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culture

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Antibiotic disc susceptibility testing

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The purpose of testing bacterial cultures for susceptibility to antibiotics *in vitro* is two-fold. First, to make a therapeutic prediction *and secondly, to define and monitor* the incidence of resistance. These two aspects are obviously interrelated but not necessarily equivalent. We feel that the controlled disc diffusion method is the technique of choice in such analyses, provided that it is performed with care. Other diffusion methods are the Kirby-Bauer Method, often used in the USA, and the method proposed by Ericsson and Sherris in their International Collaborative Study Group in 1971 (ICS method).[1] The latter method has not been widely adopted outside Sweden.

A number of novel approaches are currently being used to define susceptibility *in vitro*. These are all based on modifications of minimum inhibitory concentration[1] (MIC) determination. This highly artificial manipulation in the laboratory exposes a dormant bacterial culture to a constant amount of antibiotic. Many of the newer methods of susceptibility testing, particularly the automated methods,[2] give reproducible quantitative information, which is, of course, valuable. However, it is the intention of this article to show that while some of the novel methods may have improved precision (i.e. reproducibility), their relevance (i.e. accuracy) as far as the *patient* is concerned may be poor and the results therefore misleading. When performed carefully, disc testing provides a large amount of qualitative and quantitative information that extends well beyond the definition of susceptibility and resistance. We believe that disc testing can contribute to evaluating whether bacteria from the patient are *important* in so far as determining whether they are responsible for infections.

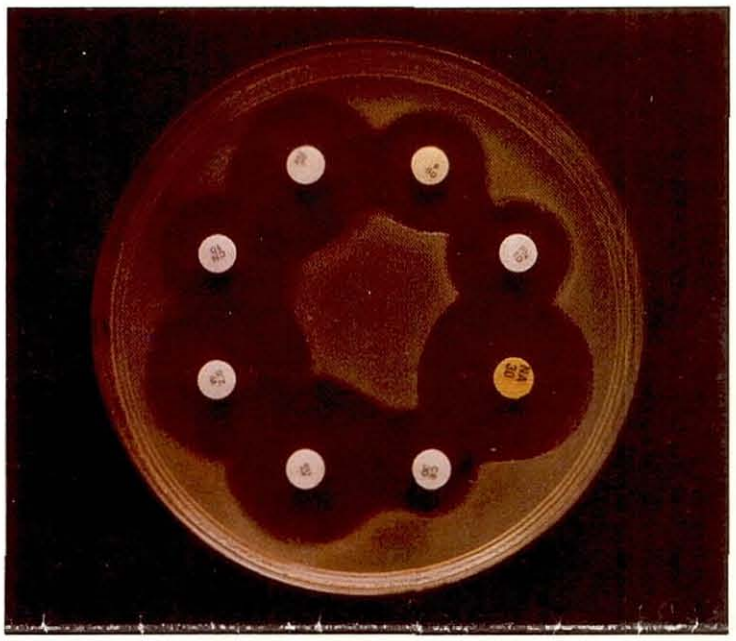

Figure 1: Optimal use of disc testing: the reverse rotary Stokes method in which the control culture is applied to the centre and the test organism to the periphery. Up to 8 discs can easily be accommodated at the interface of the cultures. The test organism is a pure culture of *E.coli* from urine and the control also *E.coli*.

Limitations of other techniques

The limitations of testing by break-point methods have received scant attention. This technique is familiar to many laboratory workers.[3] It is based on the assumption that if the culture can grow in the presence of a certain (critical) concentration of an antibiotic, therapeutic failure can be anticipated during clinical use of the drug. The break-point for the culture is derived principally from blood or tissue concentrations of the antibiotic under test following conventional doses. However, there are a very large number of host determinants that either enhance or depress the action of antibiotics.[4] For example, the composition of human serum has little resemblance to that of laboratory media. Thus, the higher the accuracy and precision of MIC determinations the greater the risk that the discrepancy between *in vitro* and *in vivo* environments will increase.[5] This is illustrated by the observation that erythromycin can be bactericidal in serum at, or just above, the MIC but is bacteristatic in artificial media, the reverse being true of amoxycillin.[5] Cephradine appears to have a potentiating effect with human blood making it equivalent to or superior to cloxacillin (an antibiotic that is very highly protein bound). Yet, if judged by conventional MIC methods, the activity of cephradine appears to be less.[6] The antibacterial activity of tetracycline can also be enhanced by human serum: strains harbouring resistance plasmids can become susceptible to tetracycline in the presence of human serum.[7,8] Union of neomycin with fatty acids *in vivo* can lead to loss of activity.[9]

Protein binding

Specific binding proteins both in serum and on cells can affect antibiotic activity. The important attribute of an antibiotic is the level of free active drug at the site of infection (usually in the tissues). The amount of drug available is inversely related to the degree of protein binding. This may be critical in some important infections where a highly-bound drug may not penetrate the site of infection adequately. Another factor can be competitive binding between, say, cloxacillin and other substances bound to serum proteins such as phenylbutazone, sodium salicylate and sulphonamides.[10,11] Increase in antibiotic activity with increase in temperature over the physiological range has been noted[12] and may be due to less protein binding at higher temperatures.

The interactions of antibiotics with neutrophils is exceedingly complex and can either enhance or antagonise antibiotic action *in vitro*; it is difficult to predict the likely clinical effect from these interactions. Break-down products of leucocytes, particularly thymidine which is released from leucocyte DNA by enzymes present in pus, will reduce the activity of trimethoprim (thymidine may also be derived from bacterial DNA degradation). Differences in pH and ionic strength can alter the activity of an antibiotic, especially in urine. Aminoglycosides are inactive at low pH while tetracyclines are unstable at neutral or high pH.[13] Proteus spp render urine alkaline, yet nitrofurantoin is less active in an alkaline urine. Aminoglycosides are also virtually useless under anaerobic conditions.

Laboratory media interactions

Similarly, there are many well-known examples of constituents of laboratory media which can affect certain antibiotic activities. Blood reduces inhibition zones of antibiotics which are highly protein bound, e.g. fusidic acid. Electrolytes affect many antibiotics, e.g. the activity of aminoglycosides against Pseudomonas varies with the amount of free Ca^{++} and Mg^{++} in the medium. Dextrose enlarges the zones produced by nitrofurantoin and ampicillin but does not alter MICs.[14] Factors which alter the pH of the medium, such as incubation with CO_2 and hence generation of H^+, may make organisms appear more resistant to erythromycin. Also, a fall in pH occurs as a result of fermentation of carbohydrates to acid.[4] The buffering action of blood added to the medium may nullify minor changes in pH. Sulphonamides and trimethoprim are affected by the presence of thymidine. The addition of lysed horse – but not human – blood neutralises this effect because it contains the enzyme thymidine phosphorylase. Where the MIC for a 'sensitive strain' is very close to the break-point used, the interpretation may vary with replicate tests. Estimations of MICs or break-point analysis is, of course, of value in identifying the proportion of bacterial isolates that have acquired resistance using appropriate criteria. This epidemiological evidence is not necessarily relevant to the particular patient. It would appear that the definition of resistance or susceptibility by break-point analysis has been derived largely by theoretical consideration. The determination of MIC *in vitro* has never been shown to be closely related to clinical outcome except possibly in the treatment of *Neisseria gonorrhoea*.

Advantages of disc testing

There is a real need for laboratory susceptibility test procedures to be based on solid clinical experience. Reading plates that contain many inocula of different organisms can be boring and difficult, so mistakes may be made. Automated reading systems are costly. On the other hand, disc testing is not expensive. One of the advantages often attributed to the automated determination of MICs is speed, a result being available in 5-6 hours. However, it is quite possible to obtain rapid susceptibility results using disc techniques by seeding the plate with a relatively heavy inoculum at, say, 9.00am and reading the result at 3.00pm. However, as in all types of rapid testing, the observations are evaluated without the full information that is generated by longer incubation, as occurs with conventional disc testing. In the latter procedure, the detection of mixed cultures, contaminants and resistant variants can all contribute to the assessment of whether the bacteria in question require treatment and, if so, whether they are amenable to therapy.

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	Gram positive		Gram negative		Urine		Anaerobes	
1	Cephradine	30μg	Cephradine	30μg	Cephradine	30μg	Cephradine	30μg
2	Erythromycin	10μg	Erythromycin	30μg	Tetracycline	200μg	Erythromycin	10μg
3	Fusidic acid	10μg	Tetracycline	30μg	Ampicillin	10μg	Penicillin G	2 IU
4	Penicillin G	2 IU	Ampicillin	10μg	Nitrofurantoin	50μg	Tetracycline	30μg
5	Neomycin	30μg	Gentamicin	10μg	Sulphamethoxazole	200μg	Ampicillin	10μg
6	Tetracycline	30μg	Trimethoprim	2.5μg	Gentamicin	10μg	Chloramphenicol	10μg
7	Gentamicin	5μg	Carbenicillin	100μg	Trimethoprim	2.5μg	Metronidazole	5μg
8	Chloramphenicol	10μg	Chloramphenicol	30μg	Carbenicillin	100μg	BLANK☆	

☆ The blank disc on the anaerobic plate is a useful control because the growth of some anaerobes may be inhibited at the edge of the plate.

Table 1 : Combinations of antibiotics used for disc susceptibility tests of different types of organisms.

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Method of disc testing

We use a modified Stokes method of disc testing (**Figure 1**).[13] Our technique uses a control susceptible organism of a species identical to or similar to the test organism. The control organism is applied by rotary application[16] to the *centre* of the plate and the test organism is applied to the *periphery* (most descriptions of the Stokes method apply the test to the centre and the control to the periphery). The inoculum should produce 'dense but not confluent growth'. This approach enables eight discs, which vary according to the nature of the organism, to be applied at the interface of control and test cultures. *Errors associated with MIC break-point and rapid automated methods are less liable to be detected because each individual well or inoculum is not internally controlled.*

We use a thymidine-deficient medium. Plates are incubated at 37°C for 24 hours. Two levels of susceptibility are normally recognised: sensitive and resistant. Intermediate criteria are reported rarely as this is of little help in selecting therapy. **Table 1** shows which combinations of discs we use for different organisms.

Mixed infections

It is axiomatic that the greater the number of different bacterial species associated with a possibly infected lesion, the less likely any of them are to be an important pathogen. Furthermore, where a large number of species of organisms abound, the prospect of their total elimination by antibiotic therapy is remote and even undesirable as it would encourage the proliferation of resistant organisms. The use of disc testing is of great value in identifying mixed populations of bacteria because of their natural variations in susceptibility to the antibiotics in the various discs.

Different zone phenomena are clearly seen with mixed infections. Recent work in Cardiff[17] has shown that the urine of patients with indwelling catheters harbours a large variety of bacterial species. It is usually the policy of laboratories to identify and perform susceptibility tests on the predominant organism. However, in patients with structural lesions infected, or colonised, by a variety of different bacteria it may well be useless trying to treat even the principal organism since the minority population of organisms will overgrow and replace the predominant species. Thus, in a culture of urine from a patient who has had a recurrent urinary infection associated with an indwelling catheter, disc testing may show the urine to contain several bacterial species, so that susceptibility is **not** reported; rather a suggestion is given that antibiotic therapy is unlikely to be effective.

In contrast, testing susceptibility by automated break-point methods will either use the predominant organism or a mixed culture. In either case susceptibility or resistance may be reported which would be misleading. The problem is aggravated if these methods are performed and reported by inexperienced staff who do not possess the information or the authority to advise that chemotherapy is not indicated.

Contamination

It is inevitable that from time to time, plates, fluids and laboratory equipment will be contaminated, e.g. animal blood is notoriously difficult to maintain in a sterile condition. The presence of contaminants is usually evident from a disc testing procedure, particularly if a fully sensitive control organism is used as in our Stokes method. In other approaches, such as automated break-point methods, contamination may not be evident, particularly when the identity of the culture cannot be assessed by the test procedure.

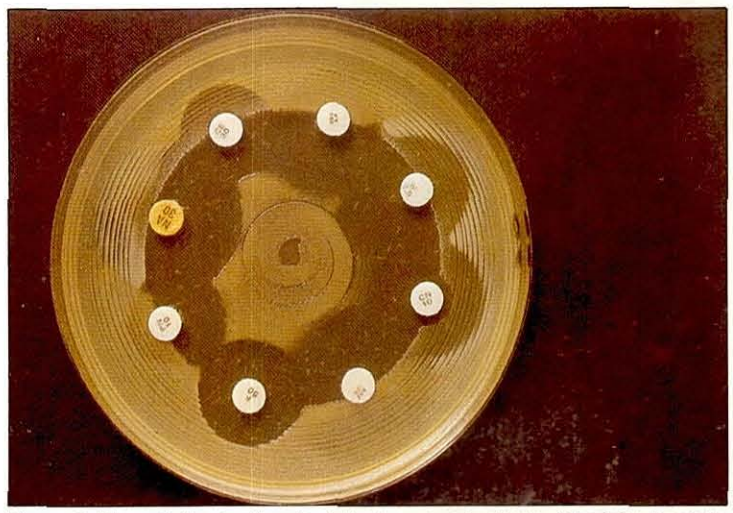

Figure 2: Use of disc testing to detect a mixed culture. The initial isolate appeared to be pure but, on testing, a minority population of other species is evident.

Swarming by Proteus spp

Confusion can arise in the interpretation of zone sizes with organisms such as Proteus spp. which swarm into inhibition zones. However, it is known that organisms taken and tested from inside these zones are no more resistant than the parent culture. Thus, swarming can be disregarded as long as there is a clear edge to the zone.

Mutants

Of the antibiotics currently in use in the UK the following three appear particularly prone to select resistant mutants *in vitro* and *in vivo:* rifampicin, fusidic acid and nalidixic acid. Resistant variants selected by the above agents are typically present in cultures at frequencies of 10^5–10^6. Many break-point or automated testing methods use an inoculum of less than 10^5 bacteria. If so, such mutants will be missed. By using a disc testing procedure with a sufficiently heavy inoculum, these mutants are obvious. The clinical significance of some of these mutants is not always certain as they may be slow-growing, particularly in the presence of antibiotic. However, it must be of value to alert the clinician to the possibility that such a culture contains these unwanted variants. On the other hand, break-point methods will give a result indicative of either *uniform susceptibility* or *uniform resistance;* the presence of a culture with a proportion of mutant bacteria will not be identified.

Antibiotic interactions: Synergy and antagonism

In order to define interactions between two or more antibiotics for a particular culture, methods based on determination of MICs are laborious. As long as appropriate controls are used it is very easy to detect interactions amongst different antibiotics by disc testing. Such controls should include the juxtaposition of discs containing the same antibiotic as well as the two different antibiotics. Without such a control it is impossible to ascertain whether the apparently enhanced zone of inhibition between two dissimilar antibiotics is due to addition or synergy.

There is, of course, uncertainty as to the clinical significance of many of these interactions. It has been shown that the synergy between trimethoprim and sulphamethoxazole, which can be demonstrated *in vitro* on thymidine-deficient media, may not fully reflect what can happen *in vivo.* This occurs when the levels of trimethoprim are sufficient to inhibit growth *in vivo* on its own. Even where antagonists are present *in vivo*, such as thymidine, they may well preferentially neutralise the action of sulphonamides rather than trimethoprim.[18,19] However, we think it is worthwhile to identify antagonistic interactions towards cultures from severely ill patients being treated with other combination therapies. Disc testing provides such a facility and is simple and effective. Of course, these can be quantified subsequently by titrations and chequerboard techniques, but this may be too cumbersome and time-consuming as a routine screen for such effects.

Inducible resistance

Most resistance that bacteria have acquired to antibiotics is due to the possession of specific sequences of DNA that determine a novel protein. This protein is often an enzyme, e.g. β-lactamase, or one of the aminoglycoside-modifying enzymes, or it may be involved with permeability as in tetracycline resistance. Erythromycin resistance is due to abnormal ribonucleoprotein.

Many of these resistance mechanisms are *inducible.* In this process the bacterium synthesises the resistance protein in very small amounts, unless the bacterium has been exposed recently to the antibiotic. After this exposure, the genes instruct the cell to produce increased amounts of resistance protein, i.e. it becomes 'switched on' or induced. The amounts of protein can increase by as much as 100-fold in the case of β-lactamase, but other induction ratios are lower. Maximum induction results from exposure of the organism to a *low* concentration of antibiotic.

In the performance of MICs or break-point susceptibility testing, the bacterium is suspended in a medium containing a high concentration of antibiotic. This concentration may well inhibit the growth of the organism before induction of resistance has occurred. In disc testing, a gradient of antibiotic concentration is established, and somewhere along the gradient there will be a concentration which is optimal for the induction of antibiotic resistance. It is reasonable to anticipate that the formation of such a gradient will also occur *in vivo* after the administration of all antibiotics. Even if an antibiotic is given intravenously, the tissue levels will rise slowly. As the process of induction often takes only a few minutes at low antibiotic concentration, then it is probable that disc susceptibility testing provides a more realistic representation of inducible resistance than break-point methods. This is particularly important with Gram-positive organisms such as *Staphylococcus aureus* where much of the antibiotic resistance is inducible: β-lactamase and macrolide resistance are important examples. We show in **Table 2** where various strains of *Staph. aureus* were tested for susceptibility to penicillin both by determination of MICs and by disc testing. As can be seen, some strains which appear very susceptible with MICs of 0.1 μg/ml do, in fact, show small zone sizes on disc testing due to inducible resistance.

Extracellularity of β-lactamase production

The majority of β-lactamases produced by Gram-negative bacteria are attached firmly to the cell surface, so little diffuses into the environment away from the bacteria. With Gram-positive organisms, up to 60-70% of β-lactamase synthesised may be released into the environment, i.e. is extracellular. When such a resistant organism is subcultured to media containing critical concentrations of antibiotics, residual β-lactamase will be diluted. Thus, when the break-point or MIC determination is performed with an organism that produces β-lactamase, it may appear more susceptible than it really is. In the setting up of disc testing, the residual β-lactamase will initially be lost into the environment, but once the organism starts growing, it will synthesise new β-lactamase and tend to grow back towards the antibiotic. This causes a sharp 'heaped-up' edge around discs of penicillin when placed on cultures producing β-lactamase.

β-lactamase-producing staphylococci	MIC (μg/l)	Zone size (mm)	β-lactamase-producing staphylococci	MIC (μg/l)	Zone size (mm)
Known susceptibility control	0.1	15mm			
1	0.5	4	11	0.1	13
2	0.5	3	12	0.75	3
3	0.5	3	13	0.5	4
4	0.5	14	14	0.5	3
5	0.75	15	15	0.5	4
6	0.1	3	16	0.25	4
7	0.1	0	17	0.75	5
8	0.1	4	18	0.5	4
9	0.1	4	19	0.75	3
10	1.0	3			

Table 2: MIC and disc testing results with various strains of *Staph. aureus*

Identification of cultures

Determination of antibiotic susceptibility by disc testing provides valuable information in the identification of the culture. This results from two phenomena: first, the opportunity to inspect a bacterial lawn and, secondly, characteristic zonal interactions between the antibiotics and the bacterial lawn. Thus, we may see pigment and plaque formation in *Pseudonomas aeruginosa*. With this organism, loss of pigment in the presence of low concentrations of some antibiotics is characteristic. Many cultures produce identifiable odours which are more obvious from a large bacterial lawn, e.g. Haemophilus spp. By use of the appropriate ranges of antibiotics in the discs, information concerning the identity of the organism can be culled. Thus, *Streptococcus faecalis* is resistant to sulphonamides and most cephalosporins. Klebsiella spp. are resistant to ampicillin, Proteus spp. are resistant to nitrofurantoin, and staphylococci are resistant to nalidixic acid. Metronidazole discs can contribute to the recognition of anaerobes. Thus, some of the discs used in susceptibility testing may not function exclusively as a means of predicting therapeutic outcome but can be of value in identifying the organism. Because it is possible to apply at least eight different discs on one standard plate, the luxury of these additional discs can be accommodated without jeopardising the susceptibility testing with the essential discs. The use of such additional plates can barely be justified in break-point techniques or in the determination of MICs.

By storing a wide variety of discs of less commonly used antibiotics, it is easy to add one of these to the plate in place of one of the routine discs if the clinician requests this information. This would be much less convenient in a laboratory using automated break-point or MIC methods.

Slowly growing resistant bacteria

If a bacterium that is resistant to an antibiotic grows slowly when it is exposed to that antibiotic, the break-point methodology and MICs may give apparently false susceptibility results. Certainly, rapid automated methods may fail to detect resistance. It is an interesting speculation whether such resistance is clinically important (i.e. are these bacteria pathogenic in the presence of the antibiotic to which they are resistant?). Certainly the possibility should be recognised. In contrast, with the disc test, even if the resistant organism grows slowly in the presence of the antibiotic, the resistance will be clear since there will be growth near the disc, although of reduced density. Examples of such resistance are lincomycin resistance in *Staph. aureus* and some streptococci and 'methicillin resistance', also in these organisms.

Instability of antibiotic discs

It is probable that most materials used for antibiotic susceptibility testing are of high quality on dispatch to the laboratory. However, opportunities for accidents are considerable during storage in the laboratory. Manufacturers' expiry dates should be observed; care must be taken that discs are stored at low temperature and that they are kept dry in tightly sealed containers. To avoid condensation, they should be removed from the refrigerator and allowed to come to room temperature before being opened. Materials can easily be inadvertently left in moist atmospheres at high temperatures. Metronidazole discs are inactivated by light[21] and therefore must be stored in the dark at all times.

If *controlled* comparative disc testing procedures are used, such as the Stokes method, then decomposition of the antibiotic in the disc should be evident from the control. However, incomplete anaerobiosis can also give apparent metronidazole resistance.[22] Thus, a separate plate with a control organism must be included in the anaerobic jar. By means of the method we have outlined earlier we can clearly identify any disc that contains unexpectedly reduced amounts of antibiotics. The presence of such errors is difficult to detect by automated methods because of the absence of an internal control.

Finally, disc testing techniques have substantial advantages of cost and, we believe, if used correctly they will aid diagnosis and reduce the requirement for chemotherapy in many patients. However, their interpretation requires experience: the reporting of susceptibility test results involves making therapeutic predictions. It follows from this that reporting should be the responsibility of a *senior* member of the laboratory. There should be adequate information on the request form indicating clinical diagnosis and previous and proposed antibiotic therapy. The results should be interpreted in conjunction with microscopy findings, e.g. presence or absence of pus cells, where relevant.

We operate an antibiotic policy in King's Lynn so that we only report susceptibility to a small number of antibiotics which we regard as the first choice in a given case. However, we also keep a record of the full range of antibiotics tested should the clinician require this information at a later date. The final report should be relevant and helpful for the treatment of *the patient* and not just his bacteria.

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PROTEIN HYDROLYSATES (Peptones)

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With papers published in 1880 and 1882, Nägeli has been credited as the first bacteriologist to discover that chemo-organotrophic organisms grow best in culture media containing a partially digested protein material which he termed 'peptone'. Peptone is a chemically indefinite term used to describe the water-soluble products obtained after hydrolysis of proteins using enzymes or strong mineral acids; hence their other name, protein hydrolysates.

The problems associated with the production of protein hydrolysates were quickly recognised and their manufacture became the concern of commercial suppliers. In fact, protein hydrolysate was the first complex culture medium ingredient to be supplied commercially.

Biochemistry of proteins

Proteins are macromolecules and are fundamental to the structure and function of all living organisms. It is therefore appropriate that the word is derived from the Greek *proteis*, meaning first. Chemically, proteins are made up of one or more chains of α-amino carboxylic acids (amino acids) consecutively linked covalently between the α-amino group of one moiety and the α-carboxylic group of the next with the elimination of water. This linkage is termed the peptide bond. Chains of three or more amino acids are termed polypeptides, whilst larger structures, with an arbitrarily determined lower molecule weight limit of 5000, are the proteins.

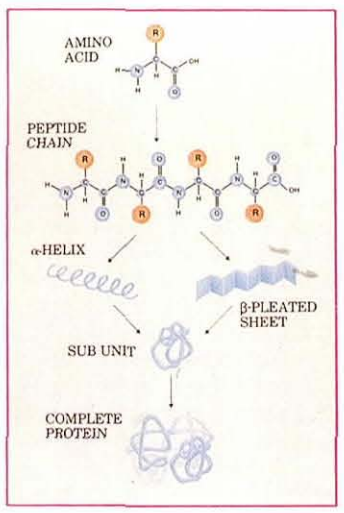

Figure 1: The structure of proteins

Only twenty amino acids commonly occur in proteins, the specific order in which they are linked being termed the *primary structure*: this sequence is remarkably consistent for each family of proteins and is determined genetically.

The *secondary structure* is maintained by the formation of intramolecular and intermolecular hydrogen bonds leading to the formation of an α-helix or beta-pleated sheet conformation, respectively (**Figure 1**). *The tertiary structure* is the way in which these structures fold on themselves to form the three-dimensional structure of the protein including, in the case of enzymes, the formation of the active site. Some proteins are formed from two or more subunits, e.g. haemoglobin; the way in which these are arranged is called the *quaternary structure*.

Hydrolysis

The hydrolysis of proteins, i.e. breaking them down into their constituent amino acids, can be achieved using strong acids, strong bases or proteolytic enzymes such as pepsin, trypsin and papain.

The proteolytic enzymes act on proteins under gentle conditions, i.e. normal pressure at 37°C. Certain heat-stable enzymes, such as papain, will hydrolyse proteins at temperatures as high as 60–70°C. This property is valuable in the production of protein hydrolysates. Proteolytic enzymes will only hydrolyse the peptide bond between specific pairs of amino acids (**Table 1**). This confers two properties: **1.** the protein is not hydrolysed to its constituent amino acids but into polypeptides of varying lengths depending on the frequency of the specific amino acid pairings; **2.** since proteins have a very consistent primary structure, the mixture of polypeptides produced after proteolytic digestion by a specific enzyme is also consistent.

Acid hydrolysis works at much higher temperatures than enzymatic hydrolysis. The process is more destructive to acid-labile amino acids. Normally, hydrochloric acid at 15% w/w is added to the *protein and heated at 110°C for* 15–18 hours. After neutralisation, the product is a black liquid with a very high salt content requiring a lot of refinement before it can be sold as the very white powder normally seen. In spite of the harsh chemical process, many peptides essential for microbial growth are still present in the product.

Enzymes	Peptide bonds attacked
Trypsin	Lys : Ala Arg : Gly
Pepsin	Leu : Val Phe : Tyr
Papain	Asn : Gln Glu : Ala Leu : Val Phe : Tyr

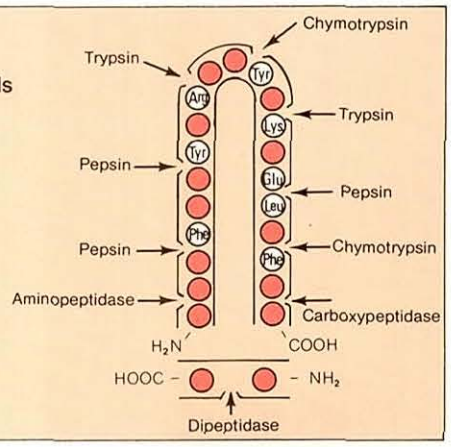

Table 1: Proteolytic enzymes such as trypsin, pepsin and papain will only break the peptide bond between specific pairs of amino acids.

Table 2: Proteins typically used for hydrolysis.

- Casein
- Milk
- Lactalbumin
- Meat
- Gelatin
- Fish
- Soya
- Yeast cells
- Cottonseed

Numerous proteins can be used for hydrolysis. Those most commonly used (**Table 2**) are chosen for the following criteria: **1.** absence of microbial toxins; **2.** ease of hydrolysis; **3.** availability in bulk. Casein, meat and soya protein are the most widely used in culture media. Fish protein is not popular because of the smell and the risk of toxic, rancid oils. Cottonseed and most other plant products have a high carbohydrate content.

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Protein hydrolysate manufacture showing hydrolysis vessels in background

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Manufacturing process

The steps in the manufacturing process are outlined in **Figure 2**. Basically, crude protein is suspended in water at the appropriate temperate. The pH is adjusted and the enzyme added, after which there is a period of digestion and agitation. Fats are separated off using high speed centrifuges and the solution is filtered. The hydrolysate is concentrated to a 'syrup' in low temperature evaporators. It is possible to hold the hydrolysates in this form for long periods at room temperature. This is an advantage to the large user as the problems of reconstituting 100kg lots of spray-dried powder (produced at the final stage) are far greater than solubilising 100kg of syrup.

Analysis of hydrolysates: laboratory specifications

The Oxoid Manual gives a table of typical analyses of protein hydrolysates available. The important factors in the table are as follows:

Moisture. Preferably, moisture should be below 5% to protect against chemical changes at high storage temperatures.
Ash. This consists of inorganic material, metals and minerals.
Chlorides (salt). These may be variable due to differing processing characteristics.
Total nitrogen. This is a very important parameter as hydrolysates may be purchased on a nitrogen-equivalent basis.
Amino nitrogen. This is the measurement of the amino groups of peptides and free amino acids. The higher the ratio of amino to total nitrogen, the greater the degree of hydrolysis.
Lipids. Clear hydrolysates will have fat levels below 0.1%.
Carbohydrates. Soya is a typical plant protein with a high carbohydrate content. Milk is high in lactose.
pH. Neutral pH values are helpful for most purposes but such products have a higher salt content than the more usual acid peptones that are available.

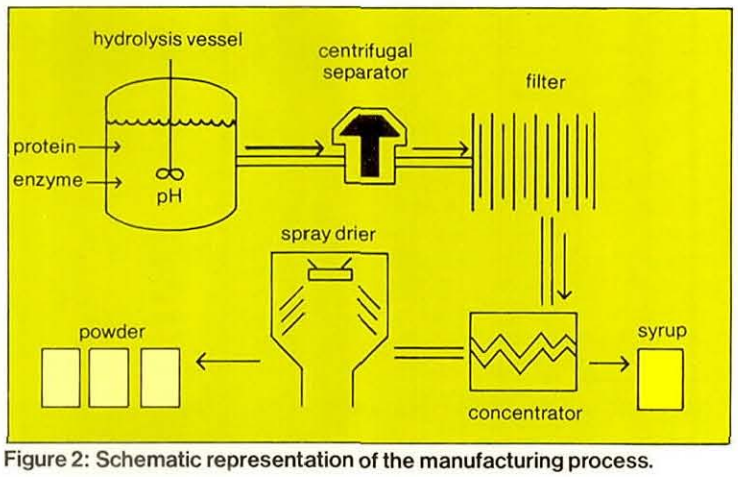

Figure 2: Schematic representation of the manufacturing process.

Amino acids. This analysis is only of limited value. It is the amount and variety of peptides present and not the amino acids that determines the value of peptones in culture media.
Metals. Sodium, potassium and calcium concentrations reflect processing characteristics. Magnesium is very important, as are a host of other trace elements. However, their prevalence in hydrolysates varies.
It is evident that protein hydrolysates can be used in many biological applications. It is not possible, therefore, for the manufacturer to carry out tests which can anticipate all the final uses. Indeed, commercial secrecy often prevents details of the applications being revealed and pre-shipment tests are usually carried out by the end-user.

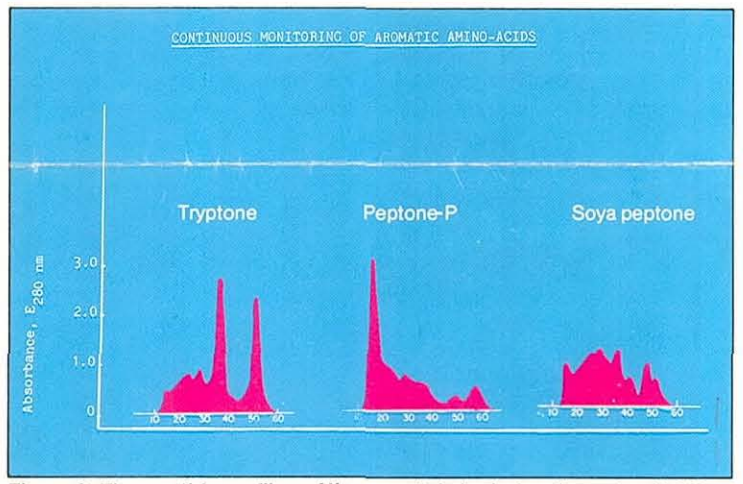

Figure 3: The peptide profiles of three protein hydrolysates using HPLC.

The most useful analysis of protein hydrolysates is the construction of a 'peptide profile', i.e. the elution profile of the polypeptides, peptides and free amino acids on a Sephadex column. The concentration of the peptides coming off the column is measured by U.V. light absorption at 280nm. Characteristic peptide profiles for **i.** trypsin, **ii.** pepsin and **iii.** papain digested protein are illustrated in **Figure 3**.

Fermentation uses for protein hydrolysates

Fermentation covers any microbial process in which the by-product is harvested rather than the organism, e.g. antibiotics, organic acids, vitamins, alcohol. These are very large scale processes and the formulation of the media can be divided into three cost-sensitive parts.

1. Lowest cost materials – dextrose/ammonium salts solution.
2. Fermentation adjuncts – corn steep liquor, fish solubles, molasses, cottonseed flour, soya flour or other plant by-products.
3. Growth factor supplements – protein hydrolysates, yeast or malt extracts.

The last group may be the most expensive but it could influence the yield of by-products to such an extent that the extra cost is amply covered. Even ammonium salts are now considered too expensive and liquid ammonia is added to the fermentation brew to provide nitrogen and to act as a pH buffer. Vaccine manufacture will have different requirements depending on the organism to be harvested. Anaerobic organisms are the most demanding of growth factors in the medium. Maximum biomass may not be the best criterion to follow as the immunity factor could be more important. In many cases a pre-purchase trial is essential.
The biotechnology of genetic engineering is producing the need to grow large volumes of DNA-altered organisms which can yield high value products such as interferon, insulin and growth hormones. These users are more concerned about the quality of the end product than about the cheapest cost: the high value of the products requires them to seek the best and most reproducible hydrolysates. This type of biotechnology is growing and will become a very significant user of protein hydrolysates.

Conclusion

Although protein hydrolysates (*peptones*) *are most commonly* associated with culture media, there are many important biotechnological applications elsewhere. Oxoid, as a primary producer of protein hydrolysates, is as concerned with these important markets as it is with culture media.

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