scale to the pilot plant (gram) scale that the major changes in approach are necessary and I shall therefore be concentrating on those techniques that can conveniently be scaled up still further to the industrial operation.

Source of enzymes

When large quantities of purified enzyme are required, the choice of starting material is of paramount importance. In broad terms mammalian tissues (bovine liver) or fluids (bovine plasma, intestinal juice, etc.) are cheapest but their enzyme content is relatively static and, more critically, outside the control of the molecular biologists. Nevertheless, if the desired

product is present in large quantities, particularly in the extracellular fluids, mammalian sources are frequently used.

Even before the advent of recombinant DNA technology, a significant proportion of large-scale enzyme production concerned enzymes of microbial origin because of the infinitely greater capacity for increasing the levels of enzyme protein synthesized by classical genetic techniques (Dixon & Postgate 1972), by continuous culture selection (Rigby *et al.* 1974), by induction (Jacob & Monod 1961)or merely by optimizing the growth conditions for the enzymes of interest. Naturally the high levels of expression of single highly prized products that are now possible with their genes cloned adjacent to good promoters will continue to swing the balance in the microbial direction.

Most of the industrially important microbial enzymes are extracellular macromolecular-degrading enzymes secreted into the culture medium by the growing organism. They are readily isolated from the culture supernatant and are usually very stable. They have been purified on a vast scale for many years (Davies 1963). An excellent recent review covers most bacterial extracellular enzymes (Priest 1977). Perhaps the perfect starting material for an enzyme of interest would be a microorganism growing in continuous culture at high dilution rate on a simple medium secreting the single protein into the effluent under conditions where it was stable. The coupling of such a source to a continuous production line would not be difficult. This ideal is rarely, if ever achieved. Regrettably the technology of gene cloning and gene expression in organisms with good secretion systems lags behind that in *Escherichia coli*, and although rapid progress in this area can be confidently expected, most of the new enzymes of interest are intracellular.

The choice of organism and the overall purification economy are critical fundamental decisions that are not always adequately considered. Enzymes from thermophiles are considerably more stable than those from mesophiles, and not merely in terms of their thermal stability. Thus, aminopeptidase I from *Bacillus stearothermophilus* is stable in 8 M urea and also in sodium dodecyl sulphage (3 g l^{-1}) (Roncari & Zuber 1969), and concentrations of aliphatic alcohols that would inactivate many mesophilic enzymes do not have an adverse effect on the activities of many enzymes from the same organism (Singleton & Amelunxen 1973). The pure enzymes therefore have better storage properties and much greater flexibility in handling than their mesophilic counterparts. An excellent example of this is glycerokinase, which is used extensively in hospital laboratories in glycerol determination. Glycerol levels before and after alkaline hydrolysis give a measure of triglyceride content, and values outside the normal range may have important clinical significance in the diagnosis of certain medical conditions, notably arteriosclerosis. Until recently glycerokinase was only available commercially from *Candida mycoderma* and *E. coli*. However, it has now been established that the enzyme can be purified easily in gram quantities from certain strains of *B. stearothermophilus* grown on glycerol and that it is two orders of magnitude more stable than the mesophilic enzyme (Comer *et al.* 1979).

Simultaneous multi-enzyme isolation

Enzyme purification is a labour-intensive, time-consuming and, therefore expensive process. When one organism produces several enzymes that are required in homogeneous form, a single multi-enzyme isolation can have significant advantages over separate purifications optimized for each individual enzyme. So far this has only been practised for enzymes needed for fundamental research. Up to 22 purified enzymes have been obtained from a single extraction

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

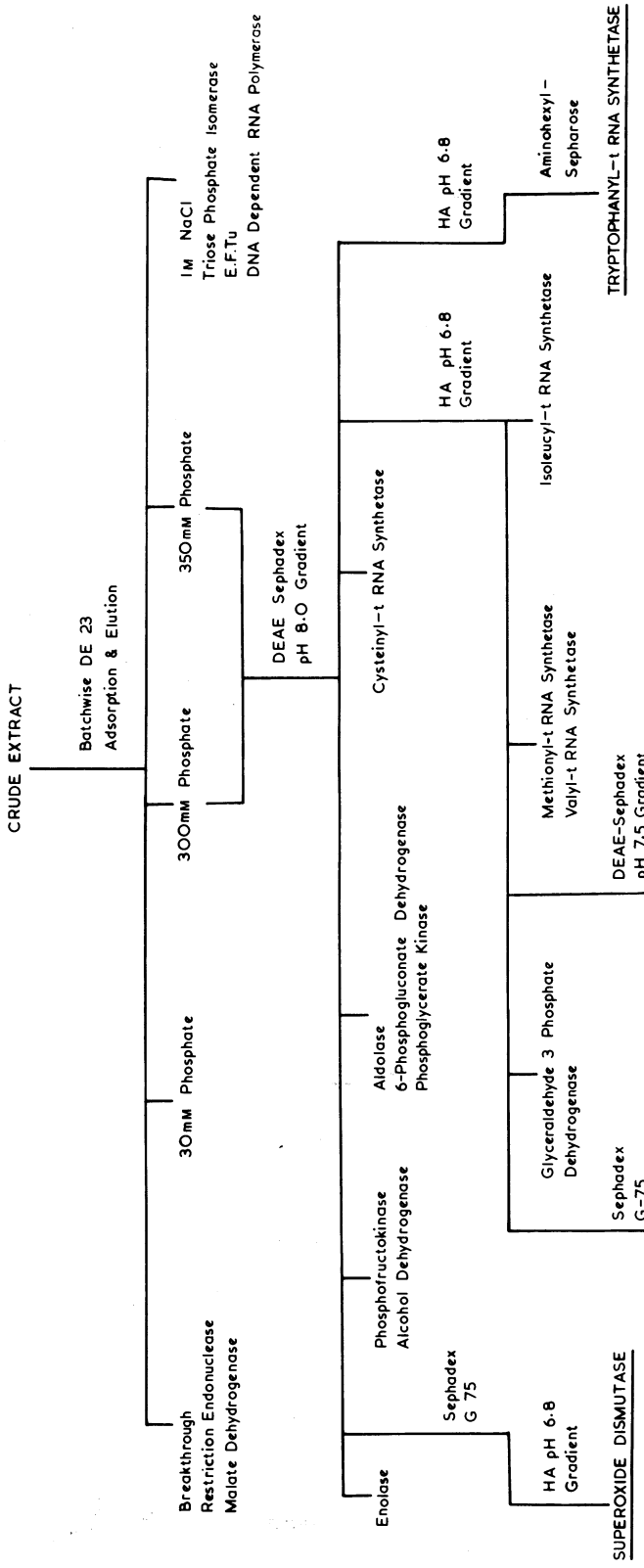

FIGURE 1. Flow diagram for a multi-enzyme preparation from *Bacillus stearothermophilus*. The four enzymes routinely obtained in homogeneous form are indicated in capitals and underlined. All the other enzymes have been purified to homogeneity on more than one occasion. Details of the two large columns are given in figure 2 (taken from Atkinson *et al.* 1979).

of 70 kg of *B. stearothermophilus* (see figure 1) (Atkinson *et al.* 1979). The overall economy of this operation is far greater than that of 22 separate purifications, even if all the individual genes had been cloned and gave high levels of expression. The process can be modified as necessary to accommodate extra unusual enzymes. Thus when the restriction endonuclease *Bst*I was required, deoxyribonuclease was omitted from the extraction buffer and an additional adsorption and elution from phosphocellulose introduced (Clarke & Hartley 1979). This enzyme is present at only 0.004% of the soluble protein in the crude extract, yet by working on this scale 38 mg of highly purified and very stable enzyme was prepared, which has kept the laboratory supplied with this useful tool for many years.

A separate preparation on the scale necessary could not have been justified but as a side product of the multi-enzyme isolation the restriction endonucleoase was obtained at relatively low cost. This philosophy, so common in the chemical industry, of maximal utilization of all by-products, must be extended to the biotechnology industry without delay.

Culture conditions and time of harvest

Besides the obvious necessity of ensuring the presence of inducers for enzymes under negative control if constitutive strains are not available, further optimizing of the culture conditions so that the yields of enzyme per unit culture volume is maximized is extremely desirable. This is not necessarily the same as maximizing the biomass of the culture. Indeed, if two different sets of conditions give the same overall yield of enzyme activity, the one with the lower cell mass yield is preferred as the degree of purification required for homogeneous enzyme will be lower. For example, *E. coli* EM 20031 (McFall 1967) grown on a simple medium produces about 5 kg wet mass of cell paste in a 400 l culture compared with about 20 kg on a fully enriched nutrient broth medium (Bruton *et al.* 1975). However, the total methionyl-tRNA synthetase activity in the two fermenters at harvest is the same. The simple medium is used for methionyl-tRNA synthetase production as it is cheaper and facilitates the purification work. Preliminary investigation on a small scale to establish these important parameters is rarely unrewarded.

Similar detailed study of the levels of the activities of all the desired enzymes and the undesirable protease activity throughout trial cultures is equally important. Even the now standard addition of protease inhibitors – diisopropylfluorosphosphate (Jansen *et al.* 1949) or phenylmethylsulphonyl fluoride (Fahrney & Gold 1963) for serine proteases, pepstatin (Umezawa *et al.* 1970) for acidic proteases, etc. – is no substitute for minimizing the levels of these degradative enzymes in the initial crude extract. With most organisms protease production, either intracellular or extracellular, starts in late logarithmic growth or early stationary phase and continues to increase. The earliest harvest point that gives adequate yields is therefore to be recommended.

Extraction procedures

Preparation of crude extracts

As indicated earlier, most new enzymes for which there is a large demand are unfortunately not extracellular. A method of breaking the cells (eukaryotic or prokaryotic) to release the enzymes is required. This rupture need not be total. Indeed there is positive advantage in making the conditions as mild as possible commensurate with satisfactory release of the enzymes to be purified. More severe treatment only complicates the subsequent purification steps by

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

adding structural proteins, membrane proteins and other undesired contaminants to the first extract, which then have to be removed.

Mammalian tissues are the simplest to disrupt and the standard laboratory-scale techniques are readily scaled up. This is not true of microbial cells. Techniques such as sonication, lysozyme treatment with or without freezing and thawing, and solid shear methods are of limited application either because of inadequate equipment for large-scale use or on the grounds of cost. The methods of choice on the large scale involve liquid shear, e.g. the Manton Gaulin homogenizer, essentially a continuous French press, which willl handle up to 100 kg per hour; solvent desiccation, mainly with acetone or toluene; or spontaneous lysis initiated in the culture by the addition of antibiotics or detergents. None of these is perfect nor applicable to all systems but most organisms can be adequately disrupted by one or more of them.

Most bacterial milieu is acidic so it is helpful if the extraction medium is well buffered slightly above the pH required for the first purification step.

Cell debris and any broken cells must be removed as efficiently as possible. With the massive volumes encountered, continuous-flow centrifuges must be used, such as the Westphalia disc bowl, but even so the crude extracts produced are not of the same clarity as achieved on the laboratory scale with high-speed batch machines such as the Beckman J2-21.

Preliminary treatments

It is at this stage, when hundreds or thousands of litres containing kilogram quantities of dissolved proteins are being treated, that large-scale work differs radically from that on the laboratory scale. In a classical purification, the first steps would be to remove the subcellular organelles–mitochondria, if present, and microsomes – by differential high-speed centrifugation and then to use an appropriate combination of precipitation techniques requiring further extensive medium-speed centrifugation – e.g. ammonium sulphate, pH and organic solvents – to achieve rapid concentration and primary fractionation. Any undesirable high salt concentrations generated would be removed swiftly by dialysis.

On the larger scale these operations are much more difficult. Continuous-flow centrifuges are barely competent for removing large concentrations of heavy cell debris from ruptured cells. For removing much lower concentrations of precipitated protein of only marginally higher density than the remaining solution, they are totally unsuitable. A further important technical consideration is that many of the precipitants used, notably ammonium sulphate at high concentration or phosphoric and sulphuric acids, are very corrosive towards even the high grade stainless steel used in the manufacture of these instruments.

Consequently procedures have been developed eliminating precipitation as far as possible. As the crude extracts are of relatively low quality and rather dilute, these procedures should, if possible, combine clarification and concentration with purification. Batchwise adsorption and elution from robust cellulose-based ion exchangers is an ideal first step. When this approach was first evaluated, very crude apparatus was used for separating the cellulose from the equilibrating buffer: home-made towelling bags in domestic spin dryers. There was, naturally, considerable frothing of all the protein solutions and some inevitable loss of material in the instruments, but nevertheless even these early trials regularly gave 80% (or more) recovery of enzymic activity. With the manufacture of large-scale basket centrifuges (e.g. Carl-Padberg), which can tackle hundreds of kilograms of solid matter (in this case the ion exchanger), the technique has been greatly refined. In a typical purification about 1 kg wet mass of diethyl-

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

aminoethyl-cellulose (Whatman DE-23) equilibrated at low ionic strength is added for each kilogram of bacterial cells disrupted. After a brief adsorption period the cellulose is collected in the basket centrifuge. It can then be treated like a bed of resin in a large filter funnel and washed sequentially *in situ* with solutions of increasing ionic strength. Quite large increments are preferred. These extracts sometimes contain cellulose 'fines' but these, unlike precipitated protein, are of high density and are readily removed by a single pass through a Sharples centrifuge. The extracts obtained are perfectly clear and ready for further purification treatments. Purification factors up to tenfold may be achieved in a single day. Note that the ribosomes have been completely ignored. They probably remain bound to the cellulose.

The process is not ideal for those enzymes that do not bind to the diethylaminoethyl-cellulose in the primary adsorption step. This breakthrough fraction is definitely turbid; but, as the proteins present must be basic, they will bind to a cation exchanger. Hence a similar batchwise adsorption and elution from either carboxymethyl-cellulose or phosphocellulose can be used to obtain a good extract with further purification from this material. The ion-exchange celluloses used in these early stages can be recycled and regenerated *in situ* in the basket centrifuge. Batches have been reused ten or more times with totally reproducible and satisfactory results.

Dialysis of these large volumes is both difficult and tedious and, if used extensively, would also be extremely expensive in buffer components such as phenylmethylsulphonyl fluoride or dithiothreitol, often required for enzyme stability. Fortunately concentration, dilution, re-concentration and redilution by using hollow-fibre ultrafiltration equipment provides a more than adequate alternative technique. Flow rates of up to 50 l h^{-1} can be achieved with the Amicon DC-50 for work on the pilot plant scale. Thus the ionic strength of 500 l of protein solution can be reduced to 1% of its starting value in 18 h if necessary. This is more than comparable with traditional dialysis, although such drastic reductions are seldom necessary. Twenty-fold is often sufficient.

Column chromatography

Perhaps surprisingly to the traditionally timid laboratory biochemist, column chromatography on the larger scale is remarkably straightforward. Steric exclusion, ion exchange, adsorption and hydrophobic chromatography are all routinely used at all scales of pilot plant and industrial operation. In the intermediate range with which I am mainly concerned, basic practical problems such as fraction collection have not yet been fully automated but pilot plants do have 24 h shift operators who in my experience are more reliable than even the very best equipment!

Two typical fractionations of *B. stearothermophilus* enzymes, one on an 80 l diethylaminoethyl-Sephadex column and the other on a 17 l hydroxyapatite column, are shown in figure 2. The separations achieved are comparable with those on 80 ml or 17 ml columns. From the elution profiles no one would remark on poor separation. Pools are of the order of one-tenth of the total elution volume and the recoveries of the enzymic activities are good.

The maintenance of high flow rates to minimize the time taken is clearly important. Improvements in the chemical design of the resins themselves have been considerable. For example diethylaminoethyl-trisacryl (LKB) gives at least fivefold better flow rates and frequently also greater protein binding capacity than the ion-exchange celluloses and Sephadexes. It is also more sturdy for recycling and sterilizing if necessary. Hydroxyapatite is a notoriously fickle chromatographic medium but very powerful for many separations. Fresh material should

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

always be used for large-scale work and we make our own by the method of Atkinson *et al.* (1973). It can be regenerated for future laboratory use but the loss of flow rate makes the material unsuitable for the large-scale work. The geometry of the hydroxyapatite column may also seem odd to the laboratory worker: the length:diameter ratio is 1:2 compared to the more standard 10:1. This is a common feature of large-scale column chromatography with gradient elution and it does not result in any loss of resolution if the packing is done with care.

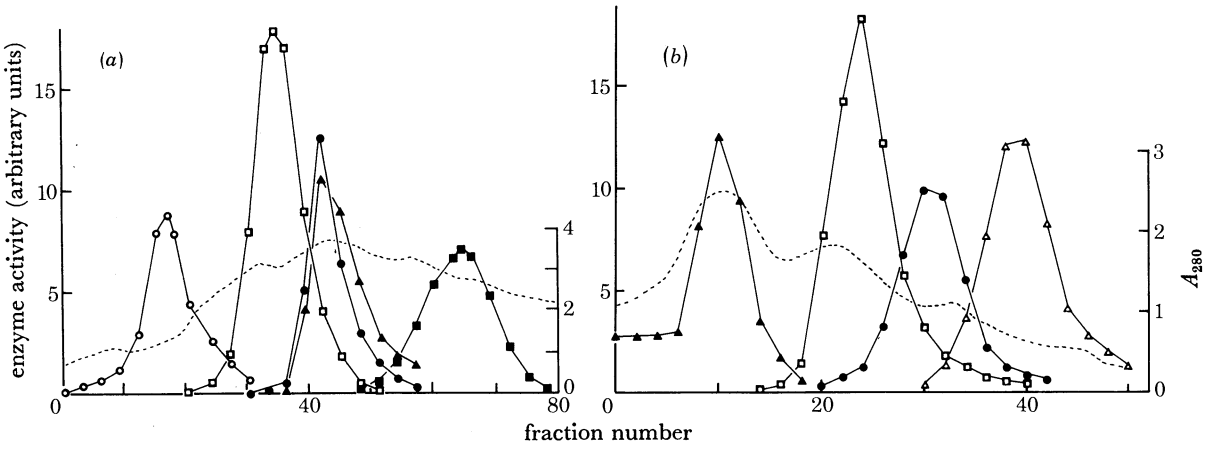

FIGURE 2. (*a*) Chromatography of a batchwise DEAE-cellulose eluate on DEAE-Sephadex: 35 l of DE-23 eluate (pH 8.0, conductivity 9 mS) was loaded on to a column (80 cm × 37 cm) of DEAE-Sephadex A50 equilibrated with 100 mM potassium phosphate, pH 8.0. The column was washed with 100 l of the same buffer and eluted with a linear gradient from 100 to 600 mM potassium phosphate, pH 8.0, followed by a wash with the finishing buffer. The total volume was 500 l and the flow rate 5 l h^{-1}. ○, Superoxide dismutase; □, aldolase; ●, tyrosyl-tRNA synthetase; ▲, rhodanese; ■, tryptophanyl-tRNA synthetase.

(*b*) Chromatography of DEAE-Sephadex eluate on hydroxyapatite: 12 l settled volume of hydroxyapatite was stirred gently with 20 l of protein solution (conductivity less than 2 mS) for 1 h. The hydroxyapatite was allowed to settle for 3 h and the supernatant removed by decantation. The hydroxyapatite was slurried in 20 l of 20 mM potassium phosphate, pH 6.8, and poured on to a prepacked bed of 4 l of fresh hydroxyapatite in a 16 cm × 37 cm column. The final 16 column was washed with 10 l of 20 mM potassium phosphate, pH 6.8, and eluted with a linear gradient from 20 to 200 mM potassium phosphate, pH 6.8. The total volume was 100 and the flow rate 2 l h^{-1}. ▲, Rhodanese; □, glyceraldehyde 3-phosphate dehydrogenase; ●, tyrosyl-tRNA synthetase; △, valyl-tRNA synthetase.

AFFINITY CHROMATOGRAPHY

Perhaps the greatest advance in laboratory protein and enzyme purification in the past decade has been the extensive use of affinity chromatography (Cuatrecasas & Anfinsen 1971; Uy & Wold 1977), and it might be expected that the technique would have wide application in large-scale and industrial work, but, in general, this is not the case. Many factors contribute to this situation.

There is an enormous variety of combinations of matrices, ligands and modes of attachment possible for even apparently simple systems. For example, nearly a dozen different ATP-agarose and NAD-agarose resins, with linkages via the ribose hydroxyls, the N^6 or the C^8, with or without spacers etc., are commercially available. The careful protection chemistry necessary to ensure precise and accurate single-site attachment so that the ligand is accessible to the enzyme is certainly not trivial. It is time-consuming and hence expensive. Frequently quite similar kinases or dehydrogenases exhibit striking differences in their binding and elution characteristics with the individual resins. The precise degree of substitution of the ligand is often critical.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Although these differences can, of course, be exploited in the purification protocols, the number of different resins that it is necessary to have available to have a good chance of success with a new enzyme is unacceptably large.

In many of the publications concerning laboratory-scale affinity chromatography, the system has been developed with homogeneous or highly enriched fractions and has not been tested at an early stage of a real practical purification scheme. Under these more demanding conditions binding capacities for the enzyme of interest are frequently significantly lower than those observed with the more highly purified material because there are other competing proteins present. Elution parameters are also more critical. Perhaps even more significant, though, is the fact that in the relatively crude fractions there are other activities that can degrade or modify the expensively fabricated affinity resin, which makes reuse hazardous. This combination of high initial manufacturing cost and unsuitability for recycling and reuse makes most of the classical affinity chromedia inappropriate for general large-scale or industrial use. Naturally there are exceptions when the product itself is so important and hence commands such a high price as to negate these essentially commercial judgements, but the large-scale worker is usually well advised to look elsewhere.

Quasi affinity chromatography

Fortunately one does not have far to look. The development of the quasi-affinity chromatography afforded by the immobilized triazine dyes originally lagged well behind the classical substrate, coenzyme or ligand work but, because the technique does not suffer from many of the disadvantages outlined above, over the past 3 years the balance has been swinging in this direction and particularly so for large-scale work.

The original observation that triggered the exploitation of these dyes was that a number of proteins exhibited anomalous elution characteristics when chromatographed on gel filtration columns in the presence of the blue dextran commonly used as a void volume marker on such columns (Haeckel *et al.* 1968; Andrews 1965). This blue dextran was the triazine dye Cibacron blue F3G-A (identical to Procion H-B) directly linked to a soluble high molecular mass dextran. When it became evident that it was the dye chromophore that was responsible for the interaction that produced the anomalous elution position (Kopperschlager *et al.* 1971), it was but a small step to immobilize Cibacron blue directly on to agarose and use this material to bind the proteins. Most of the early work concerned dehydrogenases and kinases and it was proposed that Cibacron blue could bind specifically to the 'dinucleotide fold' observed in the three-dimensional structures of many of these proteins (Thompson *et al.* 1975). It soon became apparent that this was a considerable oversimplification and that Cibacron blue could bind a wide variety of otherwise unrelated proteins (Dean & Watson 1979).

Most of the work in this area has been concentrated on this one dye, presumably because of its early commercial availability coupled to suitable matrices. However, Cibacron blue F3G-A is only one of a very large family of triazine dyes made in vast amounts as textile dyes. One manufacturer alone (I.C.I.) produces over 70 different such dyes under the trade name 'Procion'. In a survey of 32 of these dyes immobilized at relatively low dye concentrations of Sepharose 4B, Bruton & Atkinson (1979) observed that global protein-binding capacity varied from about 0.1 mg ml^{-1} to at least 2.5 mg ml^{-1}, with Cibacron blue near the middle of the range. Recently a few more resins with different dyes immobilized have become commercially available, but for the large-scale worker even this is unnecessary because the techniques for making the conjugates are very straightforward and well documented (Atkinson *et al.* 1982). The dichloro-

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

triazinyl dyes can be attached to agarose in alkaline solution in less than 2 h at elevated temperature and the monochloro dyes in a day. No noxious chemicals such as cyanogen bromide, so frequently needed for the immobilizing of ligands in conventional affinity chromatography, are needed and the triazine–agarose linkage is much more stable under a variety of conditions than the isourea formed in cyanogen bromide attachment. Thus most regular users and, I should imagine, all pilot plant and industrial users manufacture their own resins. As more workers become aware of the variety of dyes available and the ease with which they can be manipulated, I predict a continued expansion in the number of different dyes in use for enzyme purification.

A further major advantage of these dye–resins is that the triazine and the chromophore are very resistant to both chemical and enzymic degradation. Over 40 runs have been obtained from a single column in a regular purification of alcohol dehydrogenase (Lamkin & King 1976), and the reuse of the same column over several years is common in our laboratory and has been reported elsewhere (Fischer & Whitt 1979). Only the usual precautions to protect the agarose matrix from microbial degradation are necessary.

Many operational features of dye–ligand chromatography are comparable with those of classical affinity chromatography. Agarose is generally the matrix of choice for triazine immobilization, although cellulose, polyacrylamide and dextrans have been used. The degree of dye substitution must be controlled and high substitution does not necessarily correlate with high protein binding (Angal & Dean 1977). It is preferable in the manufacture of the resins to maximize the percentage of the dye covalently linked to the matrix, because the polyaromatic dye molecules have a tendency to form quite stable long hydrophobic stacks. If such stacks are present on the resin, dye not covalently linked may leach off the column in use.

Spacer arms, which have wide application in conventional affinity chromatography, are less used in triazine dye work. Most work has been done with the dyes covalently attached directly to the support by the simple procedure described above. The introduction of spacers is possible but usually necessitates the use of cyanogen bromide activation which, as discussed, it is preferable to avoid.

The elution of enzymes from the immobilized dyes is achieved by a suitable combination of steps or gradients of substrates, products, coenzymes, other ligands of the enzyme of interest, pH or merely salt concentration. The degree of selectivity possible with this latter technique is far greater than one might imagine and the elution is not directly related to simple ion-exchange phenomena. Thus neutral salts are not equipotent in their elution characteristics and the relative potency of these neutral salts is frequently independent of both ligand and protein. A correlation between the relative potency of elution and the Jones–Dole B viscosity coefficient, which is itself a measure of the entropy of the salt, has been demonstrated (Robinson *et al.* 1981).

The extension of triazine dye conjugate chromatography to systems such as receptor and membrane proteins or other particulate enzymes, which normally require the presence of non-ionic detergents, is also possible. In early experiments of this type it was generally observed that at the usual concentrations of, say, Triton X-100 the retention of protein by the dye column was significantly impaired, presumably because the dye itself became encapsulated within the Triton micelles and is thus available to bind protein. This difficulty has been overcome by 'spiking' the non-ionic detergent with a much lower concentration of an anionic detergent such as sodium deoxycholate or sodium dodecyl sulphate (Robinson *et al.* 1980).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The anionic detergent is preferentially encapsulated in the Triton micelles, leaving the dye molecules free to interact with proteins. This trick has been used with great success in the purification of a cyclic nucleotide phosphodiesterase over 50-fold from a detergent extract of bovine particulate material. Although this approach has so far only been used on the laboratory scale, it is certainly directly amenable to scaling up, affording exciting prospects for large-scale work in this difficult yet expanding area of protein purification.

TABLE 1. PURIFICATION OF 3-HYDROXYBUTYRATE DEHYDROGENASE AND MALATE DEHYDROGENASE

(The crude extract from 1 kg of *Rps. sphaeroides*, disrupted at *ca.* 50 MPa, was centrifuged and applied directly to a 1.8 l column of Procion red H-3B equilibrated with 10 mM potassium phosphate, pH 7.5. HBD† was eluted with a step of 1 M KC. and MD by a second step containing 2 mM NADH in 1 M KCl. After desalting, the HBD was absorbed on to a 800 ml column of Procion blue MX-4GD also equilibrated with 10 mM potassium phosphate, pH 7.5. The column was washed with 1 M KCl and the HBD eluted with 2 mM NADH in 1 M KCl. The MD fraction from the Procion red H-3B column was thoroughly desalted to remove all traces of NADH and bound separately to a procion blue Mx-4GD column equilibrated with 10 mM potassium phosphate, pH 7.5. The column was eluted with a 6 l linear gradient from 0 to 0.7 M KCl in the same buffer. MD eluted around 0.4 M. Data taken from Atkinson *et al.* (1982).)

	enzyme†	protein g	units	specific activity	purification	yield (%)
crude extract	HBD	76.2	6900	0.09	—	100
crude extract	MD	76.2	414000	5.4	—	100
Procion red H-3B	HBD	2.2	6800	3.1	34×	99
Procion red H-3B	MD	3.1	327000	105	19×	78
Procion blue MX-4GD	HDB	0.28	5500	19.2	213×	78
Procion blue MX-4GD	MD	0.91	265000	292	54×	64

† Abbreviations: HBD, 3-hydroxybutyrate dehydrogenase; MD, malate dehydrogenase.

The power of triazine dye chromatography in large-scale enzyme purification is best illustrated by specific examples concerning diagnostic enzymes of commercial importance. I have already referred to a classical gram-scale purification of glycerokinase from *B. stearothermophilus* (Comer *et al.* 1979), which involved five steps after the initial cell rupture. Two of these may be replaced and the overall scale increased by the use of a Procion Blue MX-3G-Sepharose 6B matrix. Approximately 60 g of protein containing 12 g of glycerokinase were applied to a 3.5 l column of the matrix equilibrated with 25 mM potassium phosphate, pH 7.5, which was then thoroughly washed with the same buffer. Ten grams of homogeneous glycerokinase were eluted at a peak concentration of 30 mg ml^{-1} by a pulse of 5 mM ATP + 5 mM $MgCl_2$ in the equilibration buffer (Atkinson *et al.* 1981).

A more sophisticated scheme involving two different dyes and both salt and cofactor elution has been employed in the purification of 3-hydroxybutyrate dehydrogenase and malate dehydrogenase from *Rhodopseudomonas sphaeroides*. 3-Hydroxybutyrate dehydrogenase is an important diagnostic tool in the estimation of ketone bodies, but the standard purification with conventional techniques involves eight steps and gives a final overall yield in the region of only 9% (Bergmeyer *et al.* 1967). In the alternative triazine-based protocol, triazine dye chromatography is the only technique used to take the two enzymes through to homogeneity with yields at least eight times higher (Atkinson *et al.* 1982) (see table 1).

These examples illustrate many of the points of triazine dye work. In both cases several dyes were evaluated before the optimal procedure was established and, interestingly, in neither case

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

is Cibacron blue F-3GA used in the final scheme. Both step and gradient elution with substrate, coenzyme or salt are employed and in neither case are spacer arms used.

Concluding remarks

Several thousand enzymes are now available commercially but most of them in surprisingly small quantities. Only about 100 are sold in kilogram amounts and fewer still, mainly extracellular proteass and amylases, are produced on a vast scale for use in washing powders, brewing, etc. The range of prices charged varies as widely as the scale of manufacture, from a few pence per gram to several hundred pounds per milligram. Gram-scale purification therefore presents genuine commercial opportunities.

It is significant that nearly all enzymes are sold by their activity rather than by mass, which is indicative of the fact that homogeneity *per se* is often neither necessary nor even desirable for many commercial uses. It is frequently important only to remove particular contaminants and there is an enormous variation in the purities of different enzyme preparations on the market. This is a fundamental difference from most requirements of academic laboratories.

A possible major disadvantage of unecessarily high levels of purity is the concomitant low protein concentration and its possible deleterious effect on storage properties. All enzymes lose activity with time, even if very slowly. Marketing a product of changing activity is difficult. Samples may have to be assayed regularly by producer or user, or both, which is wasteful of both time and precious sample. The use of thermophilic sources for diagnostic enzymes is likely to increase in the very near future because the storage and handling characteristics of their enzymes are markedly superior to their counterparts of mesophilic origin.

The techniques used in large-scale enzyme purification include both direct scaling up of laboratory processes, such as column chromatography of most types with great success, and new procedures for which large-scale equipment is more suited. Precipitation techniques are best avoided: batchwise adsorption and elution from suitable chromedia are common place. Classical affinity chromatography finds less application than might at first sight be expected, but the quasi-affinity chromatography afforded by the triazine dye conjugates shows enormous potential.

References

Andrews, P. 1965 The gel filtration behaviour of proteins related to their molecular weights over a wide range. *Biochem. J.* **96**, 595–606.

Angal, S. & Dean, P. D. G. 1977 The effect of matrix on the binding of albumin to immobilised Cibracron blue. *Biochem. J.* **167**, 301–303.

Atkinson, A., Banks, G. T., Bruton, C. J., Comer, M. J., Jakes, R., Kamalagharan, T., Whitaker, A. R. & Winter, G. P. 1979 Large-scale isolation of enzymes from *B. stearothermophilus*. *J. appl. Biochem.* **1**, 247–258.

Atkinson, A., Bradford, P. A. & Selmes, I. P. 1973 The large-scale preparation of chromatographic grade hydroxyapatite and its application in protein separation procedures. *J. appl. Chem. Biotechnol.* **23**, 517–529.

Atkinson, T., Hammond, P. M., Hartwell, R. D., Hughes, P., Scawen, M. D., Sherwood, R. F., Small, D. A. P., Bruton, C. J., Harvey, M. J. & Lowe, C. R. 1981 Triazine-dye affinity chromatography. *Biochem. Soc. Trans.* **9**, 290–293.

Atkinson, A., McArdell, J. E., Scawen, M. D., Sherwood, R. F., Small, D. A. P., Lowe, C. R. & Bruton, C. J. 1982 The potential of organic dyes as affinity ligands in protein studies. In *Affinity chromatography and related techniques* (ed. T. C. J. Gribnau, J. Visser & R. J. F. Nivard), pp. 399–410. Amsterdam. Elsevier.

Bergmeyer, K., Gawehn, K., Klotzsch, H., Krebs, H. A. & Williamson, D. H. 1967 Purification and properties of crystalline 3-hydroxybutyrate dehydrogenase from *Rhodopseudomonas sphaeroides*. *Biochem. J.* **102**, 4320–4321.

Bruton, C. J. & Atkinson, A. 1979 The binding of aminoacyl-tRNA synthetases to triazine dye conjugates. *Nucl. Acids Res.* **7**, 1579–1592.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Bruton, C. J., Jakes, R. & Atkinson, A. 1975 Gramme-scale purification of methionyl-tRNA and tyrosyl-tRNA synthetases from *E. coli*. *Eur. J. Biochem.* **59**, 327–333.
Clarke, C. M. & Hartley, B. S. 1979 Purification, properties and specificity of the restriction endonuclease from *B. stearothermophilus*. *Biochem. J.* **177**, 423–431.
Comer, M. J., Bruton, C. J. & Atkinson, A. 1979 Purification and properties of glycerokinase from *B. stearothermophilus*. *J. appl. Biochem.* **1**, 259–270.
Cuatrecasas, P. & Anfinsen, C. B. 1971 Affinity chromatography. *A. Rev. Biochem.* **40**, 259–278.
Davies, R. 1963 Microbial extracellular enzymes, their uses and some factors affecting their formation. In *Biochemistry of industrial micro-organisms* (ed. C. Rainbow & A. H. Rose), pp. 68–150. London: Academic Press.
Dean, P. D. G. & Watson, D. H. 1979 Protein purification using immobilised triazine dyes. *J. Chromatogr.* **165**, 301–319.
Dixon, R. A. & Postgate, J. R. 1972 Genetic transfer of nitrogen fixation from *K. pneumoniae* to *E. coli*. *Nature, Lond.* **237**, 103–102.
Fahrney, D. E. & Gold, A. M. 1963 Sulphonyl fluorides as inhibitors of esterases. *J. Am. chem. Soc.* **85**, 997–1000.
Fischer, S. & Whitt, G. 1979 Purification of the creatine kinase isozymes of the green sunfish with Blue-Sepharose CL-6B. *Analyt. Biochem.* **94**, 89–95.
Haeckel, R., Hess, B., Lauterborn, W. & Muster, K. H. 1968 Purification of yeast pyruvate kinase. *Hoppe-Seyler's Z. physiol. Chem.* **349**, 699–714.
Jacob, F. & Monod, J. 1961 Genetic regulatory systems in the synthesis of proteins. *J. molec. Biol.* **3**, 318–358.
Jansen, E. F., Fellows-Nutting, M. D., Jang, R. & Balls, A. K. 1949 Inhibition of the proteinase and esterase activities of trypsin and chymotrypsin by diisopropylfluorophosphate. *J. biol. Chem.* **179**, 189–199.
Kopperschlager, G., Riezel, W., Freyer, R., Lieve, S. & Hoffman, E. 1971 Wechselwirkungen der Hepephosphofructorekinase mit Dextranblau 2000. *Eur. J. Biochem.* **22**, 40–45.
Lamkin, G. & King, E. 1976 Blue Sepharose: a reusable affinity chromatography medium for purification of alcohol dehydrogenase. *Biochem. biophys. Res. Commun.* **72**, 560–565.
McFall, E. 1976 Dominance studies with stable merodiploids of *E. coli* K. 12. *J. Bact.* **94** 1982–1988.
Priest, F. G. 1977 Extracellular enzyme synthesis in the genus *Bacillus*. *Bact. Rev.* **41**, 711–753.
Rigby, P. W. J., Burleigh, B. D. & Hartley, B. S. 1974 Gene duplication in experimental enzyme evolution. *Nature, Lond.* **251**, 220–224.
Robinson, J. B., Strottmann, J. M. & Stellwagen, E. 1981 Prediction of neutral salt elution profiles for affinity chromatography. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2287–2291.
Robinson, J. B., Strottmann, J. M., Wick, D. G. & Stellwagen, E. 1980 Affinity chromatography in non-ionic detergent solutions. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5847–5851.
Roncari, G. & Zuber, H. 1969 Thermophilic aminopeptidases from *Bacillus stearothermophilus*. *Int. J. Protein Res.* **1**, 45–61.
Singleton, R. & Amelunxen, R. E. 1973 Proteins from thermophilic microorganisms. *Bact. Rev.* **37**, 320–342.
Thompson, S. T., Cass, K. H. & Stellwagen, E. 1975 Blue dextran-Sepharose: an affinity column for the dinucleotide fold in proteins. *Proc. natn. Acad. Sci. U.S.A.* **72**, 669–672.
Umezawa, H., Aoyagi, T., Morishima, H., Matsuzaki, M., Hamada, M. & Takeguchi, T. 1970 *J. Antibiot.* **23**, 259–268.
Uy, R. & Wold, F. 1977 1,4-Butanediol diglycidyl ether coupling of carbohydrates to Sepharose: affinity absorbents for lectins and glycosidases. *Analyt. Biochem.* **81**, 98–107.

Discussion

A. R. Thomson (*Biochemistry Group, A.E.R.E. Harwell, U.K.*). Dr Bruton has highlighted the question of adsorption and affinity chromatography on a large scale. Some years ago it seemed to us that there was a need for chromatographic adsorption media usable with biological macromolecules, and having better process engineering properties than the hydrophilic gels and particles such as cellulose and dextrans then available, particularly for use on an industrial scale. Accordingly we have developed techniques for fabricating rigid, porous particles from a range of inorganic powders with defined particle sizes (50–100 μm or more). These have many of the desired properties in being rigid, usable in large columns, at high rates, and being robust and chemically stable. They can be used to adsorb proteins from process fluids, extracts, cell-free fermentation fluids and possibly even whole broths. They are also non-biodegradable and reusable in columns without repouring. Furthermore, it is possible to incorporate ligands such as Cibacron blue and proteins, to give non-compressible affinity materials for use on an industrial scale.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

To respond to Professor Hartley's call for speculative comments, two interrelated thoughts occur to me. I see rDNA technology as having two further major benefits over and above the incorporation of specific genes into microorganisms.

(i) They are likely in the long run to free us from the constraint of the choice of organism. In the future it is likely that we shall be able to select whatever organism we wish to use because of its process characteristics, and then use rDNA technology to introduce the gene or genes for the product we want, thus maximizing cost-effectiveness.

(ii) As an extension of this, for proteins we should be able to *design* the process *ab initio* knowing the product we wish to make, to give maximal cost-effectiveness of the whole total process.

C. R. Lowe (*Department of Biochemistry, University of Southampton, U.K.*). I should like to ask Dr Bruton a general question concerning large-scale protein purification. How does he feel that the use of quasi-biological ligands such as immobilized triazine dyes compares, both economically and in terms of efficiency, with the use of more conventional and well tried systems such as ion-exchange resins, especially when combined with substrate elution, and at the other end of the spectrum, with immobilized polyclonal or monoclonal antibodies? Can he foresee the day when immobilized monoclonal antibodies will displace all other specific adsorbents for the elective purification of individual proteins?

C. J. Bruton. The immobilized triazine dyes compare favourably with the conventional ion-exchange resins even when they are used with substrate elution. Indeed the latter technique, although excellent in certain specific purifications notably with glycolytic enzymes, has not found particularly wide application. The purifications of malate dehydrogenases and 3-hydroxybutyrate dehydrogenase using only triazine dye columns that I described illustrate perfectly the superiority of these quasi-affinity chromedia.

The monoclonal antibodies clearly offer the ultimate solution to protein purification in terms of efficiency, but the practicality and economics are less certain. Not all proteins are good antigens and making the monoclonal antibodies is not a trivial matter. When they are available at reasonable cost, their purifying power is awesome. Their use will definitely increase dramatically but I doubt whether they will totally supersede all other specific adsorbents.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES