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Phil. Trans. R. Soc. Lond. B 1989 **324**, 497-507
doi: 10.1098/rstb.1989.0063

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Phil. Trans. R. Soc. Lond. B **324**, 497–507 (1989)
Printed in Great Britain

Biochemical engineering challenges of purifying useful proteins

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Some of the biochemical engineering issues that arise when the purification of proteins is scaled up are examined. The first question addressed is the quantity of various proteins likely to be required. Consideration is given to the order in which isolation procedures may be applied and to the selective removal on a large scale of non-proteinaceous materials. Some general problems such as the effect of mechanical forces on proteins and their complexes and aggregates are examined, together with ways of enhancing the properties of these aggregates and the methods of recovering them. The manner in which on-line process optimization can be achieved during pilot plant trials is discussed and the paper concludes with a summary of new scientific developments to which the biochemical engineer will need to respond.

Biochemical engineering is the discipline that underpins large-scale processing of biological materials and the operation of biochemical reactors (Dunnill 1987). It parallels in its breadth the discipline of chemical engineering, which performs a similar role for non-biological substances. The dramatic discoveries in biology of the last ten years have brought many new challenges to the biochemical engineer. Given the great breadth of the field, the present paper focuses only on processing and on one kind of material, the globular proteins.

No materials are more important to the community than proteins, whether as foods, antibodies, subunit vaccines or industrial and diagnostic enzymes. In addition, recombinant DNA techniques have opened up the prospect of many human proteins becoming available in quantity either for use as therapeutic agents or for research.

It is necessary to define how much of various proteins is required and what they cost because these are crucial parameters to the biochemical engineer. Figure 1 shows, very roughly, worldwide tonnages of proteins produced (in terms of pure protein), plotted against price in pounds per kilogram. Both axes are logarithmic and there is an essentially inverse relation between the production scale and price. The range of amount produced and price paid is extremely wide.

At the bottom end of the spectrum of amount produced is Factor VIII protein made at present from human plasma or placentae and vital to haemophiliacs. Human growth hormone was, until recently, derived from cadavers, but is now an rDNA product. Monoclonal antibodies are made by tissue culture. Chymosin, or rennin as it is commonly called, has traditionally been produced from calf stomach tissue. Microbial protease substitutes are widely used but will soon be challenged by recombinant chymosin. Only relatively recently has it been feasible to produce industrially enzymes that are naturally retained within the microbial cell. For example, the intracellular enzyme for penicillin deacylation is isolated on an industrial scale and used in an immobilized form (Dunnill 1980). This is partly because the higher cost of intracellular enzymes encourages extended re-use but mainly for processing convenience. About 80% of the world's semi-synthetic penicillins are made by using immobilized penicillin

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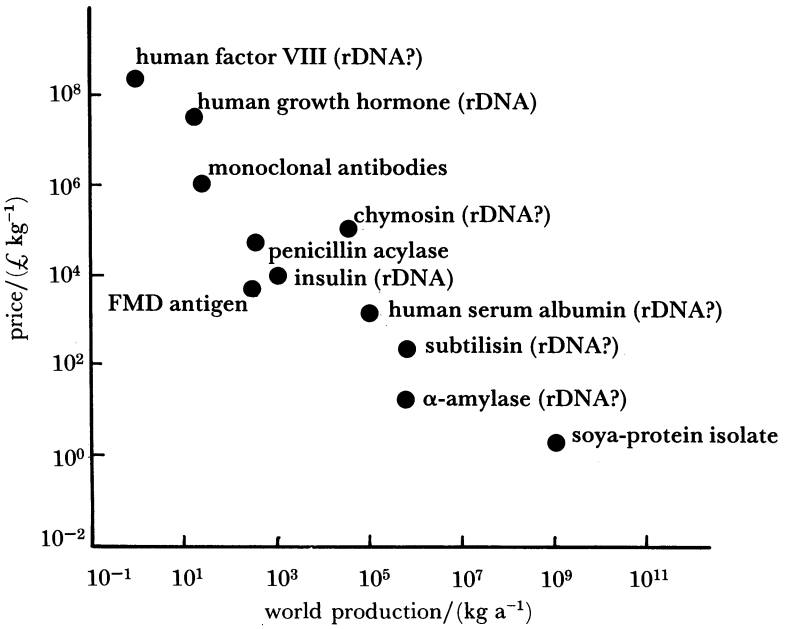

FIGURE 1. World production of important proteins against their price per kilogram. Those instances where recombinant techniques are yielding commercial products are marked with rDNA and question marks are added where this approach is still being developed.

acylase. Insulin, though still made from porcine tissue, is also available now as a recombinant human protein and already being tested are second-generation recombinant insulins with better properties gained by protein engineering techniques (Brange *et al.* 1988). Foot and mouth vaccine, represented in figure 1 in terms of protein content, is a product of tissue culture and protein-engineered subunit vaccine is in prospect. Human serum albumin is obtained from donated plasma and, being heavily heat treated, poses no risk of conveying AIDS. Several human serum albumin ribosomal DNA (rDNA) products are under development but technically and economically it will be hard to make them competitive with the plasma-derived material. Subtilisin and α-amylase are excreted naturally from microorganisms and this has made their isolation on an industrial scale easier. Finally, there is a large step on the logarithmic scale of figure 1 to the food proteins. In recent years it has been realized that proteins are not the only nutrients that the malnourished millions need, but they remain a key food component and some such as soya protein are now substantially purified to improve their functional properties.

The most striking new development is the use of recombinant DNA techniques to allow the large-scale synthesis of human proteins, which previously there would have been no prospect of producing. In spite of the inconvenience of delivering doses of protein drugs, the immediate promise of dealing with several life-threatening conditions probably will give proteins a valuable therapeutic role at least up to the next century. A much talked of example is the human enzyme tissue plasminogen activator, which dissolves blood clots. The U.S. Food and Drug Administration has approved this substance for use, but a chemically modified microbial enzyme also provides a competing approach.

With the genetic opportunities for the present in the background it is appropriate to examine some of the biochemical engineering challenges. There are a number of engineering-centred

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issues that pervade all large-scale protein purification procedures. For example, a key concern is how globular proteins and enzymes will respond to the mechanical forces to which they are subject in most process engineering equipment.

Contrary to a long-standing belief, globular proteins in solution are remarkably resistant towards mechanical forces (Thomas & Dunnill 1979). However, when the intense mixing that generates these forces is occurring in the presence of a gas–liquid interface serious damage does sometimes result (Virkar *et al.* 1981). It has long been known that proteins can be denatured at gas–liquid interfaces, so that if they are thrust by mixing to the interface and then carried away to be replaced by other protein molecules, all of the protein present may be rapidly damaged. However, by attention to design, equipment manufacturers may now avoid such problems and in many instances they can use simpler equipment than in the past. The dimensions of proteins are very small and they are evidently able to remain protected within those individual microscopic packages of fluid that are not disturbed by mixing. Proteins associated with membrane in larger complexes or precipitated aggregates of protein might be expected to be less fortunate and serious effects of mechanical shear are observed with both (Talboys & Dunnill 1985; Hoare & Dunnill 1984). Figure 2 examines the change in enzyme activity or protein-precipitate particle size with time under the influence of shear. It shows that the soluble enzyme alcohol dehydrogenase is stable to a rate of shear of 26000 s^{-1}. This was achieved under defined laminar-flow conditions by using rotating coaxial cylinders with a velocity gradient of 8 m s^{-1} exerted over a 0.3 mm gap (Virkar *et al.* 1981). The membrane-associated progesterone 11α-hydroxylase soon loses activity at only 4000 s^{-1}. (The apparent initial rise is a consequence of easier access of substrate to the fragments of membrane as they become smaller.) Protein-precipitate particles are even larger and quickly degrade at 2000 s^{-1}. Thus for membrane-associated enzymes and protein precipitates great attention to equipment and process design is needed to keep damage to a minimum on scale-up into engineering equipment. With such constraints recognized it is possible to consider the sequence of operations needed to purify proteins.

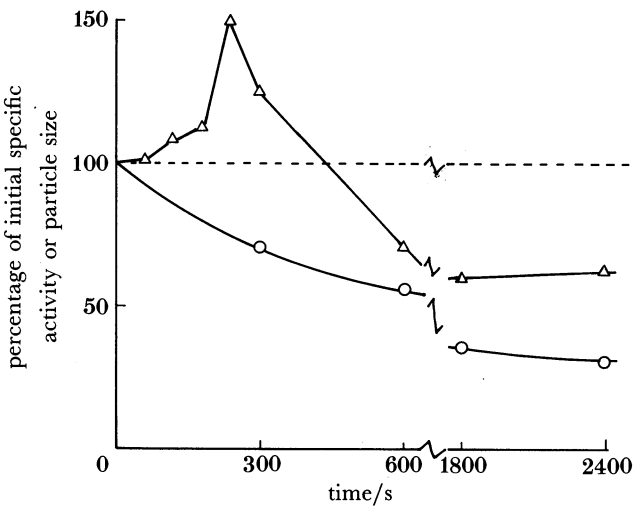

FIGURE 2. The effect of shear with time on a water soluble enzyme, alcohol dehydrogenase (---), at 26000 s^{-1}; a membrane-associated enzyme, progesterone 11α-hydroxylase (△), at 4000 s^{-1}; and soya protein precipitate (○) at 2000 s^{-1}.

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The process begins with the cell synthesizing a protein under the command of the gene: natural or engineered. The protein may either be secreted or retained within the cell. To date the majority of enzymes and proteins of interest are retained and to recover these the cells must be disrupted and the protein purified from the complex cell contents. This takes a number of stages and a key question for the biochemical engineer is whether a fixed sequence of operations can suffice. Superficially, this is not evident in reports of laboratory purifications. That is serious because a unique procedure for each protein would greatly complicate the setting up of a multi-purpose production facility. However, analysis of the literature shows that there is a pronounced tendency to a unique order in applying separation methods (Dunnill & Lilly 1972; Bonnerjea *et al.* 1986). Though analysis of laboratory methods suggests that it is possible to take a rational approach to purification that applies broadly, it is necessary to look not just at purifying one protein from another, but also at the removal of non-protein substances. A key question is where highly selective methods should first be applied in the scheme of protein purification and whether these must involve solid adsorbents. Traditionally, poorly selective purification processes have usually been applied initially and highly selective ones later. This is because in the past most selective methods have relied on adsorption, including affinity binding, and suffer from fouling and problems of scale-up. On the other hand, techniques, such as precipitation, that are relatively insensitive to impurities have been associated with poor selectivity. Recently highly selective affinity methods have been developed for precipitation; however, the key issues are whether the reagents are cheap enough to apply to the large quantities of material to be initially processed without the need for their recovery and whether they are acceptable agents to have as process aids. Many potential reagents fail on one of these two counts, but several show real promise.

Over the years the removal of the debris caused by cell disruption has exercised the thoughts of many biochemical engineers (Mosqueira *et al.* 1981; Gray *et al.* 1973; Le & Atkinson 1985). However, none of these purely physical approaches using centrifugation, filtration or microfiltration has been wholly satisfactory and two-aqueous-phase liquid–liquid extraction involves cumbersome recovery of reagents. Removal of cell debris by selective flocculation is evidently worthy of examination. It is well known that borate ions, or borax complex ions, interact with sugars having neighbouring *cis*-diol groups and it is therefore of interest whether this interaction may be strong enough to link the polysaccharide elements of appropriate cell walls in a selective flocculation reaction. The results with yeast cell debris are illustrated in figure 3, and show the extent of debris flocculation as measured by supernatant turbidity that occurs at rising borax concentrations. By 10 mM borax concentration at higher pHs, supernatant turbidities are achieved with sedimentation at 2000 *g* comparable to that achieved without borax at 10000 *g* (Bonnerjea *et al.* 1988). Remaining subcellular material is only sedimented in an ultracentrifuge at 200000 *g* and its removal will be discussed later. At the low concentration of borax needed, figure 4 shows it does not significantly precipitate protein or damage test enzymes. At 10 mM borax, protein recovery and enzyme specific activities are essentially 100%. As expected, borax has an effect on invertase, which has a carbohydrate component, but only at higher borax concentrations. As these laboratory experiments predict, the aggregated debris is easy to remove in a low-speed industrial scroll centrifuge and gives a solid of 25% dry weight; a material dry enough to crumble into granules as it leaves the centrifuge.

Borax selectively removes carbohydrate-based yeast cell debris but, as expected, the sugar

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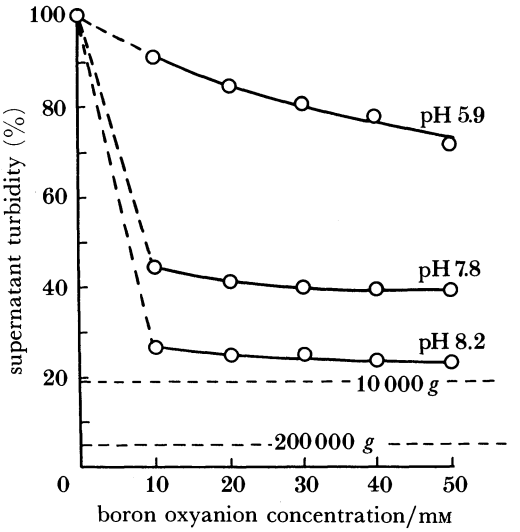

FIGURE 3. The flocculation of yeast cell debris by boron oxyanion (borax) at several pHs. The supernatant turbidity with flocculant, measured after centrifugation at 2000 *g*, is compared with that achieved without flocculant at 10000 *g* and 200000 *g*.

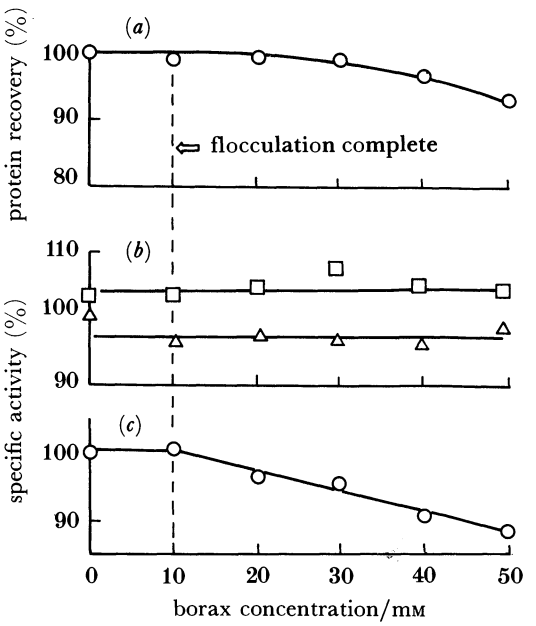

FIGURE 4. The effect of borax at increasing concentration on (*a*) protein recovery and (*b*) the recovery of three enzymes: (△), alcohol dehydrogenase; (□) fumarase and (○) invertase. Flocculation of cell debris is complete by 10 mM borax.

residues in nucleic acids are not affected and borax does not flocculate them. Nucleic acids are among the most troublesome non-protein contaminants and to precipitate them selectively the polymer polyethyleneimine has previously been applied (Atkinson & Jack 1973). However, the results shown in table 1 indicate that not only are nucleic acids largely precipitated whereas marker enzymes are unaffected, but the reagent also selectively removes lipids and colloidal protein to leave a sparkling clear liquor. The method is being refined for large-scale operation

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and the results look very promising. Just as important as the process engineering interest of these results, both borax and polyethyleneimine are reagents acceptable as process aids in the pharmaceutical sector.

TABLE 1. THE EFFECT OF POLYETHYLENEIMINE (1.5 g l^{-1}) ON BORAX PRE-CLARIFIED YEAST HOMOGENATE

substance	percentage remaining compared with control
RNA	8
DNA	5
alcohol dehydrogenase	105
glucose 6-phosphate dehydrogenase	107
lipid	2
protein less than 10^6 Da	98.5
proteinaceous colloid more than 10^6 Da	16

As well as achieving selective precipitation it is necessary also to ensure that the small and low-density particles of precipitate can be efficiently recovered on a large scale. Fortunately, it is possible to modify the properties of precipitates to enhance their sedimentation and to ensure that the precipitates survive processing with less break-up so that they are easier to recover. For example, a technique has been developed by which low-frequency vibration, about 5–10 Hz, is applied to protein precipitates immediately after their formation (Hoare *et al.* 1987). Figure 5 summarizes what is observed. As the fluid acceleration brought about by the vibration is increased the size of the smallest particles – which are the most difficult to sediment – increases to a maximum by aggregation. This end of the size spectrum is best represented by the diameter, D_{95}, which 95% of the particles exceed in size. Beyond this fluid acceleration the shear-related forces prevent the formation of the larger aggregates. The results shown in figure 5 also indicate that the particles obtained by this treatment are more robust to mechanical forces so that, after subjecting the material subsequently to intense mechanical shear, the ones vibrated at this fluid acceleration retain the largest dimensions. Low-frequency vibration also increases the density of the particles, which is the other key factor in determining whether material can be recovered in industrial centrifuges, where gravitational fields are necessarily limited. Thus selective separation can be enhanced and the properties of protein assemblages improved to aid their recovery. Subsequent particle recovery also can be made more efficient on a large scale.

Centrifugation is widely used to separate small biological particles in industry, as it is in the laboratory. However, with many cubic metres to be processed the batch rotor of the laboratory is impractical and instead industrial machines such as the disc centrifuge are used. Such is the quantity of fluid suspension to be processed that material must flow continuously into the rotor (figure 6) and, after the particles are sedimented to the edge, the separated solids and clarified fluid must both leave the rotor; the fluid from the top and the solids from slots in the rotor, which can be opened hydraulically. A set of conical discs, of which figure 6 shows a cross section, ensures that the distance solids must traverse before aggregating on a surface is minimized. Proteins entering such machines may be either in a soluble form while some other solids are being removed or they may themselves be present as aggregated particles. There has been concern that soluble proteins might be damaged during passage, owing to shear and entrapped air in the rotor, but recently we have examined this carefully and as yet find no

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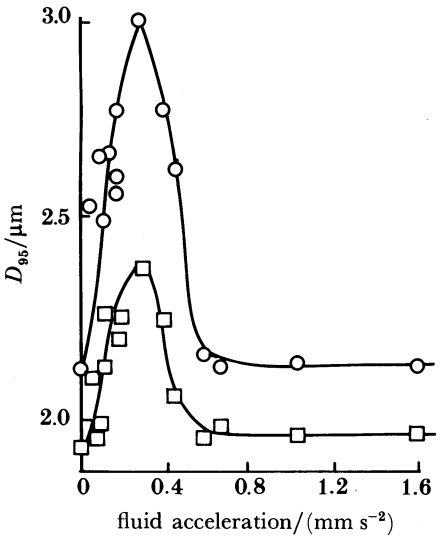

FIGURE 5. The effect of low-frequency conditioning on precipitate size and resistance to shear. The variation of particle size, D_{95}, with the fluid acceleration by low frequency vibration is indicated before subsequent intense shear (○), and after the application of capillary shear (□). Shear conditions: mean shear rate and residence time of 17000 s^{-1} and 0.065 s respectively.

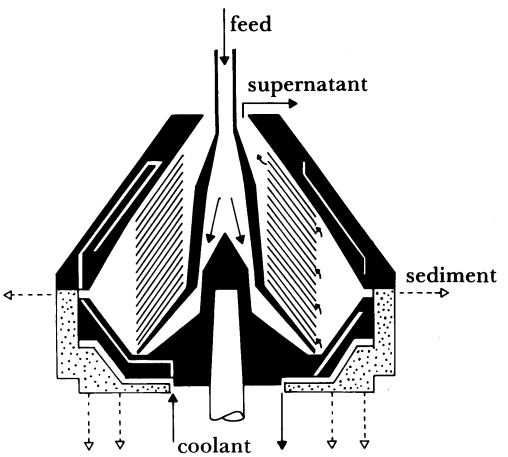

FIGURE 6. Cross section of the rotor of a cooled rotor, high speed, continuous-flow, industrial disc centrifuge. A bottom section of the rotor is lowered hydraulically to release sediment at intervals.

evidence. However, protein precipitates can be degraded by their passage through the machine. As fluid leaves the feed pipe and passes into the rotor it is rapidly accelerated and in the process is subjected to very great shear forces. The effect of this can be anticipated from the earlier data on mechanical forces. We are currently working on this problem with mechanical engineers in the centrifuge-manufacturing companies who are devising improved suspension-feed systems that will be available soon. Microfiltration represents an alternative approach in some instances, but the management of membrane fouling is still a major obstacle.

One new development where centrifugation is important is in the recovery of so-called protein inclusion bodies, insoluble particles of partly folded foreign protein that often occur when rDNA techniques are employed. They appear in cross sections of microorganisms viewed by electron microscopy as dark masses. When the cell has been disrupted the inclusion bodies

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must be separated from the resulting cell debris, but inclusion bodies are very small as figure 7 shows. This indicates the size distribution for human γ-interferon and calf prochymosin inclusion bodies represented as the percentage cumulative volume oversize against particle diameter (Taylor *et al.* 1986). The mean diameters are thus about 0.8 μm and 1.3 μm. Separate experiments showed voidages accessible to suspending fluid of 70 % for γ-interferon and 85 % for prochymosin. To recover the inclusion bodies free from the debris on a large scale requires operation of industrial disc centrifuges based on this analytical work. Figure 8 shows the performance of such a centrifuge. At low flow rate through the rotor there is good recovery of the inclusion bodies but poor removal of cell debris, which is also sedimented under these conditions. At high throughput the inclusion bodies are only partly recovered, but separation from debris is good because it is too small to sediment. The optimum lies at a flow rate of 200 l h^{-1} for this machine.

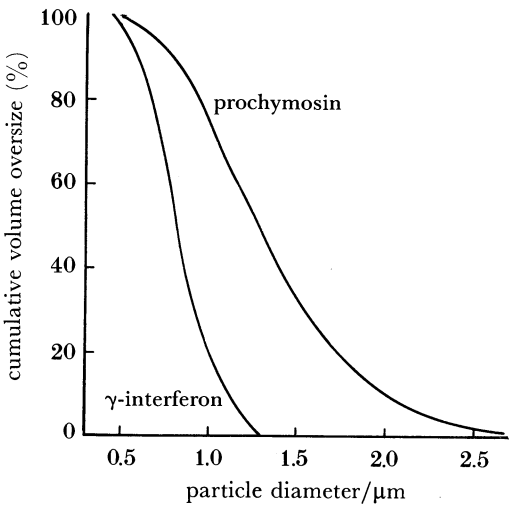

FIGURE 7. Size distribution of protein inclusion bodies containing γ-interferon and prochymosin from *Escherichia coli*.

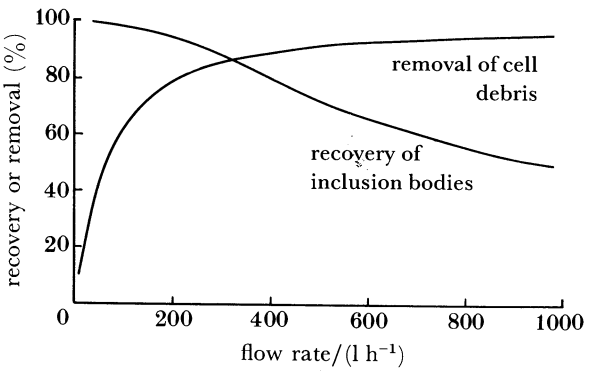

FIGURE 8. Recovery of prochymosin inclusion bodies in an industrial disc centrifuge and the extent of removal of cell debris from the inclusion bodies.

The scaling-up of enzyme and especially non-enzymic protein purification generally proceeds blind. That is, the slowness of the assay is such that a laborious and costly pilot-scale trial is conducted and only later is it learned whether the trial has been successful. If a sufficiently fast analytical procedure were available, the process could be optimized on-line. Recently the power of microprocessors and the development of new analytical methods has

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greatly improved prospects for such optimization. The principles can be illustrated through the example of purification by fractional precipitation used to separate one protein from others. When a substance such as ammonium sulphate is gradually added to a mixture of proteins, some precipitate before others. In figure 9 typical behaviour is illustrated. Enzymes, in this example alcohol dehydrogenase and glucose 6-phosphate dehydrogenase, precipitate over a narrow range whereas proteins as a whole are precipitated over a wide range of salt concentrations. Precipitation can equally well be brought about continuously to minimize time-dependent protein degradation. Then as the rate at which a stream of saturated ammonium sulphate flows into a stream containing a mixture of proteins is increased, it will begin to precipitate a proportion of the protein. If the concentration of, say, alcohol dehydrogenase can be measured very rapidly, it will be possible to detect the instant when the rising precipitant concentration just starts to remove this enzyme from solution. If the ratios of flow of the two streams are held constant, contaminating protein that precipitates at a lower concentration than 40% saturation of ammonium sulphate will be precipitated and can be separated from the process stream. A second similar operation can allow the precipitation of the alcohol dehydrogenase at a higher ammonium sulphate concentration though now, as figure 9 shows, a balance between yield and purity must be struck, because there will inevitably be some glucose 6-phosphate dehydrogenase and other proteins precipitated.

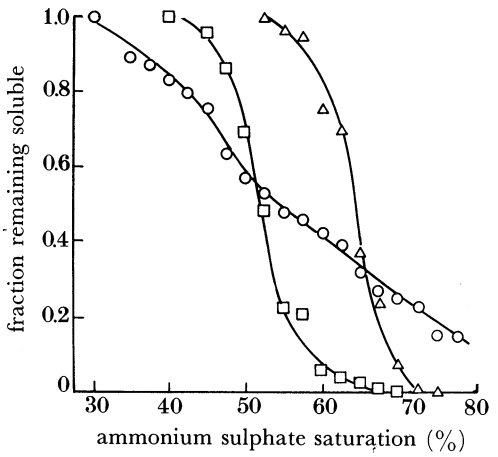

FIGURE 9. The variation with ammonium sulphate concentration of the solubility of yeast protein (○) and the yeast enzymes, alcohol dehydrogenase (□) and glucose 6-phosphate dehydrogenase (△).

Figure 10 shows diagrammatically the continuous precipitation and on-line analytical system. The cell extract and precipitant are pumped via flow meters to a mixer and a sample is led to a miniature centrifuge developed for the purpose to give a clear liquid sample. Analytical speed is important if optimization is to be rapid and the process is to be held subsequently in steady state. Flow-injection analysis methods are up to this task. Given the kind of powerful microprocessors presently available and appropriate software, the specific enzyme activity of each sample can be quickly calculated, and the yield and purity required assessed, then the pumps can be adjusted accordingly. The approach may in principle be applied to a series of purification operations and microprocessor linkage between them could mean that ultimately it will be possible to instruct the controlling system to search for the optimal conditions for purification throughout the process. We are now developing a parallel approach for non-enzymic proteins.

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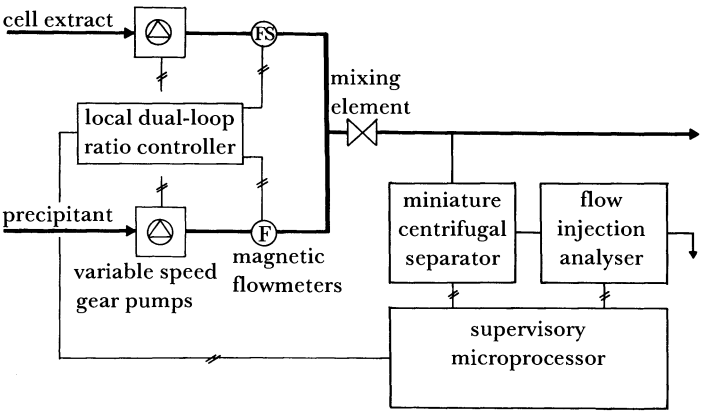

FIGURE 10. Diagrammatic representation of an on-line analytical system for measuring the extent of precipitation of a particular enzyme during continuous precipitation.

A number of developments are in prospect for large-scale protein and enzyme purification. The options opened up by genetic and protein engineering are numerous and a few are described by way of illustration.

Already, several research groups have derivatized proteins by adding nucleotides to the end of their genetic code to specify extra *C*-terminal arginine groups, so facilitating purification by selective ion-exchange binding via these very basic amino acid groups (Sassenfeld & Brewer 1984). The knowledge of how to make enzymes and proteins more stable by altering specific amino acids is developing steadily. It offers the prospect of more robust industrial catalysts and of human proteins that may function for longer periods in the body to be more effective therapeutically. The selective stabilization of one enzyme or protein in a cell would also make its purification easier. There have been reports of pairs of enzymes that are synthesized fused together by creating the genetic code for both without a termination and initiation code between. This opens up the prospect of recovering multifunctional catalysts in a single isolation procedure (Bulow 1987) or of using the dominant properties of one protein to enable the easy isolation of another (Veide *et al.* 1987). One intriguing option that genetic engineering re-opens is that of simultaneously isolating a number of intracellular proteins at one time. It is necessary to bear the cost of cell growth, cell disruption, preliminary and final purification whether or not a single protein or several are recovered, so there is an incentive to purify more. This has been tried with wild-type microorganisms but there are technical problems of recovering proteins that are generally present at fairly low levels. In addition the proportions of enzymes in the cell may be quite different from the relative market demands for each one. Genetic engineering can in principle both raise the enzyme concentrations and alter the ratios as desired. It may even be possible to derivatize or alter the form of the several enzyme products to make their separation easier.

An exciting new era is emerging in the use of proteins and enzymes. Potential health-care uses for proteins are providing a test bed for novel ideas on production and purification that may later be useful for larger-scale manufacture of industrial enzyme catalysts. Never before has the underlying basic science, whether genetics or protein crystallography, been so closely coupled to potential application. It is the role of the biochemical engineer to listen to the scientist on the one hand and the industrialist on the other and to discover ways of translating

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the exciting science into economic industrial processes. This demands research on, for example, new types of biochemical reactors and better process control as well as the downstream processing aspects discussed, but, as indicated by this single example of biochemical engineering, it is a subject full of exciting challenges.

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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