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The potential of microbial enzymes as diagnostic reagents

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There has been a rapid growth in the application of microbial enzymes for diagnosis in the area of clinical chemistry. The reasons behind this trend are discussed. Descriptions and illustrations are given of the use of microbial enzymes in the determination of metabolic marker analytes; in the assay of antibiotics, analgesic and chemotherapeutic drugs, and in the quantitation of antibody–antigen interactions.

1. Introduction

Many enzymes, from a variety of sources, are now used as diagnostic reagents in clinical chemistry for the estimation of, in particular, metabolites in blood or urine. Such metabolites as glucose, alcohol, triglycerides and cholesterol are routinely measured in many clinical laboratories, employing enzyme systems or kits to effect the analysis. Frequently only one enzyme system is available, as for example in the cholesterol oxidase determination of serum cholesterol, but in some cases alternative systems have been devised. Thus glucose analyses can be performed by using several different enzyme systems, including direct measurement employing glucose dehydrogenase or glucose oxidase, or in coupled systems with hexokinase and an even wider variety of alternative enzymes to provide a coupling system for spectrophotometric determinations.

Although enzymes used in the assay of analytes in biological fluids are produced commercially from a wide variety of animals, plants, bacteria and fungi it is the first source that has dominated this area in the past two decades. Even today the use of animal enzymes in clinical diagnosis constitutes 60 % of the total enzyme market in this field, with about 30 % coming from bacterial sources. It has been predicted, however, that in the current decade this ratio of animal:bacterial enzymes will be reversed and that the total market will increase about fivefold. Although some of the reasons for this predicted trend are specifically illustrated later in this article it is worth examining the general reasons behind it.

2. Increasing use of microbial enzymes

Although animal tissues have provided an abundance of enzymes for clinical chemistry it is technically difficult and probably unprofitable to screen a wide range of such tissues for a particular enzyme having stringent kinetic properties. Some enzymes, for example microbial antibiotic degrading or modifying enzymes, which can be used to monitor serum levels of toxic antibiotics (e.g. gentamycin), do not occur in animal species. Even if the desired protein with adequate kinetic properties should be found in the animal tissue it is impracticable to consider increasing the yields of the enzyme by genetic techniques. In contrast, both the wide range of different microbial species and the diverse range of catabolic activities displayed by

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microorganisms, such as the ubiquitous pseudomonads, means that they can often utilize novel or unusual compounds, some of which may even be toxic to higher animals, and can also provide a range of similar enzymes with various kinetic properties. In addition, standard genetic techniques can be used to produce microbial mutants with elevated enzyme levels, or enzymes with altered kinetic properties.

The extraction and purification of a required enzyme from an animal source can be complicated and costly. For instance, the variation in quantity and quality of xanthine oxidase isolated from bovine milk is known to vary, apparently with the season of the year, but in practice probably the animal's diet. Thus the source of the material is variable. Furthermore, some animal tissues are now either in short supply or becoming increasingly expensive. In contrast, microorganisms can be fermented in large quantities and under culture conditions optimized to provide maximum quantities of a specified protein. Thus they provide a reliable source of starting material, and purification régimes can be readily tailored to provide either many enzymes simultaneously or a large quantity of a specified protein (Bruton *et al.* 1975; Atkinson *et al.* 1979).

Recombinant DNA technology can be used to provide high levels of both animal and microbial enzymes by cloning the relevant gene in *Escherichia coli* or other alternative microbial hosts. Technically, however, it remains simpler to clone microbial genes, particularly those of bacterial species, than animal genes. With many antibiotic-degrading enzymes such as β-lactamases and chloramphenicol acetyl transferase. Nature has already performed the task for us; the genes expressing these enzymes occurring naturally on plasmids. The cloning of useful diagnostic proteins has therefore been confined to those from microbial species, e.g. protein A from *Staphylococcus aureus* and carboxypeptidase G_2 from *Pseudomonas* spp., already cloned in this laboratory. So far the cloning of animal genes has not spread into the clinical chemistry area and has only been used to provide the more expensive pharmaceutical or industrial proteins, e.g. human insulin, human interferon, human and bovine growth hormones. The eventual cloning of diagnostic animal proteins in bacteria, however, may remove many of the problems cited above.

In many respects, therefore, microorganisms, particularly bacteria, are a more practical source of enzymes or proteins for clinical use than animal tissue. In general terms such proteins can be used in the traditional areas of clinical chemistry such as the determination and quantitation of body metabolites, e.g. cholesterol by microbial cholesterol oxidase; the quantitation of antigen–antibody reactions; and also in other, perhaps newer areas, such as drug (analgesic and chemotherapeutic) and antibiotic assay (Atkinson *et al.* 1982).

3. Body metabolite determination

Glycerokinase is widely used in diagnostic kits in the determination of serum triglyceride levels. Concentrations of triglycerides outside the normal range have important clinical significance in the diagnosis of lipid disorders and arteriosclerosis. Glycerokinase catalyses the ATP-dependent phosphorylation of glycerol liberated from serum triglycerides by either ethanolic alkaline hydrolysis or lipase action. Spectrophotometrically, glycerol levels are then determined by coupling the glycerokinase reaction, producing α-glycerol 3-phosphate, with an NAD-dependent reaction, either α-glycerol 3-phosphate dehydrogenase or the ATP-recycling system using phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase.

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Although glycerokinase is commercially available from two microbial sources, *Candida mycoderma* and *E. coli*, both enzymes have relatively poor stability and decay on storage. Since glycerokinase is the key ingredient of kits for the determination of serum triglycerides a search has been made for a more robust and stable protein. Following a screen of thermophilic bacilli capable of utilizing glycerol as carbon source, glycerokinase from *Bacillus stearothermophilus* NCA 1503 was chosen as a promising candidate (Comer *et al.* 1979).

TABLE 1. PROPERTIES OF DIFFERENT GLYCEROKINASE ENZYMES

property	source: *B. stearothermophilus*	*E. coli*	*C. mycoderma*
1. V_{max} at 30 °C (u mg^{-1})	120	75	85
2. K_m			
(*a*) glycerol	4×10^{-5}	1×10^{-5}	3.5×10^{-5}
(*b*) ATP	6×10^{-5}	—	6×10^{-5}
(*c*) dihydroxyacetone	1×10^{-1}	5×10^{-4}	5×10^{-3}
(*d*) L-glyceraldehyde	4×10^{-2}	3×10^{-3}	2×10^{-3}
3. $t_{\frac{1}{2}}$ (min)			
(*a*) 70 °C	3	—	—
(*b*) 60 °C	310	4.5	0.72
(*c*) 20 °C	†	8.6 days	4.1 days

† No loss of activity detectable over 20 days.

Environmental selection pressure was used to produce a mutant that initially yielded about 1 % of its soluble protein as glycerokinase. Optimization of culture conditions finally gave a system producing about 2.5×10^6 units in a 400 l culture, with glycerokinase constituting about 2 % of the soluble cellular protein. Modification of the original enzyme purification protocol resulted in a three-step large-scale procedure, including an ATP-dependent elution from the pseudoaffinity matrix Procion blue MX-3G, which has been operated on the product from two 400 l cultures to give over 40 % overall yields of homogeneous glycerokinase from the starting material (Atkinson *et al.* 1981).

Glycerokinase from *B. stearothermophilus* (*B.s.*) has stability properties and some kinetic properties different from the glycerokinase enzymes of *C. mycoderma* (*C.m.*) and *E. coli* (*E.c.*) and valuable for a diagnostic enzyme (Comer *et al.* 1979). The data presented in table 1 indicates that the V_{max} of the *B.s.* enzyme is slightly greater than the *E.c.* or *C.m.* enzymes. Indeed at 60 °C, the V_{max} of the *B.s.* glycerokinase is over double that at 30 °C, while the *C.m.* and *E.c.* enzymes are too labile to measure at this temperature. The kinetic properties of all three enzymes are comparable on the primary substrates, glycerol and ATP. The *B.s.* enzyme, however, has affinities for dihydroxyacetone and L-glyceraldehyde that are orders of magnitude lower than those of the *E.c.* or *C.m.* glycerokinases. This is a major advantage in minimizing side reactions in complex environments such as serum. The half-life of the three enzymes at various temperatures indicates the much enhanced stability of the *B.s.* glycerokinase. The apparent excellent stability of the *B.s.* enzyme has been further confirmed by prolonged storage of the enzyme, no decrease in activity being observed after 4-months at ambient temperature, nor after more than a year at 4 °C.

In addition to the thermal stability properties displayed in table 1, *B.s.* glycerokinase also has enhanced chemical stability. The enzyme has a far greater tolerance to acid pH (e.g. pH 3)

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and moderate concentrations of protein-denaturing agents (e.g. sodium dodecyl sulphate, SDS) than the *E.c.* or *C.m.* enzymes. Resistance to the surface-active agent is a particularly useful property because inclusion of this agent in a diagnostic kit for the estimation of glycerol, and thereby serum triglycerides, markedly stimulates microbial lipases included to hydrolyse the triglycerides. Neither the *E.c.* nor the *C.m.* enzyme works well in this system, being sensitive to SDS, and hence triglyceride hydrolysis has to be achieved under conditions of prolonged ethanolic alkaline hydrolysis.

The properties of the thermostable *B. stearothermophilus* glycerokinase, especially its chemical robustness and storage characteristics, are therefore ideally suited for inclusion in diagnostic kits. Economically, cultures of the *B. stearothermophilus* mutant yield quantities of glycerokinase similar to those achieved from *C. mycoderma* and *E. coli*. Thus this protein, currently undergoing trials in a new diagnostic kit, may serve as a model for developing more stable reagents for use in the determination of body metabolites.

4. Antibiotic determinations

In clinical chemistry, the level of an antibacterial agent in a patient's serum or urine only requires determination when the antibiotic used is particularly toxic. Gentamycin, for example, can display toxic side-effects at serum concentrations as low as 10 μg ml^{-1}, particularly in patients with impaired renal function. Many species of bacteria produce enzymes that inactivate or degrade antibiotics, the genes coding for the synthesis of these enzymes being carried mainly on resistance plasmids (Broda 1979). Both chloramphenicol acetyl transferase and gentamycin acetyl transferase, which modify their respective antibiotic substrates by acetylation by using acetyl CoA as a source of acetyl groups, have been used to determine chloramphenicol and gentamycin concentrations, respectively, in plasma and urine. However, so far they have not been used widely for this purpose in hospital laboratories.

In contrast, the antibiotic-degrading β-lactamases, which specifically hydrolyse penicillin and cephalosporin substrates, have found wide application. In addition to the direct determination of penicillins and cephalosporins in clinical plasma and urine samples, this group of enzymes has also been used in the sterility testing of sensitive antibiotic preparations and the testing of new penicillins and cephalosporins for susceptibility to enzymic degradation. This latter application has been widely used to check the likely effectiveness of a new proposed antibiotic. The β-lactamases have also found another important use since dual therapy, with both β-lactam and aminoglycoside antibiotics being administered simultaneously, has become an increasingly common form of treatment for acute bacterial infections. In such instances β-lactamases are often used to inactivate the β-lactam antibiotics in the clinical sample before determination of the concentration of the aminoglycoside by microbiological techniques.

β-Lactamases are common in bacterial species throughout nature; such enzymes from *E. coli*, *S. aureus*, *Haemophilus influenzae* and *Pseudomonas aeruginosa* are plasmid coded, whereas those from *Enterobacter cloacae*, *Bacillus licheniformis* and *Pseudomonas cepacia* are coded for on the chromosome. Some species, such as *Bacillus cereus*, produce two β-lactamase enzymes simultaneously. The spread of antibiotic resistance through the bacterial world has meant the development of new modified penicillin and cephalosporin-antibiotics. Thus mixtures of β-lactamases, displaying a broad substrate specificity, have found favour for the treatment of clinical samples. The one used orginally was the naturally occurring mixture of two

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β-lactamases secreted into the culture medium by *B. cereus*. However, the introduction of substituted or modified lactams, which are more resistant to attack by this β-lactamase mixture, means that this enzyme preparation is now of limited value.

A comparison, shown in table 2, of the relative rates of hydrolysis of different β-lactam antibiotics by the *B. cereus* mixture of βI and βII enzymes with the *E. coli* K12 W3110 RTEM plasmid β-lactamase and the *E. cloacae* P99 β-lactamase is revealing.

TABLE 2. RELATIVE RATES OF HYDROLYSIS OF β-LACTAM ANTIBIOTICS BY VARIOUS β-LACTAMASES

(Rates are relative to benzyl penicillin.)

	enzyme		
antibiotic	*E. coli* RTEM	*Enterobacter cloacae* P99	*B. cereus* 569/H βI + βII
benzylpenicillin	100	100	100
amoxycillin	78	37	100
ampicillin	1060	71	80
phenethicillin	43	41	170
carbenicillin	21	38	64
cloxacillin	13	76	4.5
flucloxacillin	17	70	4
methicillin	21	69	26
cephalosporin C	2.3	670	6
cephalexin	0	88	0.1
cephaloridine	4	370	0.6
cephalothin	2	707	5
cepharadine	0	49	0.02
cephazolin	0.5	170	0.2
cefuroxime	0	1.0	—

The *B. cereus* β-lactamase mixture is only effective against the penicillin-based β-lactams and has little activity against the more recent cephalosporin-based β-lactams such as cephalosporin C or cephalothin. In contrast the *E. cloacae* β-lactamase is extremely active against this latter group of antibiotics but has only moderate activity against the penicillin analogues. To produce a β-lactamase mixture active against most β-lactam antibiotics, a cocktail of all three enzyme preparations was formulated from those shown in table 2. The *E. coli* W3110 RTEM enzyme was included for its very rapid rate of hydrolysis of ampicillin, the most common β-lactam used in therapy.

The mixture of *E. coli* RTEM, *E. cloacae* and *B. cereus* β-lactamases has been shown to be consistently more effective than the *B. cereus* enzyme alone, particularly in the treatment of clinical samples containing cephalosporin analogues. The mixture of the three enzyme preparations can be objected to on the basis of cost of production, in that two additional organisms have to be grown and the β-lactamase partly purified from each. The yield of *E. coli* RTEM β-lactamase from a 400 l culture (Melling & Scott 1972) expressed in units of benzyl penicillin hydrolysis is, however, over twice that of the total β-lactamase activity of *B. cereus* cultures, while the β-lactamase yield from *E. cloacae* approximately equals that from *B. cereus*. Thus a mixture of the three β-lactamase enzyme preparations, having broad β-lactam-degrading specificity, can be achieved economically.

In order both to reduce the overall number of cultures required to produce this new β-lactamase mixture and to derive a potentially interesting microbiological and genetic system

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the RTEM β-lactamase from *E. coli* W3110 has been transferred to the *E. cloacae* P99 already producing the cephalosporin-hydrolysing β-lactamases. The *Enterobacter* β-lactamase is located on the chromosome and the RTEM plasmid has been conjugated into this organism by using cephaloridine selection against *E. coli* and mecillinam selection for the acquisition of RTEM by the *Enterobacter* strain. The resultant *Enterobacter cloacae* P99 RTEM strain produces both types of β-lactamase and in at least as high yield as the parent organisms. Indeed, since the *Enterobacter cloacae* P99 strain is more vigorous than *E. coli* W3110, higher yields of the RTEM β-lactamase are achieved in this new hybrid (M. D. Scawen & N. Minton, personal communication).

The widespread occurrence of β-lactam-resistant strains of bacteria has prompted rapid developments in the antibiotic field and the introduction of new β-lactam antibiotics. As this process continues it may be necessary to include additional β-lactamase enzymes, with different specificities, in mixtures used in clinical chemistry. The inclusion, however, of β-lactamase inhibitors in pharmaceutical formulations of current β-lactam antibiotic preparations may also serve to extend the lifespan of the present β-lactam range.

5. Drug assay systems

The monitoring of drug concentrations in body fluids provides necessary information about the balance between effective therapeutic and toxic concentrations. The quantitation of such agents has been achieved by a number of different assay methodologies including chromatographic separations such as g.l.c. or h.p.l.c., spectrophotometry following extraction of the analyte from the body fluid or determination by antibody techniques in immunoassay systems such as radioimmunoassay, Elisa and Emit. Rarely, if at all, has direct enzymic determination of the analyte been applied in this field. It can be argued that finding an enzyme to degrade a specific drug may be difficult; however, almost all compounds are biodegradable and most modern drugs are merely analogues of compounds known to have been present in Nature for thousands of years. It is therefore likely that a specific microbial enzyme system can be obtained to determine the concentration of a drug in body fluid, provided that the concentration range to be determined is not unreasonable in relation to the kinetic parameters of the enzyme. Despite the current lack of effort in this area the potential advantage of assay systems based on the properties of enzymes are obvious; they should be more specific than simple photometric methods, cheaper than chromatographic and more rapid than labelled immunoassays (Atkinson *et al.* 1982).

Both chemotherapeutic drugs, such as methotrexate, and analgesic drugs, such as paracetamol, can face the clinician with a similar problem: the toxic effects of an 'overdose'. Methotrexate is currently and widely employed as a folic acid antagonist in cancer therapy. Continued circulation of methotrexate and its metabolites in high concentrations, however, can be a problem in patients whose renal clearance mechanisms are impaired. An enzyme inhibition assay has been employed to determine serum methotrexate that has both cost and speed advantages over other procedures. This involves inhibition of *Lactobacillus casei* dihydrofolate reductase (K_i for methotrexate is approximately 2 nM) by methotrexate in serum; the assay gives good sensitivity and measures concentrations down to 20 nM methotrexate (Atkinson *et al.* 1982). In the light of the advantages of enzyme assays, research has also been undertaken on the application of carboxypeptidase G2 for direct assay of methotrexate and folates (R. F. Sherwood,

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personal communication). This enzyme, produced from a variant of *Pseudomonas* ATCC 25301, has a high affinity for folic acid and its analogues including the reduced folates. The K_m for methotrexate (approximately 1 μM) suggests that the enzyme may be used to measure the compound at normal clinical concentrations and to differentiate between folate and its analogues by making use of the varying wavelengths and difference spectra of the enzyme–substrate reactions (McCullough *et al.* 1971). The enzyme cleaves glutamate from the folate analogues, and this property may be exploited in a linked assay with glutamate dehydrogenase. The yield, and therefore availability for such research, of carboxypeptidase G2 from cultures of *Pseudomonas* ATCC 24301 has so far been variable and poor. This has recently been circumvented by cloning the gene coding for expression of this enzyme into an 'expression' vector in *E. coli*, to yield over 4 % of the soluble cellular protein as carboxypeptidase G2 (Minton *et al.* 1982).

The drug paracetamol, the main active ingredient of numerous proprietary analgesic preparations many of which are commonly available without prescription, is one of the main suspects in the 20 000 cases of drug overdose admitted to British hospitals per annum. If patients with severe overdose are not treated within 10–12 h of ingestion there is a severe risk of extensive liver damage. The situation is further complicated by the presence of undesirable side-effects, associated with thereapeutic procedures used to treat paracetamol overdose. Cystaemine administration, for example, may cause ventricular tachycardia or anorexia. An accurate knowledge of serum paracetamol levels is therefore important. Since no assay was available that combined the desirable features of being rapid, accurate and sensitive, a new approach was undertaken involving the development of an enzymic assay system (Hammond *et al.* 1981; Price *et al.* 1981).

Initially a bacterium capable of using paracetamol as its sole source of carbon was sought. The ubiquitous pseudomonads yielded a strain of *Pseudomonas fluorescens* which produced an aryl acylamidase catalysing the breakdown of paracetamol (*N*-acetyl *p*-aminophenol) to acetate and *p*-aminophenol. The aryl acylamidase was purified from large-scale cultures of *Ps. fluorescens* and its kinetic parameters were evaluated. The enzyme was shown to have a K_m of 6 μM for paracetamol and thus be eminently suitable for determination of serum paracetamol in the critical range above 1 mM, i.e. 150 mg l^{-1} (Hammond *et al.* 1980).

A necessary feature of any assay system based on the hydrolysis of a compound is the development of a sensitive, specific and simple detection method for the products of the reaction. In this instance, *p*-aminophenol, not being normally found in serum, was the obvious candidate for the development of such a detection method. The chemistry of the reaction system is complex (Hammond 1982). Either Cu^{2+} or MnO_4^{2-} ions oxidize *p*-aminophenol to a quinone monoimine, which can then react with phenol analogues or substituted toluenes (possessing a free *para* position) to form an indophenol dye. Although the reaction proceeds at alkaline pH, the rate of reaction is dramatically increased by ammonia. The assay system for *p*-aminophenol therefore required only the addition of two reagents, the hydroxylated toluene, *o*-cresol, and ammoniacal copper sulphate, to result in complete colour development of the blue-indophenol dye in less than 3 min at ambient temperature. The only likely chemical agents that would also form an indophenol dye with the quinone monoimine of *p*-aminophenol are phenol and 8-hydroxyquinoline, neither of which is likely to be found in a patient's serum (Atkinson *et al.* 1980).

The final assay procedure for serum paracetamol estimation was developed to involve firstly incubation of serum with two units of arylacylamidase for 5 min at ambient temperature,

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followed by the addition of *o*-cresol and ammoniacal copper sulphate either separately or as a mixed reagent; 3 min after reagent addition the colour intensity, which is stable for several hours, can be determined at 615 nm (Hammond 1982). In preliminary trials the assay has performed well, as indicated by the data shown in table 3.

This highly sensitive and precise assay was evaluated in three hospital laboratories, all of which routinely carry out serum paracetamol determinations. Both correlation coefficients and robustness of the technique were excellent. Two other features of the system are worth noting.

TABLE 3. ARYL ACYLAMIDASE-BASED SERUM PARACETAMOL ASSAY

1. linearity	0–400 mg l^{-1} paracetamol
2. sensitivity over critical range	0.6 A_{615} at 150 mg l^{-1} paracetamol
3. effects of dilution	ten-fold dilution gives a linear relation with absorbance
4. interference:	
(*a*) human metabolites of paracetamol	none
(*b*) other analgesics found, with paracetamol, in proprietary formulation	none
5. correlation coefficient:	
(*a*) with g.l.c.	> 0.99
with h.p.l.c.	> 0.99
(*b*) between three hospital laboratories† routinely carrying out paracetamol assay	0.98, 0.93 and 0.92

† Dr C. P. Price, Department of Clinical Biochemistry, Addenbrooke's Hospital, Cambridge; Dr B. Widdop, The Poisons Unit, New Cross Hospital, London; Dr D. Burnett, Department of Clinical Chemistry, St Albans City Hospital, St Albans.

First, the assay is economic. A 400 l culture of *Ps. fluorescens* yields 4×10^5 units of enzyme, from which 1.6×10^5 units can be routinely and partly purified to give an aryl acylamidase preparation suitable for use in serum paracetamol estimations; only two units are needed per estimation. Secondly, the assay procedure is suitable for immobilization on 'dip-sticks', research on which is currently in progress.

The mechanism by which specificity was achieved in the above system is important and possibly creates principles for the development of other systems. Specificity is initially imparted to the system by the aryl acylamidase in that only non-peptide carbon–nitrogen bonds of amides will react. Specificity is again imparted in that only paracetamol in serum will yield *p*-aminophenol on enzymic hydrolysis. Finally the chemical reaction between *p*-aminophenol and *o*-cresol to form an indophenol dye is itself specific. The ability to impart specificity at each stage of the assay system has been critical in the formulation of the serum paracetamol assay and clearly demonstrates the potential of microbial enzymes in the development of drug assay systems.

6. ANTIGEN–ANTIBODY DETERMINATIONS

One of the major problems in assessing antibody formation in a suspect patient is a reliable and accurate method of quantitating the antigen-based determination of antibody levels. In this respect protein A, a protein that can be isolated from the cell wall of *Staphylococcus*

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aureus, may be unique in that it can form the basis of a 'universal probe' for determining most antigen–antibody interactions. The characteristic biological property of protein A is its ability to tightly interact with, and form precipitates with, a wide variety of IgG molecules from several species. In the human species protein A reacts specifically with subclasses 1, 2 and 4 of IgG, binding two moles of IgG per mole (Kronvall & Williams 1969; Hjelm *et al.* 1975). Some reactions with human IgA subclass 2 and IgM have also been detected. Since the interaction between protein A and IgG occurs only at the IgG F_c region, the IgG F_{ab} binding site remains unaffected and is available for antigen binding. Suitable labelling of protein A, with an enzyme marker for example, therefore creates a mechanism by which most antigen–antibody reactions can be quantitated.

Protein A can be isolated from the cell walls of *S. aureus* Cowan strain I following digestion of the whole cells with lysostaphin, an enzyme prepared on the commercial scale from *Staphylococcus staphylolyticus*. After lysostaphin digestion, the bulk of the protein can be removed

TABLE 4. ENZYME–PROTEIN-A CONJUGATES

(RTEM β-lactamase from *E. coli* K12 W3110 RTEM; ENT β-lactamase from *E. cloacae* P99; SPDP and MBS are defined in the text.)

conjugate	method	molecular mass/kDa	protein A: enzyme ratio	yield (%)
HRP	periodate	130	2:1	9–20
RTEM lactamase	SPDP/MBS	120	1:2	9–20
RTEM lactamase	SPDP	120	1:2	80
ENT lactamase	SPDP	—	—	20

by acidification of the digest to pH 3.5, leaving protein A in the supernatant. Protein A can then be finally purified by homogeneity in one additional step by either affinity chromatography on IgG-Sepharose or hydrophobic chromatography on octyl-Sepharose. Yields of approximately 1 g pure protein A per kilogram *S. aureus* cell paste have been achieved by this method (M. D. Scawen, personal communication). Alternatively, protein A can be isolated from the culture fluid of methicillin-resistant strains of *S. aureus*, which excrete this protein into the culture medium, instead of allowing its accumulation in the cell wall (Lindmark *et al.* 1977).

As demonstrated in table 4, several enzyme–protein-A conjugates can be prepared by a variety of methods (M. J. Taylor & M. D. Scawen, personal communication). The glycosyl groups of horse radish peroxidase (HRP) have been coupled to protein A by using periodate coupling. The resultant HRP–protein-A conjugate was only recovered in relatively low yield, however, approximately 9 –20 % for both enzymes and immunological activity (IgG binding), independent of variations in the proportions and concentrations of HRP and protein A in the reaction mixture. Assessment of the structure of the conjugate by both enzyme and protein A determination and SDS–polyacrylamide gel electrophoresis (SDS–p.a.g.e.) indicates that the protein-A:HRP ratio in the conjugate is 2:1 with a molecular mass of about 120–130 kDA, in agreement with the known size of HRP (40 kDa) and protein A (42 kDa). A disadvantage of HRP–protein-A conjugates in determining antibody–antigen interactions, however, is that several enzymes in serum can react with hydrogen peroxide and *o*-phenylenediamine, giving high backgrounds in such immunoassay systems.

Microbial β-lactamase does not, however, suffer from this disadvantage. In addition a

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relatively new substrate PADAC (a pyridine-2-azo-p-dimethyl anilide derivative of cephalosporin), is available for assaying β-lactamase levels. This violet substrate, which turns yellow on hydrolysis, has very high extinction coefficients, $E_{570} = 58 \times 10^2$ and $E_{460} = 36 \times 10^2$. Unlike Nitrocefin, another β-lactamase substrate, PADAC does not interact of change colour with any component of human serum. Thus microbial β-lactamase is an excellent candidate for protein A conjugates.

β-Lactamase–protein-A conjugates have been prepared by using SPDP (*N*-succinimidyl-3-(2-pyridyldithio)propionate), a heterobifunctional cross-linking agent that acylates protein amino groups. Thus thiol groups have been introduced into protein A by treatment with SDPD under mild conditions, followed by reduction with dithiothreitol. Two different methods have then been used to couple *E. coli* RTEM β-lactamase. In the first method (SPDP–MBS), β-lactamase is reacted with MBS (maleimido benzoyl-*N*-hydroxysuccinimide ester), which acylates amino groups; reaction of the MBS-β-lactamase derivative with thiolated protein A gives a conjugate bridged by a thioether linkage. In the second method (SPDP), the β-lactamase is treated with SDPD and then reacted with thiolated protein A, liberating pyridine-2-thione and forming a conjugate bridged by disulphide bonds. The β-lactamase from *E. cloacae* has also been reacted with thiolated protein A employing this latter reaction system. The results are presented in table 4.

Both the SPDP–MBS and SPDP coupling methods gave protein conjugates with protein-A: RTEM-β-lactamase ratios of 1:2 as determined by enzyme assay and protein A determination. SDS–p.a.g.e. (in the absence of reducing agent) confirmed these data and gave approximate molecular masses of 120 kDa in both cases. Because strong reducing agents cleavede sulphide bonds, SPDP-linked conjugates were expected to yield, and indeed gave, two bands corresponding to protein A (42 kDa) and β-lactamase (35 kDa) respectively on SDS–p.a.g.e. in the presence of 2-mercaptoethanol. Only the SPDP coupling of RTEM β-lactamase with protein A, however, gave high recoveries of both IgG-binding capacity and enzyme activity. Although this conjugate is only linked by disulphide bridges it has proved to be quite stable on both storage and in use in assay systems. One interesting property of *all* the enzyme–protein-A conjugates is that they exhibit anomalous behaviour on gel filtration, under normal conditions of pH and ionic strength. On agarose–acrylamide copolymers such as Ultrogel, the HRP–protein-A conjugate chromatographs at a molecular mass equivalent to 77 kDa instead of 130 kDa. The β-lactamase–protein-A conjugate is even more bizarre, chromatographing at a molecular mass equivalent to only 22 kDa. This retardation phenomena is currently being examined on other gel filtration matrices such as dextran (Sephadex) and copolymers other than Ultrogel.

The method of using these conjugates, which have been prepared on the 50 mg protein scale, is as follows: specific antigen immobilized in a well on a polystyrene plate is incubated with a patient's serum. Serum IgG antibody to the antigen reacts with the immobilized antigen by binding at the F_{ab} binding site. The F_c region of the IgG molecules, bound to the immobilized antigen by the F_{ab} site, is then probed with a protein-A–lactamase conjugate. The immobilized complex of antigen–IgG–protein-A–lactamase is then assayed with PADAC to determine the amount of lactamase and thus protein A bound, thereby estimating IgG antibody to the antigen present in the serum sample. In preliminary trials with viral antigens plus monoclonal IgG and viral antigens plus serum the protein-A–lactamase conjugates have performed well.

There is one further method for producing protein-A–lactamase conjugates, namely by genetic construction of a hybrid protein. The protein A gene from *S. aureus* has recently been

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cloned and expressed in *E. coli* in this laboratory (S. Jones & C. Duggleby, unpublished data). Since only the N-terminal region of protein A is required for binding to the IgG F_c region, the C-terminal region of protein A being solely required for attachment to the *S. aureus* cell wall, it should be possible to fuse the N-terminal region of the protein A gene with either the whole or the necessary part of a β-lactamase gene by using recombinant DNA technology. Several β-lactamase genes have been studied and DNA nucleotide sequences established that would allow such a hybrid protein to be constructed, including the RTEM-like β-lactamase from the *E. coli* plasmid pBR322, and the chromosomal β-lactamase from *Bacillus licheniformis*, which has been cloned into several plasmids.

7. The future

Where are the trends in microbial enzyme technology leading in the area of clinical chemistry? Undoubtedly enzyme immobilization techniques will lead to a greater use of 'dip-sticks' or bio-sensor probes for rapid metabolite or drug determination in emergency situations. An increased demand for precision and even more assay systems will also lead to greater automation. Greater sensitivity will also be demanded and it is this final area that gives much scope for immediate developments.

Perhaps the most sensitive enzyme system that can be envisaged is one measuring the release of a single photon. The coupling of current and as yet undeveloped assay systems to either the firefly luciferase enzyme (which can be used in ATP-coupled systems) or microbial luciferase (NAD- and FMN-coupled systems), both of which emit photons, could yield remarkable sensitivity. In preliminary tests such enzymes have allowed the determination of a metabolite, e.g. malate, at levels as low as 10^{-12} to 10^{-14} M. The microbial luciferase system from *Photobacterium fischeri*, naturally constitutes 3–5 % of the soluble protein of the cells and consists of two enzymes: a luciferase and an FMN oxidoreductase.

The coupling of microbial luciferase and FMN oxidoreductase enzyme systems to present systems is already under research and perhaps points to another of the trends in the application of microbial enzymes in clinical chemistry.

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Discussion

T. K. Sundaram (*Department of Biochemistry, UMIST, Manchester, U.K.*). Did Professor Atkinson imply that laboratory workers can develop resistance to antibiotics simply by doing genetic engineering experiments?

T. Atkinson. Anyone working with antibiotic-resistant organisms may become infected and thus develop antibiotic-resistant flora by plasmid transfer. This, however, is more likely with 'wild-type' strains than the relatively crippled and esoteric strains that we work with in the laboratory, which have a low survival capability. A survey of the antibiotic-resistant flora of domestic animals, which is very large, indicates that large reservoirs of antibiotic-resistant microbial species already exist in nature. I feel that infection and acquisition of antibiotic resistance in human flora is more likely through this and other such routes than via the laboratory.

D. S. Secher (*M.R.C. Laboratory of Molecular Biology, Cambridge, U.K.*). The idea of constructing by recombinant DNA techniques a hybrid molecule between protein A and a bacterial marker enzyme is a beautiful one. How difficult does Professor Atkinson think it will be to construct such a hybrid so that it folds correctly to give both the binding activity of the protein A half and the catalytic activity of the enzyme half?

T. Atkinson. This may not be as difficult as it first appears. Firstly, only the N-terminal region of protein A is required for IgG binding activity; secondly, native protein A is a relatively extended molecule. Certainly chemically bonded conjugates between protein A and β-lactamase, retaining both IgG-binding and enzymic activity, can be synthesized in high yield, as we have already demonstrated. I can therefore see no reason in principle why a genetically constructed hybrid protein possessing both activities cannot be constructed.

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