AN INVESTIGATION OF THE TERTIARY STRUCTURE OF BOVINE PLASMA ALBUMIN.

by

LAURENCE J. McINTYRE

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Department of Biochemistry,
University of Glasgow.

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CONTAINS

PULLOUTS

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ABBREVIATIONS.

BPA Bovine Plasma Albumin.

NET 0.1 M N-ethylmorpholine titrated to pH 8.0 with acetic acid.

All other abbreviations used are listed in "Policy of the Journal and Instructions to Authors" of the Biochemical Journal.

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PROLOGUE

I never saw a Purple Cow
  I never hope to see one;
But I can tell you, anyhow,
  I'd rather see than be one.

Frank Gelett Burgess

1. INTRODUCTION

1.1. THE TERTIARY STRUCTURE OF PROTEINS.

"A protein molecule may be compared to an animal in having a three-dimensional anatomy laid out to a definite plan, rigid in some parts and flexible in others, with perhaps some minor variations in different individuals of the same species. The nature of this anatomy constitutes the central problem of protein chemistry (Perutz, 1962). The basis of this thesis is an attempt to dissect the anatomy of one protein, bovine plasma albumin (BPA).

The tertiary structure of a protein is the manner in which the peptide chain is folded upon itself to produce a more ordered molecule. An understanding of this aspect of protein chemistry is essential in order to explain why a protein can perform a given function. Tertiary structure is maintained by secondary forces which are generally considered as electrostatic and hydrophobic interactions, hydrogen bonding, van der Waals forces and disulphide bridges.

A large amount of evidence has been accumulated (Anfinsen, 1972) which indicates that the tertiary structure of a protein is determined by the nature and order of amino acids in its peptide chain. As a result of this, one possible method, in the future, of determining the tertiary structure of a protein will probably be the prediction of the shape of a protein by analysis of its primary structure. This method is, however, still at a very early stage of development and will suffer for some time from insufficient data on which to base its models (Robson and Pain, 1971). At the present time, therefore, other methods have to be employed in the study of the anatomy of proteins.

The method which gives the most direct evidence of the structure of a protein is x-ray crystallography. To date nearly thirty

proteins have had their structures solved to near-atomic resolution using this technique and many more are under study.

1.2. X-RAY CRYSTALLOGRAPHY.

The basis of this technique is that when x-rays are shone through a crystal, diffraction occurs and a three-dimensional lattice of reflections is obtained. If the size of the lattice and the intensity and phases of the reflections are known, then the shape of the protein can be calculated. In the case of proteins, in order to measure the phases of the reflections it is usually necessary to form crystals in which a heavy atom is bound to the molecule. Comparison of the diffraction pattern from this type of crystal with the normal pattern permits calculation of the phases of the reflections.

The major limitation of x-ray crystallography is in obtaining well-ordered suitably-sized crystals, for, as yet, little is known about the factors governing the formation of protein crystals. The degree of order of the crystal is very important as it is this, more than anything else, which limits the resolution of the final electron density map.

Another problem of this technique is that the conformation of proteins in crystals might be different from that in solution. Two lines of evidence have dispelled this concern : (1) the fact that related proteins have similar shapes although they have been crystallised in very different conditions. One example of this is the similarity of ribonuclease A, crystallised from ammonium sulphate solution (Wyckoff et al., 1967), and ribonuclease A, crystallised from a 55% alcohol solution (Kartha et al., 1967). Several other groups of proteins confirm this observation (Perutz, 1969). (2) Many enzymes still combine with substrates and inhibitors when crystallized (Doscher and Richards, 1963; Sigler et al, 1966) albeit at a reduced

rate, in most cases.

A further problem of x-ray crystallography, at present, is that, as larger proteins are studied, it is becoming difficult, in some cases, to handle the large amounts of data involved. However, even at a low level of resolution this technique can produce the overall shape of a protein, the location of binding sites, the molecular weight and the molecular symmetry.

Although x-ray crystallography is the best method of obtaining direct evidence of protein structure, it provides little information about the involvement of the tertiary structure in the function of the molecule as the system being studied is not a dynamic one. In order to overcome this problem it is necessary to resort to less direct methods of structural analysis which can be applied to a protein in a more physiological situation. Such methods can be divided, to some extent, into physical and chemical techniques. Most of the physical techniques are based on the absorbance of energy by different groups of the molecule. The nature of this absorbance reflects the properties of the groups under study.

In the following section several relevant physical methods are described. The first of these are those based on the absorbance of circularly polarised light by proteins.

1.3. PHYSICAL INVESTIGATION OF PROTEIN STRUCTURE.

1.3.1. Circular Dichroism and Optical Rotatory Dispersion.

Optical activity of proteins may be examined in two different ways. The first is optical rotatory dispersion which measures the dependence of optical rotation on wavelength and the second is circular dichroism which measures the difference between the absorption of left and right circularly polarised light. Circular dichroism and optical rotatory dispersion are different manifestations of the same phenomenon and are related mathematically (Moscowitz, 1962). In most cases circular dichroism is the preferred method as it yields discrete spectral bands unlike optical rotatory dispersion which gives rise to bands of infinite width.

In studying the circular dichroism of protein, information about the tertiary structure is principally obtained from the bands corresponding to the amino acid side chains. These may reflect features of both the secondary and tertiary structures. These bands occur in the spectral region below 330 nm. Below 250 nm the side-chain bands are more difficult to study, because of the presence of bands due to the peptide backbone. The main bands which are present are due to tryptophan, tyrosine, phenylalanine and histidine residues as well as disulphide bridges. The size of a band and its position will depend on the conformation of the peptide backbone, the environment of the side-chain and the ionization of the residue in the case of tyrosine and histidine. As a result the side-chain circular dichroic spectra of protein are usually very complex (Beychok *et al*, 1966) because of the overlap of closely positioned bands. At present insufficient information is available from model

systems, and other sources, to permit a detailed analysis of such spectra, but once the data are available it is hoped that the complexity of the circular dichroic spectra of proteins will provide accurate results concerning their tertiary structure. Circular dichroism is, however, currently being used to study, in detail, changes in tertiary structure which have been characterised using other methods (Hess, 1969). One example of this is the way in which the change in the circular dichroic spectrum of chymotrypsinogen, caused by its activation, can be analysed on the basis of x-ray data and related to the movement of specific residues (McCann et al., 1969).

A physical method which has required less study than circular dichroism for its results to be capable of detailed analysis and which is as a result, a more advanced and probably more productive method of investigating the tertiary structure of proteins is fluorescence spectroscopy.

1.3.2. Fluorescence.

The basis of fluorescence is that certain molecules absorb energy from incident light and move from a ground state, which is the lowest energy state of a molecule, to an excited state. Excitation occurs from the lowest vibrational level of the ground state to the various vibrational levels of the excited state. In solution the excess vibrational energy is lost by thermal exchange with the solvent so that within about 10^{-12}s the molecule goes to the lowest vibrational level of the excited state, from which emission of fluorescence occurs as the molecule returns to one of the vibrational levels of the ground state. In moving to the

lowest vibrational energy of the ground state further energy is lost. Since vibrational energy is lost in the excited state, the energy drop on emission of fluorescence will always be less than or equal to the energy absorbed in excitation. As a result the wavelength of fluorescence is normally longer than that of excitation. Only one fluorescence band is observed as fluorescence always occurs from one energy level.

Three types of fluorescent groups are used in protein studies. Firstly there are the three fluorescent amino acids which occur naturally in proteins: tryptophan, tyrosine and phenylalanine. Secondly there are certain enzyme cofactors which will act as fluorescent chromophores. The third type of fluorescent molecule used is one which can be artificially attached to the protein. The advantage of the last group is that the area of the protein to be investigated can be selected more precisely.

Fluorescent probes can be used to measure the polarity of their particular location as this affects the position of the emission spectral band (Stryer, 1965). A further application of fluorescence is in measuring the distance between groups within a protein, as the proximity of two groups can be related to the efficiency with which energy is transferred between their excited states (Stryer & Haugland, 1967). A final method in which fluorescence can be applied to an investigation of the tertiary structure of proteins is in measuring the decay of fluorescence polarization. In this case the protein solution is subjected to polarized radiation. This causes the fluorescent chromophores which are parallel to the direction of polarization to be preferentially excited. The intensities of fluorescence parallel and perpendicular to the plane of the exciting

irradiation are measured as a function of time. From this, information on the rigidity of the protein and its molecular shape can be obtained. It can be seen, therefore, that fluorescence techniques can be employed as very useful probes of protein structure.

The next two methods to be considered are at present still very much at the developmental stage and sufficient data have not been gathered to permit them to be used to a large extent in the study of the tertiary structure of proteins. They do, however, possess great potential for development as sensitive techniques.

1.3.3. Nuclear Magnetic Resonance (N.M.R.) and Electron Paramagnetic Resonance (E.P.R.)

N.M.R. is a very sensitive method of investigating the structure of proteins in solution. It is a form of spectroscopy based on the absorbance of radio frequency electromagnetic radiation by atomic nuclei, having nuclear spin (I) greater than zero, when placed in a strong magnetic field. The absorption occurs when the nuclei move from a lower to a higher energy level.

An N.M.R. spectrum can provide the following information about the species of atom being studied :- (1) the chemical bonding of the atom, (2) the number of identical nuclei in neighbouring groups, (3) the number of nuclei in a given chemical configuration and (4) the mobility of the nuclei and their exchange between different chemical environments.

By far the most commonly studied nucleus in this field is the hydrogen nucleus ($I = \frac{1}{2}$). A high protein concentration is required for good sensitivity in this case. Another problem is that, as the size of the protein increases (above 20,000 MW.)

its mobility in the solution decreases leading to loss of resolution of the N.M.R. spectrum. Even when studying smaller proteins the spectrum obtained is very complex, but sufficient data are now becoming available to permit analysis of the results (Metcalfe, 1970). Several methods exist of simplifying the N.M.R. study of proteins : (1) by the use of proteins in which all but a few amino acids are deuterated. The observed resonances will then be due to the undeuterated residues (Markley et al., 1968). (2) The changes in the N.M.R. spectrum of a small molecule when it is bound to a protein can also be studied (Raferty et al., 1969). (3) As about 30% of enzymes require a metal for their action, the spectrum of the metal ion in the protein, where possible, is another source of information (Mildvan and Cohn, 1970). One good example of the application of N.M.R. to the study of protein structure is in the investigation of the binding sites for sugar molecules on lysozyme (Raftery et al., 1968).

E.P.R. is the absorbance of microwave frequency electromagnetic radiation by unpaired electrons when placed in a strong magnetic field. In this case the absorption is also due to a movement between energy levels. The spectrum obtained will give much more localised information than that of N.M.R. as atoms containing free electrons are not spread throughout a protein. The use of E.P.R. in structural studies on proteins is confined to two areas: (1) metal atoms containing an unpaired electron (Malmstrom et al., 1968) and (2) the study of spectral changes occurring when a small molecule containing an unpaired electron is attached to a protein (McConnell, 1971). Although magnetic resonance techniques are still being developed they have the potential to supply a large amount of detailed

information about the tertiary structure of proteins.

Another method of spectroscopy used in the study of protein structure is a technique which utilizes one of the better-known types of absorbance found in proteins - ultraviolet absorbance.

1.3.4. Ultraviolet difference spectroscopy.

One way of overcoming the complexity of the ultraviolet absorbance spectra of proteins is to measure the difference in absorbance of identical concentrations of a protein in different environments. In this way all common features of the spectra are cancelled out and only those chromophores whose absorption has been changed by the alteration or perturbation of the environment will be evident as positive or negative bands. The most extensively studied residues in this type of experiment are tryptophans and tyrosines.

A sophisticated application of this technique has been developed by Laskowski (1966) and is known as solvent perturbation spectroscopy. In this, the protein environment is perturbed by addition of a non-aqueous component. Only chromophores present on the surface of the protein will have altered absorbances while those in the hydrophobic interior will be unchanged. If the size of the perturbant is altered the accessibility of chromophores in crevices in the protein can be investigated.

At present the topography of a number of proteins has been studied using this technique and it has been shown that although tryptophans and tyrosines are normally hydrophobic at least some of them are generally exposed to the solvent (Herskovits and Sorensen, 1968).

1.3.5. Other Techniques.

There are physical techniques available to the protein chemist other than those mentioned above for the investigation of the tertiary structure of proteins. These do not give detailed information but rather give an idea of the general shape of the protein, whether it is rod-like, spherical, ellipsoid etc. The most common methods of providing this type of data are light-scattering, viscosity, ultracentrifugation and electron microscopy.

The physical methods discussed so far can provide quite detailed information about the general structure and, in some cases, the relationship between structure and function of a protein. However, the methods described in the following section, the chemical techniques of investigating protein structure, can often provide more specific information about the tertiary structure of proteins, especially the active site of an enzyme.

1.4. CHEMICAL INVESTIGATION OF TERTIARY STRUCTURE.

1.4.1. Chemical Modification.

One useful application of chemical methods in the investigation of protein structure is in intramolecular crosslinking. By using a bifunctional reagent to react with two residues on the protein's surface, groups which are close neighbours can be identified and the distance between such groups can be calculated, in some cases, by breaking down the modified protein and studying the peptides obtained (Wald, 1967).

Some more localised information about the structure of proteins can be obtained by chemically altering the active site of an enzyme. Consideration of the effect of the modification on the activity of the enzyme leads to a great deal of data about the groups involved in, and the structure around the active site of an enzyme. A more sophisticated application of this method is affinity labelling (Wofsy et al., 1962) in which the modifying reagent is structurally similar to the enzyme substrate and, as a result, generally produces a modification only at the active site. This method was used in a study of the protease chymotrypsin in which the affinity label L - (1 - tosylamido - 2 - phenyl) ethyl chloromethyl ketone reacted only with His 57 of the enzyme, implying that this residue was part of the active site (Ong et al., 1965).

The above two techniques are both very specialised applications of one of the main chemical methods of investigating the shape of proteins, chemical modification.

The first aspect which must be considered in a study of the chemical modification of proteins is the factors which affect the

reactivity of the functional groups on the protein, and of the reagent used:

1) The polarity of the localised area of the reaction.

Since the reactivity of a functional group is measured by its nucleophilicity, which is often related to its basicity, the pK of the group is an important factor. However, it is difficult to estimate the local polarity of a functional group from its pK as this is also affected by other factors.

2) The proximity of charged species.

These can alter the pK of the functional group and can also attract or repel the modifying reagent (French et al., 1963)

3) The steric effect.

This can be of two types, either hindrance of the reaction due to the presence of a bulky residue near the functional group on the protein surface (Cohen and Jones, 1963) or inaccessibility of the functional group due to being buried in the hydrophobic interior of the protein.

4) Preferential adsorption.

The rate of chemical modification can be altered if the modifying reagent is preferentially adsorbed to a particular region of the protein (Fonda and Anderson, 1969).

There are other factors which could influence the reactivity of functional groups in proteins but little, as yet, is known about them. These include charge transfer, covalent bond formation, metal chelation

and freedom of rotation of the functional group.

The factors listed above illustrate that the reactivity of a functional group is very dependent on its environment. If the individual reactivities of a given type of functional group are studied using chemical modification, each group will provide some data about the structure of its own particular location. As a result, information can be deduced about the general tertiary structure of the protein.

Any deductions that are made must be viewed with the knowledge that there are several limitations inherent in the technique of chemical modification. One such limitation is the fact that few chemical modifiers are totally specific. Another problem is that all modifications will cause some change in the protein structure. Furthermore, it is also difficult to assume that the effect of a modifying compound will not vary from one protein to another.

Once a protein has undergone chemical modification, the extent of the reaction is often measured, after isolation of the protein, by methods such as isotopic label or absorbance of the modifying species. Identification of the modified residues usually requires degradation of the protein and amino acid analysis. For this reason, modifications which give rise to unstable products can be unsuitable. It is also difficult to produce a stable modification which will emerge as a discrete peak on amino acid analysis.

1.4.2. Results of Chemical Modification.

As long as the limitations of chemical modification mentioned above are acknowledged and the results obtained are, therefore, treated with due caution, a large amount of data can be collected

concerning the degree of exposure of reactive groups in proteins.

One way in which this problem can be studied is by reaction of the protein under constant conditions with an excess of reagent for a suitable time and then determination of the degree of modification of a certain type of residue. As the molar ratio of reagent to protein used is increased the number of groups modified will increase to a maximum value and no further, as those groups which are buried in the protein will not react at any concentration of reagent. In this way, the number of such groups in the protein can be calculated.

A second method of obtaining information from chemical modification is to follow the rate of modification with a given reagent. The curve obtained can be resolved into a series of first-order slopes which will provide a rate constant for the modification of, and an idea of the number of different sub-sets in a given type of functional group (Ray and Koshland, 1962). An example of chemical modification is seen in the work of Gounaris and Ottesen (1965) who found that only ten of the eleven lysine residues in subtilisin could be succinylated. X-ray studies of the protein (Wright et al., 1969) show that only ten of the lysine residues lie on the surface of the molecule. This could explain the different reactivities observed.

The classification of methods of investigating protein structure into physical and chemical techniques is often an arbitrary decision. The last method to be described, hydrogen isotope exchange, has been included under chemical techniques because the method of analysis of the results obtained is similar to that of chemical modification.

1.4.3. Hydrogen Isotope Exchange.

This technique of structural analysis is based on the fact that

hydrogen atoms in a protein in solution are continually exchanged with hydrogen atoms in the solvent. Those bound to carbon atoms exchange too slowly to be measured under normal experimental conditions while most of those bound to sulphur, oxygen and nitrogen atoms exchange too quickly to be detected. In fact only amide hydrogens exchange at an intermediate rate and can be observed without interference from other exchanging species. The reason for studying the exchange of amide hydrogens is that because they are linked to the peptide backbone of the protein their exposure to the solvent depends on the conformation of the backbone, and the degree of exposure is reflected in the exchange rates.

The basic method of measuring the rate of exchange of hydrogen atoms in a protein is to permit the protein to exchange its protons with one of the isotopes of hydrogen, either deuterium or tritium. The amount of exchange which has occurred in a given time can then be determined either by direct measurement of the amount of isotope in the protein, using infra-red or N.M.R. techniques, or by separating, from a sample taken at that time, the solvent and protein, and analysing either of them to obtain the amount of isotope in the protein.

One commonly used type of tritium exchange is the technique devised by Englander (1963) in which protein is separated from the tritiated buffer by gel filtration.

Several attempts have been made to analyse hydrogen exchange curves in terms of different classes of exchanging hydrogen atoms (Ottesen, 1971). Unfortunately there is insufficient information in such curves to permit a detailed analysis of the types of peptide backbone present in the protein. The major use of hydrogen exchange is in the detection of changes in the tertiary structure of a protein,

due to some alteration of its environment.

One important fact which emerges from a survey of the techniques for the study of protein structure, both physical and chemical, is that, because each method involves a different principle it is much more rewarding to study a protein using a variety of methods rather than just one. This approach is, in fact, synergistic, for when when the results of one method are viewed in the light of results from a second method, the information which can be deduced is greatly increased.

Having considered the many methods of investigating the tertiary structure of proteins, it would be useful to describe, to some extent, the information available on the properties of the protein under study in this thesis before going on to formulate a way in which its structure may be investigated.

1.5. PLASMA ALBUMIN.

1.5.1. Occuwence and Purification.

Plasma albumin is found in the blood of reptiles and all higher species (Peters, 1970). It is, however, difficult to be rigorous about this as the physical and chemical properties of plasma albumin vary slightly from species to species and since the protein has no defined function, its classification can sometimes prove troublesome.

Albumin is by far the most abundant protein in blood, normal levels in mammals being about 60 - 70 g/l (Engle and Woods, 1960). Because of this high concentration and the ease of isolation of the protein, albumin was one of the first proteins to be studied. This is reflected in the similarity of its name to the word albumen, an early name for proteins. In fact albumin was first studied by Liebig and Mülder in the late 1830's when the term "protein" had just been introduced, and by 1894 a procedure had been drawn up for the crystallisation of horse plasma albumin (Gürber, 1894). From that time, until the present day, albumin has been the subject of more research than almost any other protein.

Albumin is prepared commercially by a variation of the cold-ethanol method devised by Cohn et al., (1950) during World War II. This method involves the fractionation of plasma proteins by precipitation with ethanol at low temperatures. In the course of the fractionation, pH, temperature, ionic strength and ethanol concentration are all varied to produce a good separation. Using this method albumin can be precipitated in about 96% purity.

Albumin produced in this manner can then be used to prepare the

crystalline form of the protein. Crystallization occurs most readily from concentrated solutions at a pH just on the alkaline side of the isoelectric point of the protein.

1.5.2. Metabolism

Although albumin is usually associated with plasma, a slightly higher total weight is, in fact, found in the interstitial fluid of mammals than in the blood and, to some degree, albumin permeates all extracellular fluids and is found in all secretions and excretions (Schultze and Heremans, 1966). Albumin synthesis appears to take place in the liver and amounts to about a third of the protein production of that organ (Schultze and Heremans, 1966). There is some evidence (Peters *et al.*, 1968) that the synthesis of plasma albumin may be regulated by altering the number of liver cells which are actually producing the protein rather than altering the rate of synthesis.

Although the catabolism of circulating albumin is by no means completely resolved, the evidence available suggests that the protein is broken down by nearly all of the tissues of the body as a source of amino acids in addition to those derived from the plasma (Radovich *et al.*, 1963). The rates of synthesis and catabolism are such that about one-twentieth of the total albumin mass is destroyed and replaced daily. Compared with other plasma proteins, it has quite a long half-life but the mass of protein involved is very much larger in the case of albumin. This turnover rate may be significant in a control process but it is difficult to say; just as it is difficult to see why tissues require to break down albumin as another source of amino acids.

The regulation of albumin synthesis and catabolism is affected

by three main factors: (a) the plasma albumin level, (b) dietary protein intake and (c) the action of certain hormones (Peters, 1970). Abnormal levels of albumin in plasma are in many cases closely related to disease or injury (Meindok, 1967).

1.5.3. Physical and Chemical Properties.

BPA is a one-chain globular protein. The generally accepted value for its molecular weight is around 65,000 (Koenig and Perrings, 1952) although estimates have gone as high as 70,000 in some cases. Sedimentation and diffusion data suggest that the molecule is a prolate ellipsoid of revolution with a major axis of 14 nm and a minor one of 4 nm (Squire et al., 1968). The amino acid composition of BPA is shown in Table 1. The hydrogen ion titration curve for the protein measures the ionizable groups on the molecule. From the amino acid analysis it appears that almost all the possible groups are capable of ionizing as the number of titratable groups in BPA (Tanford et al., 1955) are 1 α-carboxyl, 99 β-, γ-carboxyl, 16 imidazole, 1 α-amino, 57 ϵ-amino, 19 phenolic and 22 guanidine residues. One anomalous factor in the hydrogen ion titration is that the β- and γ-carboxyl groups and the ϵ-lysine groups have an abnormally low intrinsic pK while the value for the phenolic groups is higher than expected.

The primary sequence of BPA is, at present, under study. One group of workers has produced what is virtually the complete sequence of the protein but one or two regions remain to be characterised (Brown et al., 1971). King and Spencer (1972) are also in the process of sequencing the peptides they obtain on cleavage of BPA with cyanogen bromide. It appears probable that the complete amino acid sequence of BPA will be resolved in the near future.

TABLE I.

The Amino Acid Composition of BPA.

The amino acid analysis of BPA is taken from the results of King and Spencer (1970). The number of residues of each amino acid per mole of BPA is expressed in terms of the nearest integer and is the average of three determinations. The composition shown here is in close agreement with other literature values (Stein and Moore, 1949; Spahr and Edsall, 1964),

AMINO ACID	RESIDUES PER MOL BPA
Lysine	58
Histidine	17
(Ammonia)	(27)
Arginine	23
Aspartic acid	54
Threonine	32
Serine	26
Glutamic acid	80
Proline	28
Glycine	15
Alanine	44
Half-cystine	35
Valine	35
Methionine	4
Isoleucine	14
Leucine	58
Tyrosine	19
Phenylalanine	26
Tryptophan	2
Total	570

1.5.4. The Sulphydryl Group.

One interesting aspect of the protein BPA is that only about two-thirds of the albumin molecules in a typical preparation have a measurable thiol group (Hughes, 1950). According to the amino acid analysis, if one assumes that the maximum number of disulphide bridges are formed, every molecule should have one free sulphydryl group. The albumin molecules containing a free sulphydryl group were separated from the remaining molecules by Hughes (1947). This was achieved by forming a dimer of the protein containing the thiol group using mercury. After separation, the mercury can be removed by reduction. As a result the albumin containing a free sulphydryl group is known as mercaptalbumin. Jensen (1959) and others have also purified mercaptalbumin by chromatography on DEAE cellulose.

The cause of the variation in the sulphydryl content of BPA was shown by Andersson (1966) to be the fact that in one-third of the molecules the thiol group is masked by disulphide bridge formation with cysteine and, to a lesser extent, glutathione.

1.5.5. Low - pH Behaviour.

Another unusual characteristic of the BPA molecule is its abnormal behaviour at low pH. Evidence from a wide variety of physical studies (Tanford, 1950; Weber, 1952; Yang and Foster, 1954) suggested that the protein molecule underwent a molecular expansion below pH4. Harrington *et al.* (1956) showed that the results could not be explained by dissociation of BPA into smaller molecules at acid pH and that the expansion could be repressed

by increasing ionic strength suggesting that it was electrostatic in origin. Tanford and Buzzell (1956) proposed that the expansion took place through an intermediate form. The accepted model, at present, is that, as the pH is lowered, the native form of BPA (called N) isomerises to an intermediate form (called F) with no change in hydrodynamic volume and that it is the F form which undergoes molecular expansion as the pH is lowered further (Aoki and Foster, 1957). Foster and Clark (1962) concluded that the anomalous titration behaviour of BPA, originally thought to be due to the acid expansion, was, in fact, related to the N→F transition. They also showed that the transition involved the normalization of the abnormally low pKs of up to fifty carboxyl groups in the protein. The cause of the unusual pKs is uncertain but may be connected with a second isomerization of the molecule at alkali pH, for which there is some evidence (Harmsen et al., 1971: Leonard et al., 1962).

1.5.6. Heterogeneity.

Another aspect of BPA which has been studied in great detail is the heterogeneity of the protein. Apart from the more obvious types of heterogeneity of BPA which are the presence of a sulphydryl group (1.5.4.), polymerisation (Andersson, 1966) and the N and F forms (1.5.5.), there exist slight differences in albumin molecules which are much more difficult to detect. This has been called the micro-heterogeneity (Colwin et al., 1954) of BPA. The phenomenon can be detected by chromatography on DEAE cellulose (Noel and Hunter, 1972), solubility studies (Foster et al., 1965) and equilibrium salting out (Wong and Foster, 1969) as well as several other sensitive physical

techniques. Although the majority of the microheterogeneity appears to reside in the non-mercaptalbumin component (Kaplan and Foster, 1971), Hagenmaier and Foster (1971) showed that microheterogeneity can be demonstrated in mercaptalbumin using solubility studies and reversible boundary spreading.

There are several possible explanations for this observed microheterogeneity: (1) anions bound to the BPA. This is becoming less likely as purification techniques for the protein are improved. (2) Alteration of the protein side-chains e.g. acetylation deamidation or amidation (Spencer and King, 1971). (3) Intramolecular disulphide interchange. There is a larger amount of evidence for this possibility (Andersson, 1966). It is suggested that the free sulphydryl group (1.5.4.) catalyses the interchange. (4) Differences in the primary structure of the protein. At present there are no data to support this suggestion.

It has been shown that the microheterogeneity is not due to differences between individuals (Spencer and King, 1971) and the most probable explanation is a combination of the factors listed above.

1.5.7. Ion-binding.

BPA has the ability to bind a wider range of compounds than probably any other protein. Some examples of this property are listed in Table 2. The most significant of these interactions are : (1) the binding of copper ions, for which the initial binding site is well characterised (Peters, 1970), (2) the binding of bilirubin, (3) the binding of fatty acids and (4) the binding of L - tryptophan. It is also interesting to note that, in the case of human albumin, acetyl

TABLE 2.

Substances which Bind to HPA.

The list of substances which will bind to HPA is taken from an article by Putnam (1965). The list does not claim to be complete. It is merely representative of the diversity of such compounds.

Ca^{++}, Cu^{++}, Zn^{++}

Bilirubin

Uric Acid

Vitamin C

Acetylcholine

Cholinesterase

Adenosine

Aureomycin

Barbiturates

Chloromycetin

Digitonin

Fatty Acids

Suramin

Atebrine

Erythrocytes

Neosalvarsan

Penicillin

Salicylate

Sulphonamides

Streptomycin

Histamine

Triiodothyronine

Thyroxine

Tryptophan

salicylate (aspirin) not only binds to the protein but also acetylates a specific lysine residue (Hawkins et al, 1969).

1.5.8. Function.

The function of BPA has not yet been clarified but it seems probable that the protein does, in fact, have several functions. Plasma albumin is not essential for life as some rare individuals can survive with, apparently, no such protein in their blood (Watson, 1965). Despite this the protein does appear to have several physiological functions: (1) it accounts for about 80% of the colloid osmotic pressure in blood and also contributes largely to the Donnan effect of blood due to its high negative charge, (2) BPA has an important transport function in blood due to its wide capacity for binding small molecules. (1.5.7.), (3) BPA is also an important source of amino acids as it is broken down in large amounts throughout the body (1.5.2.). These are the main properties attributed to BPA but further functions for this protein may yet be discovered.

1.6. THE TERTIARY STRUCTURE OF BPA.

The one aspect of BPA which has not been discussed so far is the tertiary structure of the protein. A study of this problem forms the basis of this thesis.

Although the binding sites of several of the small molecules which interact with BPA have been characterised to some extent (Peters, 1970; Noel and Hunter, 1972), the only information about the general tertiary structure of the protein has come from two main sources; (1) the formulation of models for the protein based on physical measurements, and (2) the cleavage of the intact protein using mild conditions.

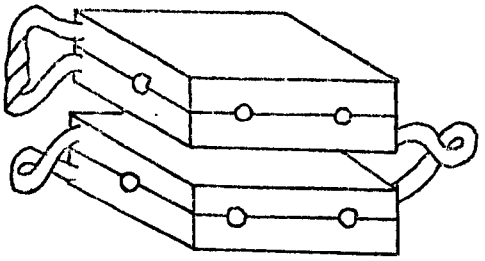
One of the first models proposed for the structure of BPA is that of Foster (1960) based on electrophoretic, titration and detergent binding measurements (Fig. 1). The model consists of four "pseudosub-units" or globular regions linked by lengths of the peptide chain. In the F form of the BPA (1.5.5.) the hydrophobic surfaces are exposed. The holes around the edge of the model represent the 10 or 12 strong detergent binding sites which are destroyed in the F form. Although some of the observations on which the model was based have since been withdrawn (Foster et al., 1965) there are still sufficient data to support a model of two or more pseudosub-units (Adkins and Foster, 1965). A second model obtained from physical studies is that of Bloomfield (1966) which is based on hydrodynamic and low-angle x-ray scattering data (Fig. 2). This model consists of three spherical regions with the dimensions shown (Fig. 2).

The type of model which can be obtained by studying the products obtained on cleavage of the BPA by various methods is shown in Fig. 3.

FIGURE 1.

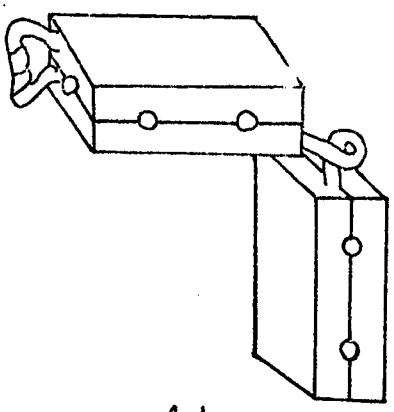
The Tertiary Structure of BPA (I).

This model for the tertiary structure of BPA was proposed by Foster (1960). F' represents an intermediate form which can be detected electrophoretically.



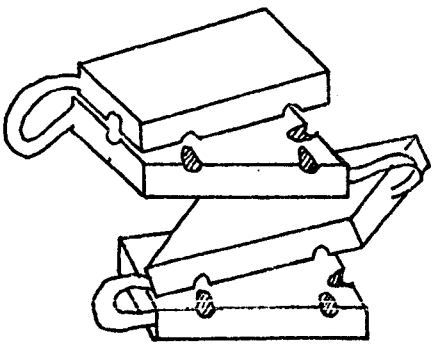
N

H^+ OH^-



F'

H^+ OH^-



F

FIGURE 2.

The Tertiary Structure of BPA (II).

Bloomfield (1966) based the calculations for this model for the tertiary structure of BPA on hydrodynamic and low-angle x-ray scattering data. The dimensions of the pseudosub-units are shown.

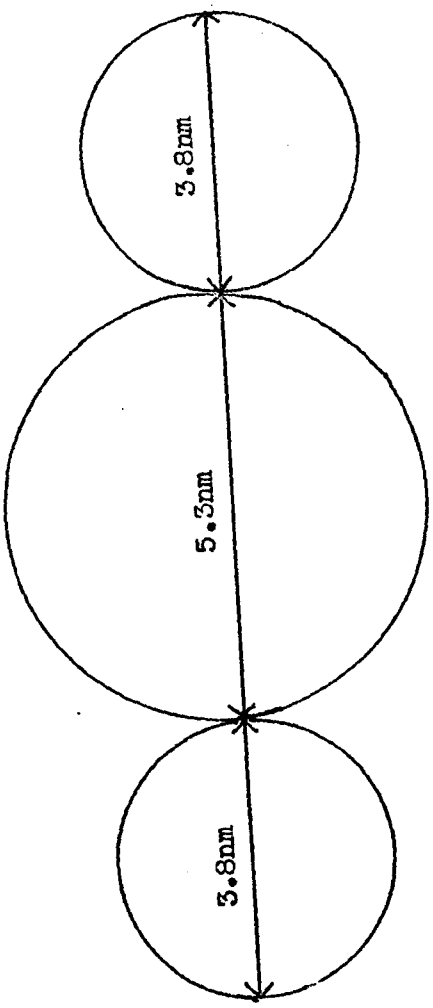
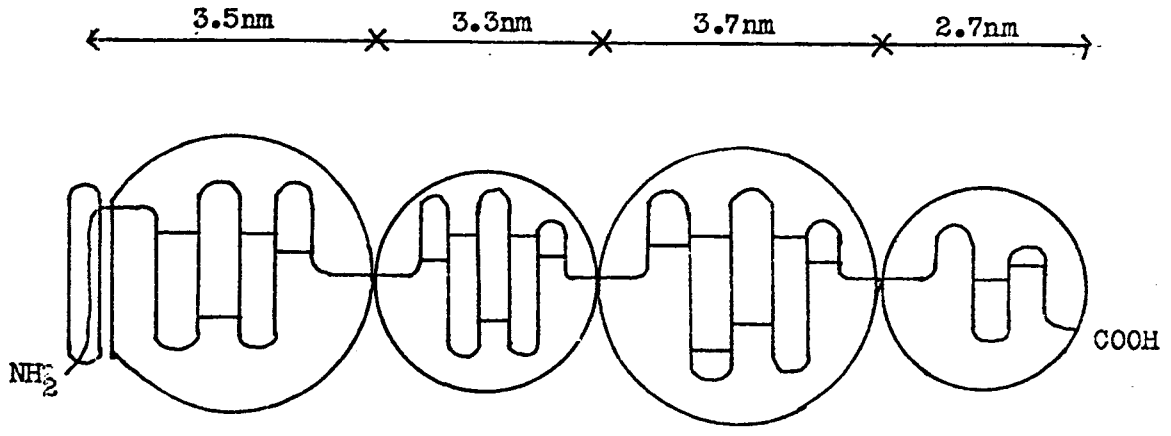

3.8nm
5.3nm
3.8nm

FIGURE 3.

The Tertiary Structure of BPA (III).

This model for the tertiary structure of BPA is taken from the work of Peters (1970). The dimensions of the proposed pseudosubunits are shown above the diagram and the number of amino acids in each is shown below.


3.5nm
3.3nm
3.7nm
2.7nm
NH2
COOH
172
137
190
76

This model again has a pseudosub-unit structure and is based on the results observed by several workers (Peters, 1970).

In order to try and obtain further information about the general tertiary structure of BPA it was decided to investigate the reactivity of certain functional groups on the protein using the method of chemical modification (1.4.). It was further hoped that the methodology developed in this way would be applicable to the study of the structure of other proteins.

The theory put forward is that, allowing the limitations of chemical modification mentioned earlier (1.4.), if a group in a protein is chemically modified by a reagent which is unable to penetrate the interior of the protein then such a group will lie on the outside of the protein. If the modified residue can then be located in the primary sequence of the protein an idea can be obtained of the tertiary structure of the molecule from the distribution of modified groups, if a reasonable number are present. Further information can be obtained about the binding sites of various molecules on the protein by observing whether their presence alters the distribution of modified groups or not.

It was decided to use the reagent Dns-Cl in this investigation for several reasons: (1) Dns-Cl reacts with the ϵ-lysine amino group, the o-tyrosine hydroxyl group and the imidazole group of histidine in proteins. It will also react with the α-amino group of the protein and thiol groups if present (Gray, 1967). The reactivity of Dns-Cl, is, therefore, reasonably selective but it will modify sufficient groups to provide useful information about the relative reactivities of the functional residues, (2) the reaction of Dns-Cl with amino acids has already been well characterised (Gray, 1967; Gros and Labouesse, 1969), (3) peptides containing modified residues can be easily identified by

their fluorescence or absorbance at 330 nm, (4) the fluorescence of the Dns group can be used to quantitate the amount of modified amino acid present, (5) the modified residues are easily identified (Woods and Wang, 1967) and (6) the Dns group appears unlikely to penetrate the interior of the protein for it has been shown that if this happens it involves a large, easily detected change in the structure of the protein (Okabe and Takagi, 1971). The reasons outlined above suggested that Dns-Cl would be a suitable reagent for the investigation of the tertiary structure of BPA by the technique of chemical modification.

The work which follows falls into three main areas, all of which have to be investigated before the information obtained can be used to form an impression of the tertiary structure of BPA. Firstly the reaction between Dns-Cl and the protein has to be studied in order to determine the number of residues modified and the effect of experimental conditions on that number. Secondly it must be shown that extensive modification of the protein does not alter the structure of the protein to a significant extent. Finally the modified residues must be located in the primary sequence of the protein. Once these problems have been solved, the data obtained can then be used in an attempt to probe the tertiary structure of BPA.

2. MATERIALS AND METHODS

2.1. MATERIALS.

2.1.1. Reagents.

All reagents used were Analar grade unless otherwise stated. In all experiments distilled, deionised water was used. This was obtained by passing distilled water directly through an Elgastat deioniser (Elga Products, Ltd., Railway Place, London, S.W.19, U.K.). Dns-Cl and standard Dns-amino acids were purchased from B.D.H. Chemicals, Ltd., Poole, Dorset, BH12 4NN, U.K. This company also supplied the Aristar grade acetone which was used in all experiments. Analar pyridine was redistilled over ninhydrin before use. N-ethyl morpholine was also redistilled prior to use.

2.1.2. Dialysis and Filtration Apparatus.

Dialysis was carried out in Visking tubing obtained from the Scientific Instrument Centre Ltd., 1 Leeke St., London, W.C.1, U.K. The tubing was boiled before use for $\frac{1}{2}$ h in 0.05 NaOH containing 2.5 g/l EDTA to remove impurities. The tubing was then thoroughly washed and stored in distilled, deionised water. Protein solutions were concentrated in a Diaflo ultrafiltration apparatus using a PM10 membrane (Amicon Ltd., 57 Queens Rd., High Wycombe, Bucks., U.K.).

2.1.3. Chromatographic media.

Sephadex G10 and G50 fine were obtained from Pharmacia Fine Chemicals AB, Upsalla, Sweden. Whatman No. 1 Chromatography paper (46 cm x 57 cm) was purchased from W. & R. Balston Ltd., Maidstone, Kent, U.K. Analytical grade anion exchange resin, AG2 - x8, 100-200 mesh, was supplied in the chloride form by Bio Rad

Laboratories, Richmond, California, U.S.A. B.D.H. Chemicals Ltd., Poole, Dorset, BH12 4NN, U.K., supplied polyamide layers. All resins and gels were prepared for use in accordance with the manufacturers' instructions.

2.1.4. Proteins.

The proteins used, and the companies which provided them are listed below:

Protein	Batch No.	Supplier
Crystallised BPA	SLI370,TE0370	Armour Pharmaceutical Co. Ltd., Eastbourne, U.K.
TPCK-Trypsin	91C	Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.
α- Chymotrypsin	CD1 8LK	as above
Pepsin, crystallized	23	Pentex Biochemicals, Kankakee, Illinois 60901, U.S.A.
Thermolysin, 3x crystallised	200077	Calbiochem, San Diego Calif. 92112, U.S.A.
Bacitracin		as above
Insulin	crystalline	Sigma London Chemical Co.Ltd., Norbiton Station Yard, Kingston-upon-Thames, Surrey, KT2 7BH, U.K.
Ribonuclease A	Type X-A	as above

2.1.5. Radiochemicals.

Tritiated Dns-Cl (7.0Ci/mmol) and tritiated water (0.36 mCi/m mole) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

2.1.6. Instrumentation.

Absorption spectra were measured using a Cary 15 recording spectrophotometer (Cary Instruments, 2724 South Peck Rd., Monrovia, Calif. 91016, U.S.A.) or a Pye Unicam SP8000 ultraviolet recording spectrophotometer (Pye Unicam Ltd., Cambridge, U.K.). Other absorption measurements were carried out on a Beckman DB spectrophotometer (Beckman Instruments Ltd., Glenrothes, Fife, U.K.). Circular dichroism was measured using a Cary 60 spectropolarimeter (Cary Instruments, 2724 South Peck Rd., Monrovia, Calif. 91016, U.S.A.). Fluorescence studies were carried out on a Hitachi Perkin-Elmer MPF - 2A fluorescence spectrophotometer (Perkin-Elmer, Norwalk, Connecticut, U.S.A.). All pH measurements were obtained using a Pye Unicam model 290 pH meter (Pye Unicam Ltd., Cambridge, U.K.). Electrophoresis was carried out using a 10KV cooled plate electrophoresis unit (Miles Hivolt Ltd., Shoreham, Sussex, U.K.) Fluorescent derivatives were visualised, on paper, using ultraviolet irradiation produced by a Chromatolite portable ultraviolet lamp (Hanovia Lamps, Slough, U.K.). All scintillation counting was carried out on an Isocap 300 liquid scintillation system (Nuclear-Chicago Corporation, 333 East Howard Ave., Des Plaines, Illinois 60018, U.S.A.).

2.2. METHODS

2.2.1. Preparation of Dns - BPA.

The chemical modification of BPA using Dns-Cl was performed using the method of Weber (1953). The essential features of this method are outlined in Fig. 4. After preparation, the protein conjugate was stored at - 10°C in the lyophilised form or in frozen solution.

2.2.2. Determination of the Molar Ratio of Dns Groups to BPA.

Three methods of determining the number of Dns groups bound to each molecule of BPA were tested in order to find a suitable one. The techniques used are listed in Table 3. All the methods are based on the direct determination of the relative concentrations of Dns groups and of BPA in a given sample.

The first method was based on the observation by Lagunoff and Ottolenghi (1966) that ratio of the absorbance of the Dns group at its maximum (310 - 330 nm) to that at 280 nm was close to 2. This can be seen from the absorbance spectrum of the Dns group (Fig. 5). In this case the absorbance maximum is about 315 nm but on binding of the reagent to protein the maximum shifts to 330 nm. From this observation the following expression was deduced for the number of Dns groups bound per molecule of BPA.

$$\frac{E_{330nm}}{E_{280nm} - \frac{1}{2} E_{330nm}} \times \frac{\varepsilon_B}{\varepsilon_D}$$

where E is the absorbance at the wavelength indicated by the subindices,

FIGURE 4.

Preparation of Dns - BPA.

Dns-BPA was prepared according to the method of Weber (1953) with some modification. The method illustrated used a weight of Dns-Cl which gave the maximum degree of labelling.

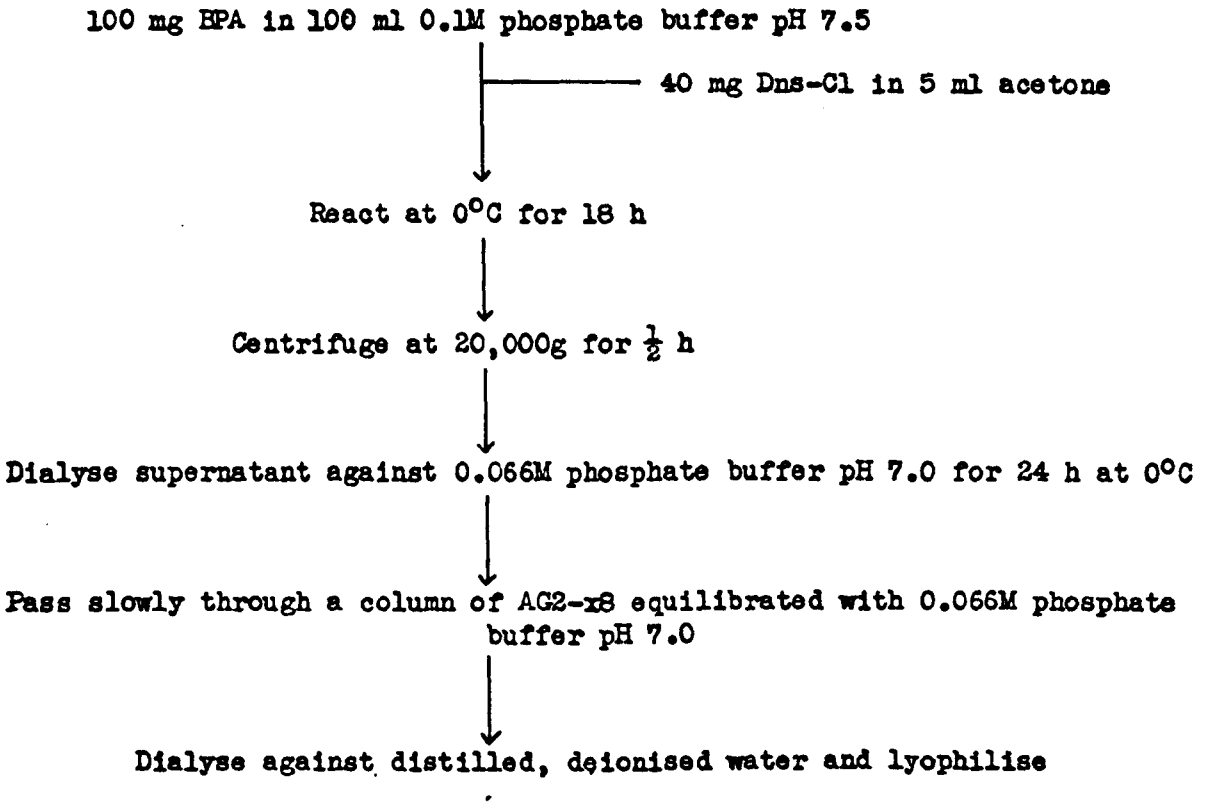

100 mg BPA in 100 ml 0.1M phosphate buffer pH 7.5
40 mg Dns-Cl in 5 ml acetone
React at 0°C for 18 h
Centrifuge at 20,000g for ½ h
Dialyse supernatant against 0.066M phosphate buffer pH 7.0 for 24 h at 0°C
Pass slowly through a column of AG2-x8 equilibrated with 0.066M phosphate buffer pH 7.0
Dialyse against distilled, deionised water and lyophilise

TABLE 3.

Assays for the Molar Ratio of Dns Groups to BPA.

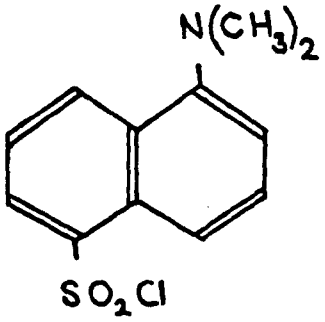
Several different techniques were examined in order to produce an accurate assay for the molar ratio of Dns groups to BPA in the modified protein.

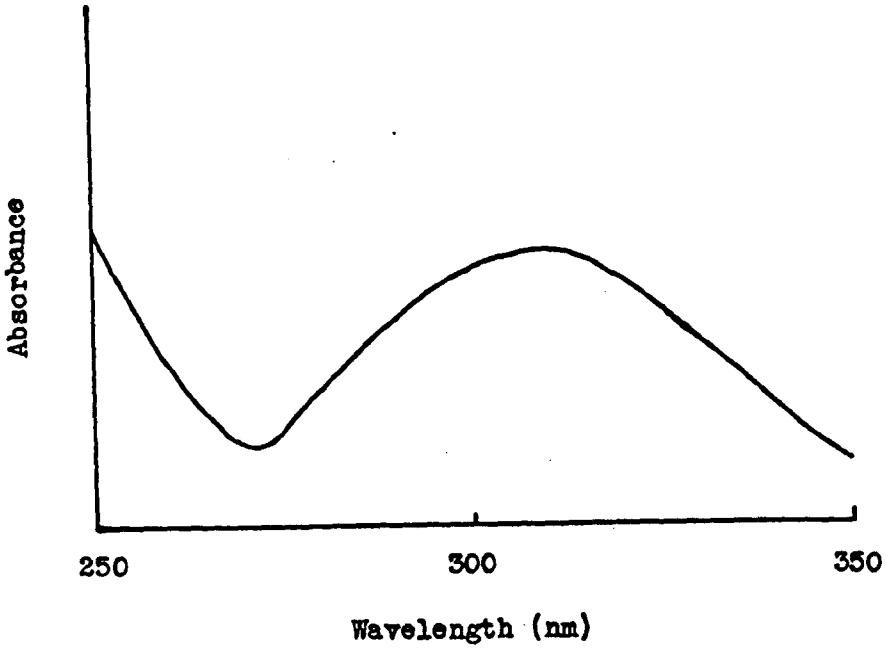
MEASUREMENT OF CONCN. OF DNS GROUPS	MEASUREMENT OF CONCN. OF BPA
1. E_{330}	E_{280}
2. E_{330}	Biuret
3. Tritiated Dns - Cl	Biuret

FIGURE 5.

The Absorbance Spectrum of the Dns Group.

The spectrum is taken from the results of Laguanoff and Ottolenghi (1966). The structure of the reagent Dns-Cl is shown above the diagram.


N(CH3)2
SO2Cl


Absorbance
250
300
350
Wavelength (nm)

ε_B is the molar extinction coefficient of BPA at 280 nm and ε_D is the molar extinction coefficient of the Dns group at 330 nm (Okabe and Takagi, 1971). In this way, the protein concentration was measured by the absorbance at 280 nm, corrected for the contribution of the Dns group, and the concentration of the latter species was measured by absorbance at 330 nm where there is no interference from the protein absorbance.

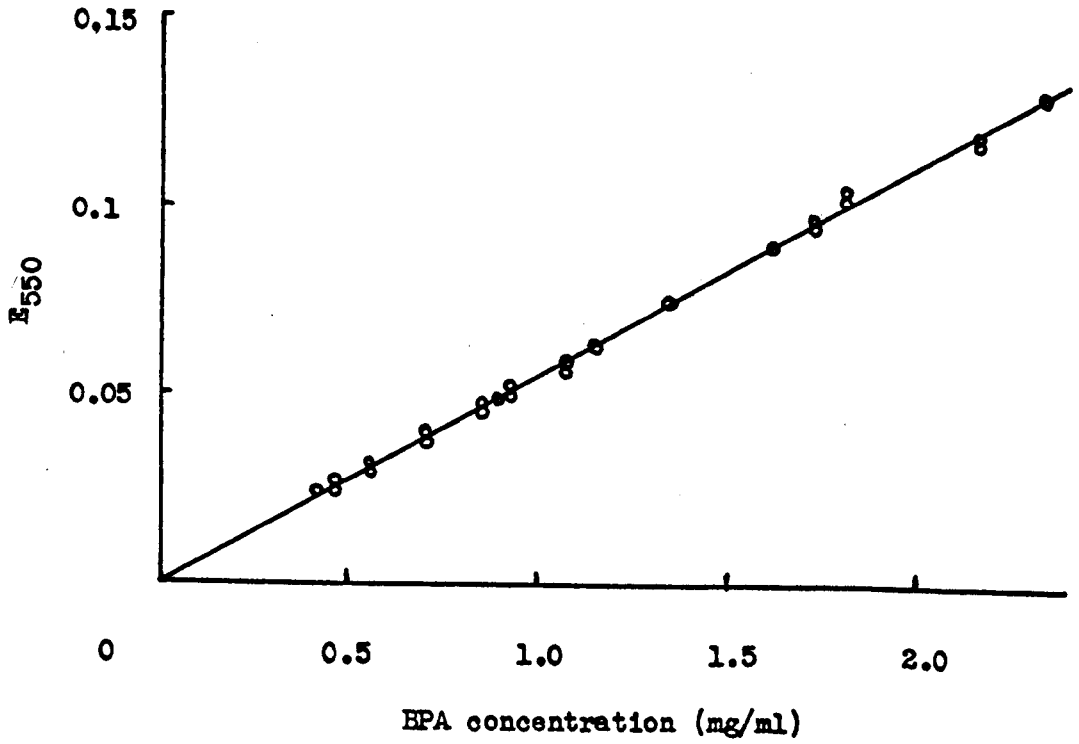
All measurements using this method were performed on Dns-BPA dissolved in NET buffer pH8.0. In this buffer, ε_B was measured and found to be 4.114×10^4 litre $mol^{-1}cm^{-1}$. Okabe and Takagi (1971) suggested a value, for ε_D, of 4.3×10^3 litre $mol^{-1}cm^{-1}$. However, Hartley and Massey (1956) proposed a value for ε_D of 3.0×10^3 litre $mol^{-1}cm^{-1}$ based on studies with Dns-ovalbumin conjugates. This appears to be a more accurate value as the former is based on studies with simple molecules (Weber, 1952).

The second method in Table 3 involved measurement of the concentration of Dns groups as in the previous method. The protein concentration, however, was measured by the biuret method which, in this case, involved mixing 1 ml of sample with 4 ml of biuret reagent and reading the absorbance at 550 nm of the mixture after 30 min at room temperature. The biuret reagent consisted of 1.5 g of $CuSO_4$. 5 H_2O and 6 g of sodium potassium tartrate dissolved in 500 ml of water to which was added 300 ml of 2.5 M NaOH. This solution was then made up to 1 l before use. The standard curve obtained using this method is shown in Fig. 6. The protein concentration was measured using this technique because it has been shown (Lagunoff and Ottolenghi, 1966) that the Dns group will interfere with other methods of protein estimation but has no effect in this case.

FIGURE 6.

Standard Curve for Biuret Method.

BPA was dissolved in NET buffer pH8.0 to give samples of known protein concentration. These were then tested according to the biuret method of determining protein concentration. After the reaction the samples were measured for absorbance at 550 nm.


0.15
0.1
0.05
0
E_{550}
0.5
1.0
1.5
2.0
EPA concentration (mg/ml)

The final way in which the number of Dns groups bound to BPA could be assayed was by measuring the protein concentration by the biuret method and measuring the number of Dns groups by a radiochemical method. In the last case a small amount of tritiated Dns-Cl was added to non-radioactive Dns-Cl, before mixing with BPA, to give a known specific activity. The Dns-BPA was then prepared as usual (2.2.1.) and an aliquot of known protein concentration was taken for scintillation counting (2.2.3). The concentration of Dns groups could then be calculated from the activity of the sample.

One other possible method of determining the degree of binding of Dns groups to BPA would be to devise an assay system based on the fluorescent properties of Dns compounds. Such an assay would prove difficult to use in the system under study as the fluorescent properties of the Dns groups are dependent to a large extent on the environment of the particular group (Chen, 1967; Parker et al., 1967) and it would be unlikely that all the Dns groups bound to BPA would occupy equivalent positions. As a result such an assay system was not investigated.

2.2.3. Scintillation Counting.

All scintillation counting was carried out on an Isocap 300 liquid scintillation system. 200 µl of sample was mixed with 10 ml of scintillation fluid and counted for 10 min. against an external standard. The number of counts obtained for each tritiated sample were converted to d.p.m. using a quench curve prepared earlier. The scintillator used consisted of 0.5% 2,5 - diphenyloxazole in toluene mixed with 2 - methoxyethanol in the proportions 60 : 40 (v/v). In some cases, because of the colour quenching due to high concentrations of Dns groups, the measurement of d.p.m. was carried out using an internal standard.

2.2.4. Tritium Exchange.

The tritium exchange method used was the two-column technique of Englander (1963). Using this technique of tritium exchange the amount of tritium remaining in the protein after a given time of exchange with non-radioactive buffer is measured by separating the free tritium from that bound to protein and determining the radioactivity of the latter. It was decided to use Sephadex G10 instead of the G25 normally used in an attempt to obtain a faster separation of the protein and tritiated water. One large column (6.0 cm x 2.5 cm) and five smaller columns (6.0 cm x 1.0 cm) were set up, equilibrated with NET buffer pH 8.0. BPA or Dns-BPA was then dissolved in 0.1 M N-ethylmorpholine pH 9.6 to give a solution containing 4 mg/ml protein. 1 ml of this solution was added to 100 μl of tritiated water (20 mCi/ml) and incubated at 25°C for 20 hr in order to achieve a complete tritiation of all labile hydrogens.

When tritiation was complete the pH of the solution was adjusted to pH 8.0 with 10% (v/v) acetic acid. The sample was then transferred to the large Sephadex column which was eluted at 120 ml/h and 1 ml fractions were collected manually. The fractions were measured for absorbance at 280 nm and those containing protein were pooled, after 200 μl of each had been removed for scintillation counting, and incubated at 25°C once more.

At a series of time intervals from 0.5 h to 24 h after the initial separation, 300 μl of the sample were placed on one of the smaller columns, eluted with NET buffer pH 8.0 and 300 μl fractions were collected. The protein concentration of each fraction was determined from the absorbance at 280 nm and 200 μl of those containing protein were taken for the measurement of radioactivity. In each experiment, the E_{280} of the protein was calculated using an aliquot

of the original solution, after adjusting the pH to 8.0 as before. The protein concentration was measured by the buiret method.

The number of tritium-labeled hydrogens per mole of protein (N) is calculated from the equation

$$N = \frac{110.8 \times \varepsilon_p}{Co} \times \frac{C}{A}$$

where ε_p is the molar extinction coefficient of the protein, Co is the radioactivity of the incubation mixture measured in d.p.m. per 200 μl, A is the absorbance of the sample, C is the radioactivity of the sample, measured as for Co and 110.8 is the molar concentration of hydrogen in water.

2.2.5. Circular Dichroism.

All circular dichroism measurements were carried out on a Cary 60 spectropolarimeter using a 0.5 mm path length, cylindrical quartz cell. The protein solutions studied were made up in 0.1M phosphate pH 8.0 to a concentration of approximately 0.075% (w/v). The sensitivity control was set such that a full-scale deflection corresponded to 0.1° ellipticity. All the samples studied were scanned at 25°C at a chart speed of 9 nm/min. The spectra were measured in duplicate and the average values have been reported. The circular dichroism was calibrated with d-camphorsulphonic acid, assuming a circular dichroic absorption coefficient of 2.2 at 290 nm.

2.2.6. Fluorescence

Samples for fluorescence measurement were dissolved in 5% (v/v) pyridine and then studied using a Hitachi Perkin-Elmer fluorescence

spectrophotometer. The sample chamber was maintained at a temperature of 28°C for all experiments by circulation of water from a thermostatted bath. In the experiments where fluorescence was used as a means of measuring the amount of fluorescent material in peptide hydrolysates, the spectrophotometer was calibrated with a standard sample of N^{ε}- Dns lysine (1 nmol/3ml), A 1 cm x 1 cm quartz cell was used in all fluorescence measurements.

2.2.7. Aminoethylation.

The aminoethylation of BPA and modified BPA was carried out by a modification of the method of Cole (1967). The method was altered because it was difficult to dissolve the BPA in the 8M urea, 1 M Tris buffer used in the original technique.

100 mg BPA were dissolved in 2.5 ml 4 M Tris buffer pH 8.6. This solution was then added to 7.5 ml 12M urea and nitrogen was bubbled through the solution for 20 min. Then 100 µl β-mercaptoethanol were added and the solution was left at room temperature under nitrogen for four hours. Three 0.2 ml aliquots of ethylenimine were added at 10 min. intervals and the reaction was allowed to proceed for a further hour. The reagents were removed by repeated dialysis. Amino acid analysis of the aminoethyl BPA showed that between 80% and 90% of the cysteine residues had been converted to aminoethylcysteine residues. As it is difficult to obtain a 100% recovery of aminoethylcysteine on amino acid analysis, the actual extent of conversion is probably nearer 100% than indicated by this method.

2.2.8. Performic Acid Oxidation.

Performic acid was prepared by mixing 5 volumes of 30% (v/v)

hydrogen peroxide with 95 volumes of 99% (v/v) formic acid. The reaction mixture was then left at 25°C for 2 h in a closed container. 10 ml of performic acid and a solution of 3μmol BPA in 5 ml 99% (v/v) formic acid, 1 ml methanol, were then permitted to stand at -5°C for 30 min. The solutions were mixed and allowed to react at -5°C for 150 min. The reaction was terminated by the addition of 400 ml of ice-water followed by lyophilisation. This process was repeated once more.

2.2.9. Amino Acid Analysis.

For amino acid analysis, 1 mg of protein was dissolved in 2 ml 5.7 M (constant boiling) hydrochloric acid. The sample was then hydrolysed for 22 h at 110°C in an evacuated, sealed tube. After hydrolysis, the sample was dried, redissolved in distilled, deionized water and analysed on a Jeol JLC 5 AH amino acid analyser (Japan Electron Optics Laboratory Co. Ltd., Chiyoda-du, Tokyo) using a two-column system and step-wise elution.

2.2.10. Proteolytic Digestion.

Aminoethyl Dns-BPA was broken down by several different proteases in order to find a method suitable for the production of peptides containing the Dns groups. The conditions used in each case are outlined below:

(1) Chymotrypsin.

100 mg of protein was dissolved in 10 ml of 1% (w/v) ammonium bicarbonate. 1 mg of chymotrypsin dissolved in 1 ml of 0.001 M hydrochloric acid was added to this solution. The pH was adjusted to

8.0 and the reaction allowed to take place at 25°C for 6 h with stirring. The solution was then centrifuged to remove any precipitate and the reaction was stopped by the addition of 1 ml of glacial acetic acid.

(2) Pepsin.

100 mg of protein was dissolved in 11 ml 5% (v/v) formic acid and to this was added 10 mg pepsin dissolved in 1 ml of the same solvent. The reaction mixture was then left at 37°C for 17 h with stirring.

(3) Trypsin and Thermolysin.

Both these proteases were allowed to attack the protein under identical conditions. 100 mg of protein was dissolved in 10 ml NET buffer pH 8.0. To this was added 2.0 mg of protease dissolved in the same buffer. The aminoethyl Dns-BPA was digested, with stirring, for 17 h at 37°C.

In all the above cases the reaction mixture was lyophilised on completion of the reaction.

2.2.11. Chromatography of Peptides.

In order to obtain an estimate of the molecular weight distribution of the peptides produced by peptic digestion of aminoethyl Dns-BPA, gel chromatography was carried out. A column (115 cm x 1 cm) of Sephadex G50 fine was set up, equilibrated with 5% (v/v) formic acid and eluted at 35 ml per hour. 1 ml fractions were collected. The column was calibrated with the following molecular weight markers, detecting the presence of protein in the eluate by its absorbance at 280 nm:

	Molecular weight
Ribonuclease A	13,683
Insulin	5,733
Bacitracin	1,411
Tryptophan	204

20 mg of aminoethyl Dns-HPA which had been digested with pepsin (2.2.10.) was then applied to the column and chromatographed. In this case, the protein concentration of the fractions was determined by the buiret method (2.2.2.).

2.2.12. Peptide Analysis.

The mixture of peptides produced by peptic digestion of aminoethyl HPA (2.2.10.) was fractionated by ion-exchange chromatography on Chromobead P resin using a Technicon peptide analyser (Technicon Instruments Ltd., Hamilton Close, Houndmills, Basingstoke, Hants.). 3μmol of the mixture was applied to the column and eluted at 27 ml per hour with one of two gradients of pyridine-acetate buffers. The first gradient gave a rise from 0.2 M to 2.0 M pyridine-acetate and from pH 3.1 to pH 6.5 while the second went from 0.1 M to 2.0 M buffer and from pH 2.75 to pH 6.5. 4.5 ml fractions were collected. As the eluate emerged from the column, a small amount was continuously monitored by the analytical system for protein concentration. This was achieved by measuring the colour produced on reaction of the sample with ninhydrin, before and after hydrolysis. The fractions were also tested for absorbance at 330 nm which indicates the presence of Dns groups. The relative fluorescence of the samples was checked by spotting an aliquot of each on Whatman No. 1 paper and comparing the spots under an ultraviolet lamp. The fractions containing Dns-peptides were pooled and lyophilised.

2.2.13. Electrophoresis.

The electrophoretic separation of peptides was carried out on a water-cooled flat-bed apparatus. The samples were applied to an origin on a sheet of Whatman No. 1 paper along with two standard mixtures of known amino acids. The paper was then wetted with the buffer being used and electrophoresis was carried out, normally at 3,000V, for a given time. At the end of the separation the paper was dried in a fume cupboard.

2.2.14. Staining and Elution of Peptides.

Peptides were visualised, after electrophoresis, by staining with a ninhydrin-cadmium acetate reagent. The paper was dipped in a solution containing 100 ml ninhydrin (1% (w/v) in acetone) and 15 ml of a cadmium acetate solution made by dissolving 5g cadmium acetate in 750 ml 33% (v/v) acetic acid. The paper was then dried for 20 min at 60°C and the ninhydrin positive spots were easily seen.

In the case of preparative paper electrophoresis, the areas containing Dns-peptides were visualised under an ultraviolet lamp, cut out and eluted from the paper using 5% (v/v) pyridine.

2.2.15. Identification of Dns-Amino Acids.

Dns-amino acids were identified by thin layer chromatography on polyamide sheets (5 cm x 5 cm) using the solvent systems of Woods and Wang (1967). The positions of unknown Dns-amino acids were compared with those of standard Dns-amino acids run in the same system.

3. RESULTS

It has been pointed out earlier (1.6.) that the study of the tertiary structure of BPA using chemical modification by Dns-Cl falls into three areas: (1) a study must be made of the factors influencing the reaction of the protein with Dns-Cl and the extent of the modification occurring. (2) The tertiary structure of Dns-BPA must be examined in order toshow that the reaction has not significantly altered the original shape of the protein, otherwise any deductions made about the modified protein cannot be related to its native structure. (3) In order to make such deductions the residues with which the Dns-Cl has reacted must be isolated, identified and located in the primary structure of the protein.

To simplify the presentation of the results obtained in the course of this research they will be grouped under these three areas of investigation.

3.1. THE REACTION OF DNS-CL WITH BPA.

3.1.1. The Preparation of Dns-BPA.

The method used for all preparations of Dns-BPA was basically that of Weber (1953). The only modification normally made to this method was to greatly increase the amount of Dns-Cl used, although in some experiments several different factors in the reaction were altered. The steps involved in the preparation of Dns-BPA are outlined in Figure 4. When the Dns-Cl solution was added to the reaction mixture a very fine suspension of the reagent was formed as Dns-Cl is virtually insoluble in water. As the reaction proceeded the turbidity gradually cleared. When large volumes were being used in the reaction, the solution was normally stirred. However,

whether the mixture was stirred or not had no influence on the degree of modification. The centrifugation step was included in the preparation of Dns-BPA to remove any remaining traces of the insoluble Dns-Cl. It was thought, at one point, that this step might lead to erroneous results for the molar ratio of Dns groups to BPA in the product if heavily-modified protein precipitated in the course of the reaction. This was shown not to be the case by measuring the protein concentration in the initial solution and after the centrifugation, using the biuret method. The remaining steps in the preparation were involved in the removal of any adsorbed reagent molecules from the protein. BPA does, in fact, appear to adsorb the reagent molecules more strongly than most proteins (Weber, 1952). Non-covalently bound Dns groups, which will be in the sulphonic acid form, can be removed from the protein by ion-exchange chromatography using a column of AG2 - x8 resin. In preparations where the volume involved is large, the ion-exchange process can be equally well carried out using a batch technique.

Since it was essential, from the point of view of all subsequent experiments, that all the adsorbed Dns-OH was removed from the modified protein, two methods of testing for complete removal were used. The first of these was devised by Weber (1953). He separated the free Dns-OH from the Dns-BPA using paper chromatography with a solvent system consisting of ethanol and 0.2M acetate buffer pH 4.8 (55:45, v/v). In this system, the free Dns groups had an R_F value of 0.9 while Dns-BPA did not move from the origin. The spots present on the chromatogram could be easily located under an ultraviolet light. Using this method

the presence of free Dns groups in the Dns-BPA preparation can be simply detected.

The second method used was designed in an attempt to ensure that no Dns-OH was absorbed in the hydrophobic sites known to exist in BPA (Jonas and Weber, 1971). 20 mg of Dns-BPA was dissolved in 10 ml NET buffer pH 8.0 which was then made 8 M with respect to urea. The solution was filtered in an Amicon ultrafiltration cell containing a PM 10 membrane. The fluorescence of the filtrate was compared with that of a standard solution of Dns-Cl in the same solvent. Only free Dns-OH will be able to pass through the membrane as molecules larger than 10,000 molecular weight will be retained. The amount of fluorescent material released was less than 5% of the total fluorescence of the Dns-BPA. This corresponds to the release of less than one Dns group per molecule of BPA.

Both the methods outlined above indicated that the Dns-BPA was free from a significant amount of adsorbed reagent. Once prepared, the Dns-BPA can be stored, either in the lyophilised state or frozen as a solution in buffer pH 8.0, for long periods of time, at -10°C, with no detectable alteration.

3.1.2. Determination of the Molar Ratio of Dns Groups to BPA in the Modified Protein.

Several methods were examined in order to find a suitable technique for measurement of the molar ratio of Dns groups to BPA. These have been outlined previously (2.2.2.). To ensure that the effect of increasing amounts of bound Dns groups was taken into consideration each of the assay methods was carried out on the samples obtained from an experiment in which the amount of Dns-Cl in the reaction mixture was

gradually increased over a wide concentration range (1-250 mol Dns-Cl per mol BPA).

The first method to be attempted was that used by Okabe and Takagi (1971) in their studies on Dns-lysozyme. This method relied entirely on the ultraviolet absorbances of the Dns-group and of BPA (2.2.2.). The results obtained using this method are shown in Figure 7.

Since the above technique depended on the absorbances of the compounds involved and because these could alter as more Dns groups were bound to BPA, due to intramolecular interactions, it was felt that other methods of measuring the molar ratio of Dns groups to BPA should be investigated.

The second method examined (Table 3) eliminated one of the absorbance measurements by determining the protein concentration using the biuret method (2.2.2.). Use of this method assumed that the absorbance of the Dns group at 330 nm was not affected by an increase in the molar ratio of Dns groups to BPA. Although this assumption could prove invalid the method merited investigation because of its simplicity. The results obtained using this method are also shown in Figure 7. There was a large difference between the results from this method and those of the previous method, suggesting that the absorbance at 280 nm of Dns-BPA could not be used to accurately determine the protein concentration of the sample.

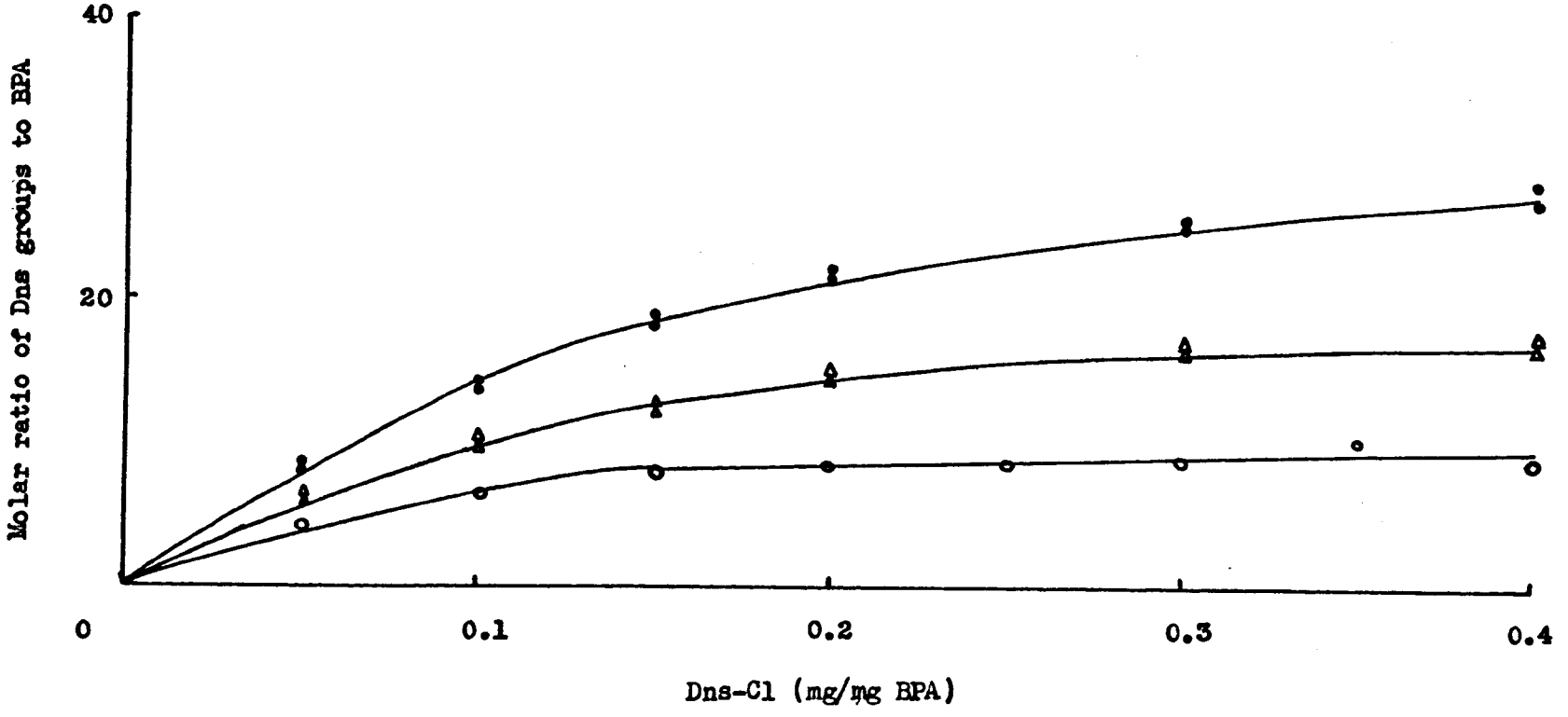
The third method outlined in Table 3 was devised in order to remove the requirement to measure the concentration of Dns groups by their absorbance at 330 nm. In this case, the concentration of protein was still measured using the biuret method but the concentration of Dns groups was measured by using radioactive Dns-Cl in the reaction

FIGURE 7.

Determination of the Molar Ratio of Dns Groups to BPA in Dns-BPA.

The three methods outlined previously (2.2.2.) for the determination of the number of Dns groups which bind per molecule of BPA were applied to samples obtained in an experiment to study the effect of the concentration of Dns-Cl in the reaction mixture on the molar ratio of Dns groups to BPA. This permits study of the influence of increasing amounts of Dns groups on each assay method.

	Method of assay	
	Dns group concn.	BPA concn.
•——•	E 330	Biuret
A——A	Tritiated Dns-Cl	Biuret
o——o	E 330	E 280


40
20
0
0.1
0.2
0.3
0.4
Molar ratio of Dns groups to BPA
Dns-Cl (mg/mg BPA)

mixture and counting the radioactivity present in the modified protein. The results obtained using this method of measurement are also shown in Figure 7.

In the assay technique using radioactive Dns-Cl outlined above the d.p.m. values for the samples of Dns-BPA were calculated from the c.p.m. values using an external standard source of radioactivity to measure the quenching of the sample and comparing the result with a solvent quench curve prepared earlier. However, since the Dns groups are coloured, there is a possibility of quenching in addition to that caused by the solvent because the coloured compound can absorb the light emitted by the scintillator. It is generally assumed that colour quenching follows the curve obtained for solvent quenching. If this is the case, the d.p.m. values obtained using the external standard method will be correct. To ensure that this is, in fact, what happens in the system under consideration the following experiment was carried out. Several samples were selected for which the d.p.m. values had already been calculated using the external standard method and the solvent quench curve. To each sample was added a known amount of tritiated toluene. The volume added was very small (5 μl) and, as such, should not alter the quenching characteristics of the sample. The samples were then counted again and, from the increase in c.p.m. obtained, the efficiency of counting of the sample and its d.p.m. value could be worked out. The results obtained are shown in Table 4. These indicate that any colour quenching due to the Dns groups must follow the solvent quench curve as the differences between the values obtained by the two methods of calculating d.p.m. are well within experimental error. On the basis of this, the d.p.m. values obtained

TABLE 4.

A Comparison of Counting Methods for Dns-BPA.

In order to examine the effect of colour quenching of Dns groups in Dns-BPA prepared from tritiated Dns-Cl, two methods of calculating the d.p.m. values were applied to the same samples. In the first method, the external standard method, an external standard source of radioactivity was used to measure the quenching of the sample and from this the efficiency of the counting was calculated using a solvent quench curve prepared previously. The samples were then used for the second method, the internal standard method, in which the efficiency of counting was measured by adding a known amount of radioactivity to the samples and counting them again.

SAMPLE	D.P.M.	
	INTERNAL STANDARD	EXTERNAL STANDARD
1	5129	5286
2	11376	11301
3	20694	21811
4	45656	44257

by the external standard method can be taken as correct.

A comparison of the third assay method, outlined above, with the second method described in Table 3 demonstrates that the absorbance, at 330 nm, of the Dns groups cannot be used to calculate their concentration in the determination of the molar ratio of Dns groups to BPA in the modified protein. This is probably because the molar extinction coefficient of the Dns groups at 330 nm is altered as the degree of modification of the protein is increased. The most likely cause of such a change is interactions between the Dns groups or between the Dns groups and the protein (4.1.).

Due to its methods of measurement, the third technique was, by far, the most accurate of the three tested. However, in order to avoid excessive use of radiochemicals and to provide a simpler method of assay it was decided to use the second method described (Table 3) as the results obtained could be readily converted to the true values using the graph shown in Figure 7. This method had the added advantage that it also gave the true protein concentration of the sample, unlike the first technique.

3.1.3. The Effect of Different Concentrations of Dns-Cl in the Reaction Mixture on the Degree of Modification.

Having devised a suitable method for determining the molar ratio of Dns groups to BPA in Dns-BPA, the next step in a study of the reaction of Dns-Cl with BPA is to investigate the factors which affect the degree of modification of the protein. This is necessary if any structural information is to be derived from the residues which are modified and those which are not. It must be shown that less than complete modification is not due to the reaction conditions but is due, instead, to the structure of the protein.

It was thought that one important influence on the molar ratio of Dns groups to BPA in the modified protein would be the relative concentrations of the reagents in the initial reaction. In order to study this, the degree of modification obtained was measured for samples of Dns-BPA prepared using different concentrations of Dns-Cl in the reaction mixture. The results obtained are shown in Figure 8. This shows that as the concentration of Dns-Cl added to BPA is increased to about 70 moles per mole BPA, the degree of modification increases. The curve then becomes horizontal and even using much higher initial concentrations of Dns-Cl has no effect on the degree of modification produced. This tends to indicate that all the possible reactive residues in the protein are not equivalent.

3.1.4. The Wavelength of Maximum Absorbance of the Dns Group in the Modified Protein.

Since the assay used to calculate the degree of modification depends on the absorbance of the Dns group at 330 nm, it is essential that the wavelength of maximum absorbance of the group does not vary significantly as more Dns groups are bound to the protein. This was examined for a number of different molar ratios of Dns groups to BPA (Fig. 9). Although the results indicate that the wavelength of maximum absorbance is around 335 nm for most of the samples examined, it was decided to continue to assay the Dns groups by measuring their absorbance at 330 nm as the peak of absorbance in this region is comparatively broad and any errors introduced by readings taken at a slightly lower wavelength than the maximum will be negligible.

3.1.5. The Effect of Reaction Time on the Degree of Modification.

Another important experimental factor which could affect the molar

FIGURE 8.

The Effect of Different Molar Ratios of Dns-Cl to BPA on the Degree of Modification.

Flasks were set up containing 100 mg BPA dissolved in 100 ml 0.1 M phosphate buffer pH 7.5. To each was added a different weight of Dns-Cl dissolved in 5 ml acetone. The reaction was then allowed to proceed and the modified protein isolated as described earlier (Fig. 4). The molar ratio of Dns groups to BPA in the modified protein was measured using three samples from each reaction mixture.

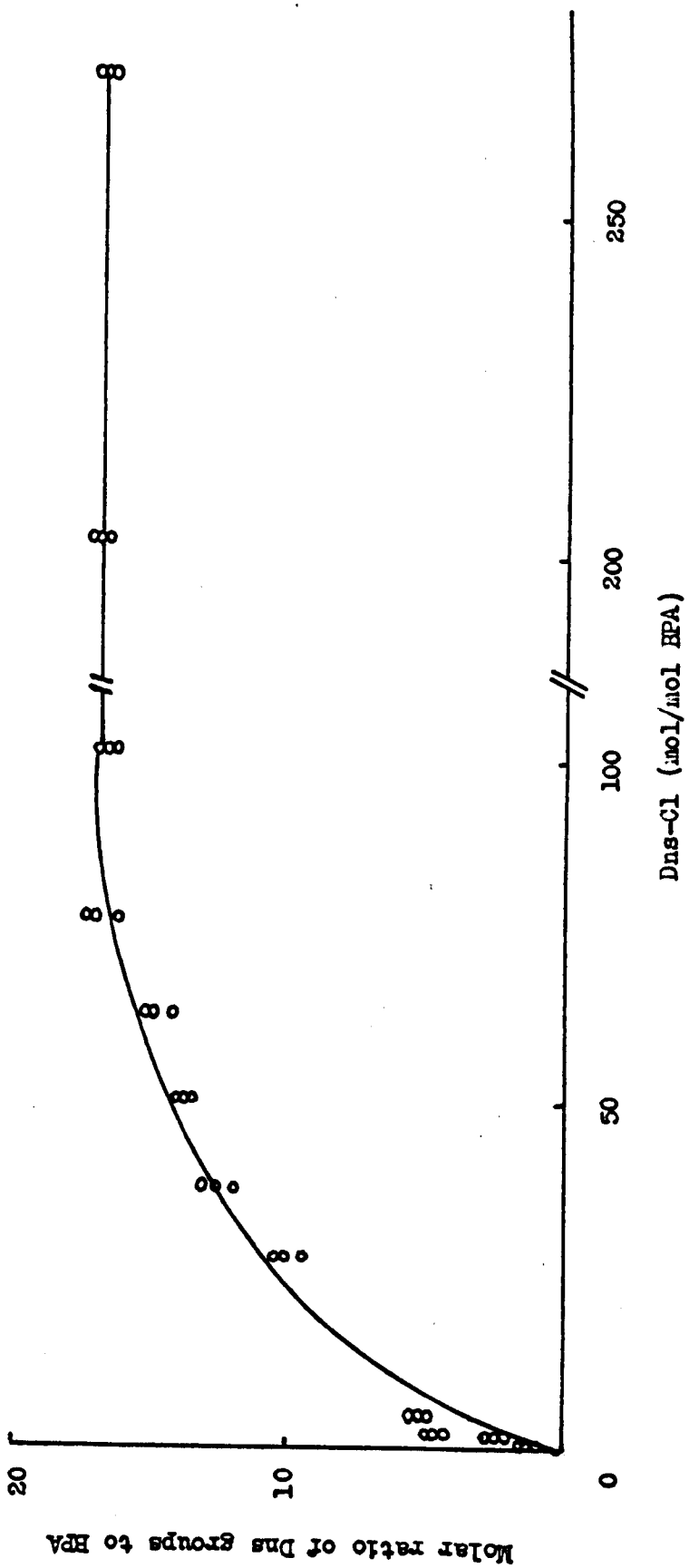
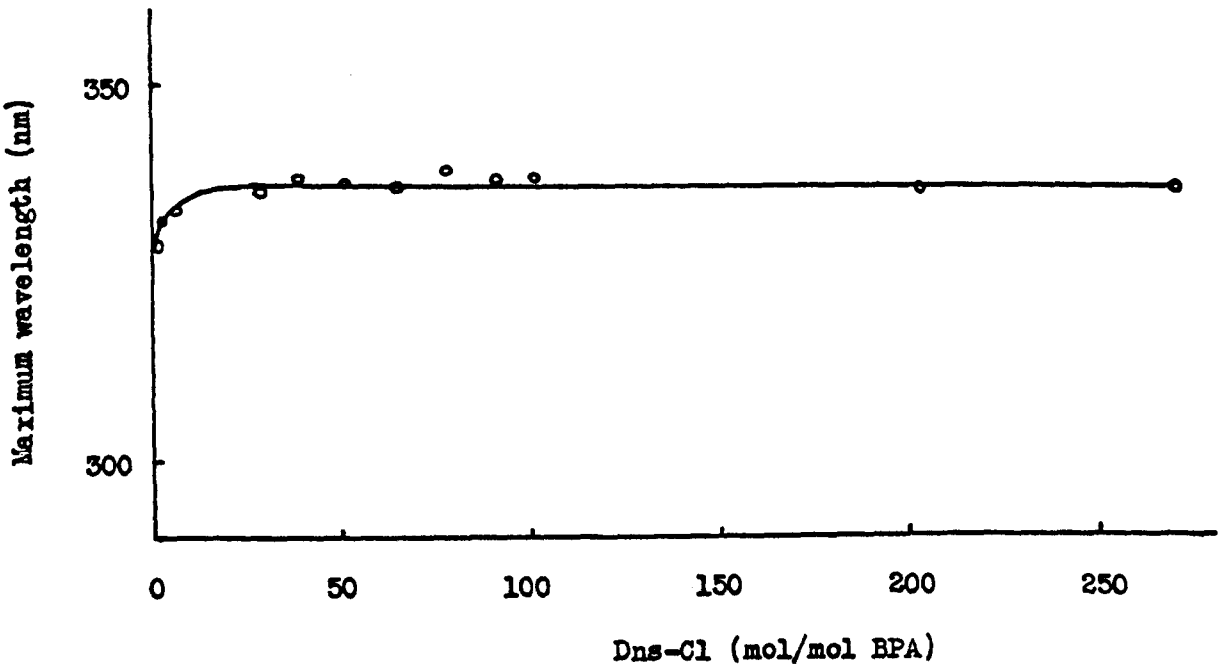

Molar ratio of Dns groups to HPA
20
10
0
50
100
200
250
Dns-Cl (mol/mol HPA)

FIGURE 9.

The Effect of the Degree of Modification on the Wavelength of Maximum Absorbance of the Dns Group.

In the course of the previous experiment (Fig. 8), the wavelength of maximum absorbance of the Dns group was measured for each sample of Dns-HPA produced by a given weight of Dns-Cl in the reaction mixture. Measurements were confined to the peak occuring between 300 nm and 350 nm.


Maximum wavelength (nm)
350
300
0
50
100
150
200
250
Dns-Cl (mol/mol BPA)

ratio of Dns groups to BPA is the length of time of the reaction. This was examined by setting up a preparation of Dns-BPA and taking samples at various times after starting the reaction (Fig. 10). The results show that initially about ten molecules of Dns-Cl react very rapidly with BPA. This is followed by a slower increase in the degree of modification over the next ten hours to attain the maximum value. The effect of the time of the reaction on the ratio of Dns groups to BPA could suggest that there are two different subsets of functional groups on the protein which vary in the rate at which they react with Dns-Cl (1.4.2.). However, it is difficult to obtain any detailed information from the results available.

3.1.6. Other Factors which Alter the Degree of Modification.

Several preparations of Dns-BPA were carried out in which experimental parameters, other than those discussed above, were altered in order to study their influence on the molar ratio of Dns groups to BPA in the modified protein. In this set of experiments, because a greater degree of modification may be obtained than that shown in Figure 7, and as this would make it impossible to use the assay decided on previously (3.1.2.), the radiochemical method of determining the molar ratio of Dns groups to BPA was used (2.2.2.). The results obtained are shown in Table 5.

The effect of increasing the pH of the reaction mixture is shown in the first preparation outlined in Table 5. In this case, the pH of the phosphate buffer used in the experiment was pH 9.1. After the reaction had been carried out, the product was found to contain about 25 moles of Dns groups per mole of BPA. Although carrying

FIGURE 10.

The Effect of Reaction Time on the Degree of Modification.

Dns-BPA was prepared as described previously (Fig. 4). At several time intervals after the addition of the Dns-Cl, a 5 ml sample of the reaction mixture was removed and the modified protein was isolated. The molar ratio of Dns groups to BPA was measured in the samples of Dns-BPA prepared in this way.

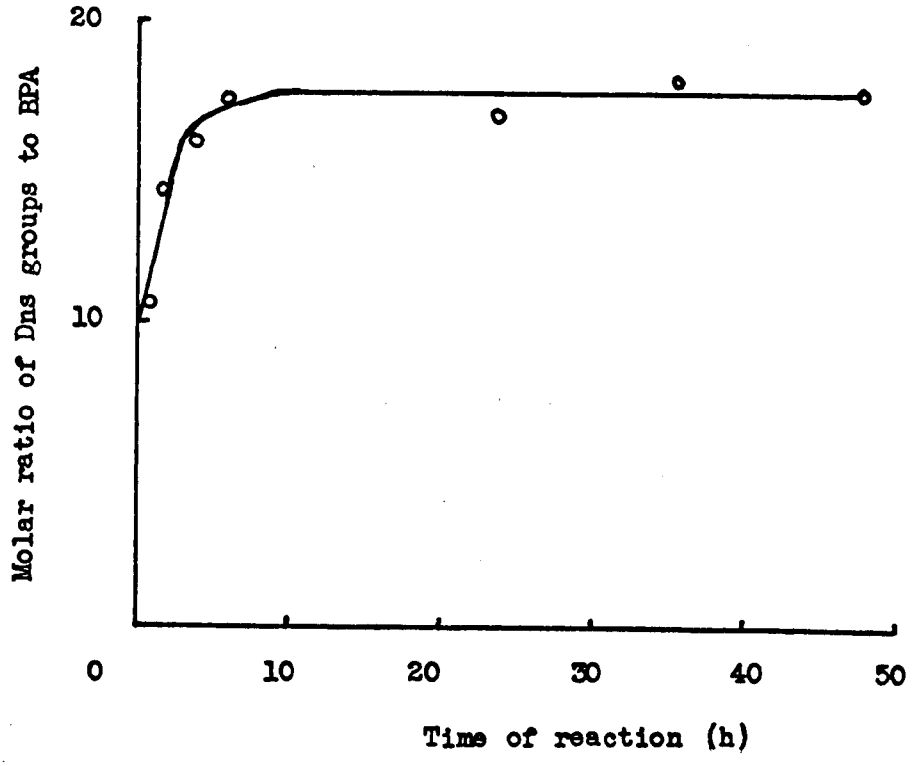

20
10
0
10
20
30
40
50
Molar ratio of Dns groups to BPA
Time of reaction (h)

TABLE 5.

Other Factors which Alter the Degree of Modification.

In order to examine the effect of several factors on the degree of modification of BPA using Dns-Cl, a number of different reaction mixtures were set up in which one experimental parameter was varied in each case. The reaction was then carried out and the modified protein isolated as described earlier (Fig. 4) unless otherwise stated. The molar ratio of Dns groups to BPA in the isolated protein was measured.

REACTION CONDITIONS	MOLAR RATIO OF DNS GROUPS TO BPA	
1. Reaction carried out at pH 9.1	26.3	24.3
2. Concentrations of BPA and Dns-Cl reduced by half	19.6	19.7
3. Reaction carried out at 25°C	21.9	22.6
4. Reaction carried out in 8M urea buffer	6.1	5.8
5. Reaction carried out in 6M guanidine hydrochloride buffer	10.7	10.8
6. Reaction carried out in 1% (w/v) sodium dodecyl sulphate buffer	15.4	15.5
7. Reaction carried out using performic acid oxidised BPA	17.4	16.3
8. Reaction carried out using the conditions of Gros and Labouesse (1969)	80.9	81.4

out the reaction at a higher pH gave a greater degree of modification, it was felt that it would be more meaningful to study the structure of the protein under the conditions of the original preparation procedure (Fig. 4), as the pH used was much nearer physiological pH. A second, and perhaps more important reason for studying the protein at the lower pH is that a considerable amount of evidence is accumulating which indicates that BPA undergoes a structural change, similar to the N→F transition (1.5.5.), at alkaline pH (Leonard et al., 1963). As a result, structural studies at pH 9.1, for example, may lead to erroneous conclusions about the native shape of the protein.

Preparation 2 in Table 5 demonstrates the effect of reagent concentration and reaction volume on the extent of the chemical modification. It was felt that, since the Dns-Cl is only very sparingly soluble in water, its solubility in the reaction mixture might alter the ratio of Dns groups to BPA in the Dns-BPA. In preparation 2, the concentrations of the protein and the Dns-Cl were halved. The number of Dns groups bound to each molecule of BPA was only slightly increased and it was felt that the increase did not justify using much more dilute reaction mixtures as it would be more difficult to handle the larger volumes.

The third experimental parameter which was altered in this series of experiments was that of temperature. The results of preparation 3 in Table 5 show the effect of raising the temperature at which the reaction was carried out. The higher temperature gave rise to a slightly higher molar ratio of Dns groups to BPA in the product. Despite this, it was felt that carrying out the reaction at room temperature had little extra advantage, as at the higher temperature

there was a possibility that intramolecular transfer of the Dns-groups after the original reaction might occur, and cause a change in the protein structure. Transacetylation, for example, can occur in proteins after modification (Cohen, 1970). Although carrying out the reaction at 0°C does not preclude such a transfer, it might reduce its extent and, since the increase in the number of Dns-groups bound at room temperature is only slight, it was decided to continue to prepare Dns-BPA at 0°C.

All the remaining preparations in Table 5 were carried out in an attempt to achieve the modification of all the reactive groups present in the BPA molecule. Preparations 4,5 and 6 were carried out under conditions which can cause denaturation of proteins. In all three cases a lower molar ratio of Dns groups to BPA in the product is obtained in the presence of denaturing agents than in their absence. Preparation 7 demonstrates that, if Dns-Cl is reacted with a sample of BPA in which the disulphide bridges have been broken by performic acid oxidation (2.2.8.), the degree of modification is the same as that obtained using the native protein. In the final preparation technique used (Table 5) the reaction was carried out using the conditions of Gros and Labouesse (1969). In this method, the pH of the reaction mixture drops from pH 9.4 to pH 8.7 during the course of the reaction. The modification is carried out at room temperature, and the reaction mixture contains 4M urea and 25% (v/v) dimethylformamide. It appears, from the results, that this method will bring about nearly complete modification of all the reactive groups in the protein since, on the basis of the amino acid analysis of BPA (1.5.3.), the number of groups which can react with Dns-Cl can

be estimated as about 95.

The final set of experiments in this section, outlined below, was not directly concerned with an investigation of the reaction of Dns-Cl with BPA but it may help explain some of the results observed earlier (4.1.). For this reason it is included in this section.

3.1.7. The Fluorescence of Dns-BPA.

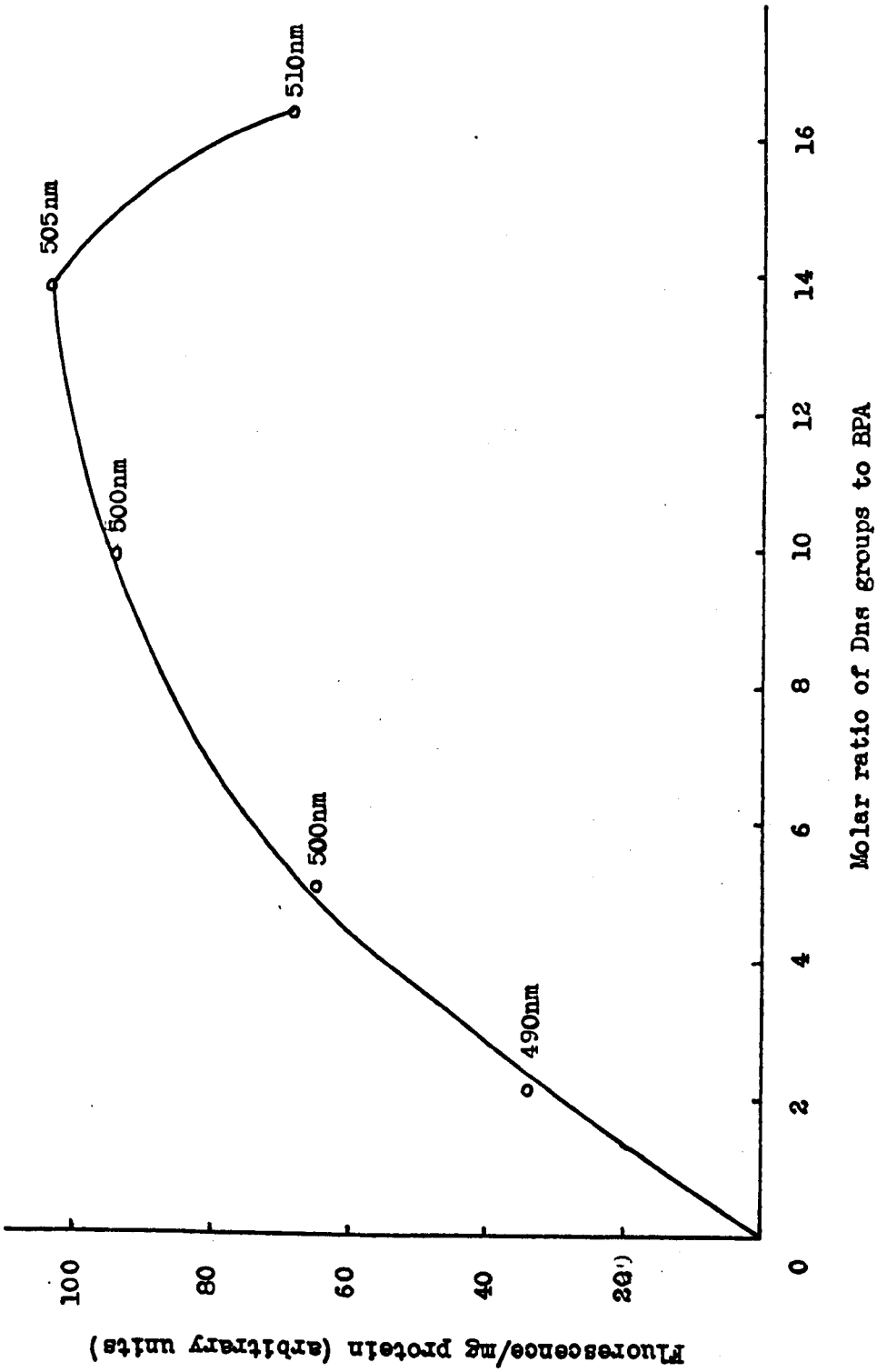
As part of an investigation of the fluorescent properties of Dns-BPA, the maximum fluorescence emission and its wavelength were measured on several samples of Dns-BPA which had different molar ratios of Dns groups to BPA (Fig. 11). In all cases the protein concentration was the same. The wavelength of the exciting light was 285 nm, the maximum fluorescence emission occurring around 500 nm. This is well separated from the fluorescence due to the protein, which is present around 340 nm. Thus the fluorescence observed at 500 nm can be attributed solely to the Dns groups. It was found that as the molar ratio of Dns groups to BPA increased the fluorescence did likewise up to a point, but then began to decrease again. At the same time, the wavelength of maximum fluorescence moved gradually to longer wavelengths as the degree of modification increased.

This concludes the section of experiments designed to investigate the factors influencing the reaction of BPA with Dns-Cl and the extent of the modification occurring. Now that the best method of preparing suitably modified protein has been elucidated, before any deductions about the structure of the protein can be made from the manner in which the Dns-Cl reacts with BPA, it is essential to demonstrate that the native shape of the protein is not significantly altered by the

FIGURE 11.

Fluorescence of Dns - BPA.

The maximum wavelength of fluorescence emission and the fluorescence intensity at maximum wavelength were measured for several samples of Dns - BPA, each of which contained a different molar ratio of Dns groups to BPA. The exciting light had a wavelength of 285 nm. The samples used all had the same protein concentration. The wavelength of maximum emission is noted beside each point on the graph.


Fluorescence/mg protein (arbitrary units)
100
80
60
40
20
0
2
4
6
8
10
12
14
16
Molar ratio of Dns groups to BPA
490nm
500nm
500nm
505nm
510nm

Dns groups bound to it. The following section deals with this aspect of the study.

3.2. STRUCTURAL CHANGES ON THE FORMATION OF Dns-BPA.

The basic problem in studying what, if any, structural changes occur on the formation of Dns-BPA by the reaction of Dns-Cl with BPA is in deciding which methods to use in the investigation. Most of the methods which could be utilised have been discussed in the introduction to this thesis. X-ray crystallography can be ruled out as a possible technique because of the time involved and also because, as yet, nobody appears to have been able to even produce suitable crystals of BPA, let alone the modified protein. Since the binding of such relatively high amounts of Dns groups to the protein results in a large increase in its molecular weight (about 5,000 daltons) and, presumably, a concomitant increase in molecular volume, hydrodynamic techniques would also be unsuitable for the detection of changes in structure occuring on modification of the protein. The absorbance of the Dns groups in the regions in which protein chromophores also absorb would probably complicate any attempt to use difference spectroscopy in this investigation and thus it also was ruled out. Fluorescence techniques were not employed for a similar reason as the Dns groups are fluorescent and would make such a method very complex. The only suitable methods remaining are circular dichroism and optical rotatory dispersion, resonance spectroscopy and hydrogen isotope exchange. It was decided to use at least two different techniques for the study of any changes in the tertiary structure of the protein as it is difficult to predict the exact influence of the bound Dns groups on any one technique. However, if two completely unrelated methods showed that there was no significant change in the shape of the protein, it is virtually impossible to

imagine that in both cases the effect of the Dns groups would be to exactly nullify the observable changes due to a structural alteration. As a result, it was decided to use circular dichroism and tritium exchange to study the effect of binding Dns groups to BPA on the protein's tertiary structure.

3.2.1. The Calibration of Tritium Exchange Columns.

The technique of tritium exchange used was that of Englander (1963) which has been described previously (2.2.4.). Since the method was altered from the original in that Sephadex G10 was used instead of G25 it was essential to ensure that under the conditions used the system would separate the protein from tritiated water. Firstly, several separate samples of protein were passed through each column to ensure that the BPA emerged in the same fractions on each run. Samples of tritiated water were then passed through the columns and their elution positions noted by measuring the radioactivity of each fraction. The results showed that the BPA and tritiated water were, in fact, separated by the system (Fig. 12). These findings were confirmed by the results obtained once the experimental runs were carried out.

3.2.2. Tritium Exchange.

A typical example of the results obtained in a tritium exchange experiment with BPA is shown in Figure 13. It is evident that, when the tritiated protein was allowed to exchange with non-radioactive buffer after the initial separation, the amount of radioactivity associated with the protein gradually decreased. From such a set of results, the number tritium atoms remaining per mole of BPA (N) was calculated using the equation:-

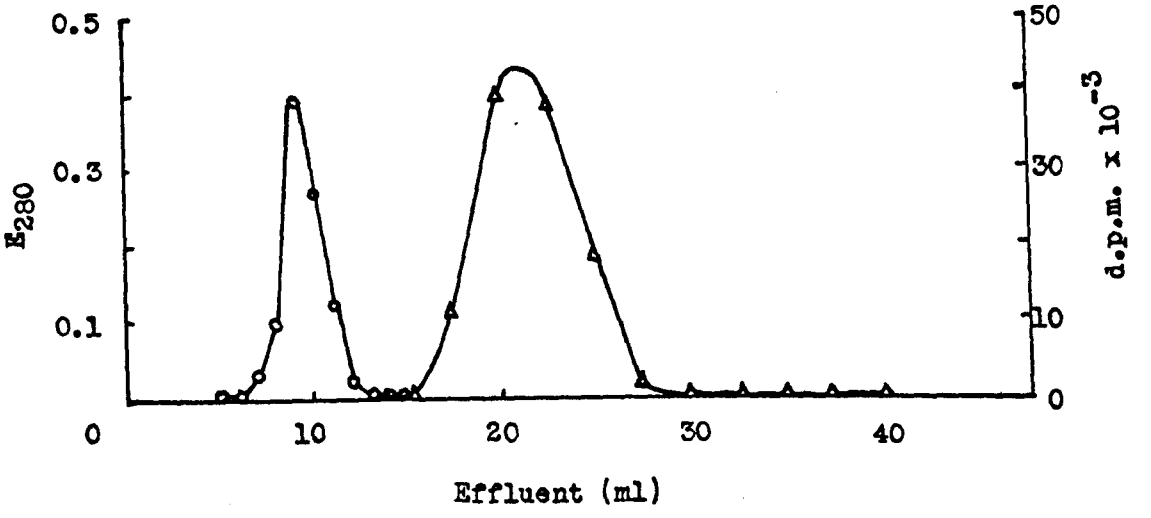
FIGURE 12.

Calibration of Tritium Exchange Columns.

In order to ensure that the columns of G10 Sephadex used in tritium exchange will separate free tritiated water from BPA under the conditions used, a sample of the protein and a sample of tritiated water were passed separately through the columns. The columns are equilibrated with and eluted with NET buffer pH 8.0. The effluents from the columns were monitored for absorbance at 280 nm to detect the BPA and for radioactivity to detect the tritiated water.

o——o A_{280}

▲——▲ Radioactivity


0.5
0.3
0.1
E_{280}
0
10
20
30
40
Effluent (ml)
50
30
10
0
d.p.m. x 10^{-3}

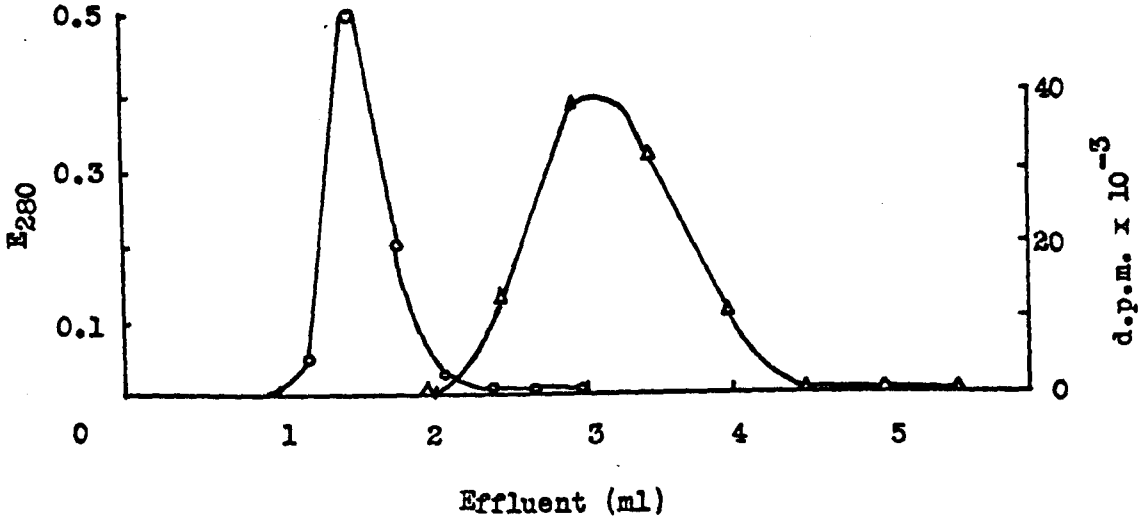

0.5
0.3
0.1
E_{280}
0
1
2
3
4
5
Effluent (ml)
40
20
0
d.p.m. x 10^{-3}

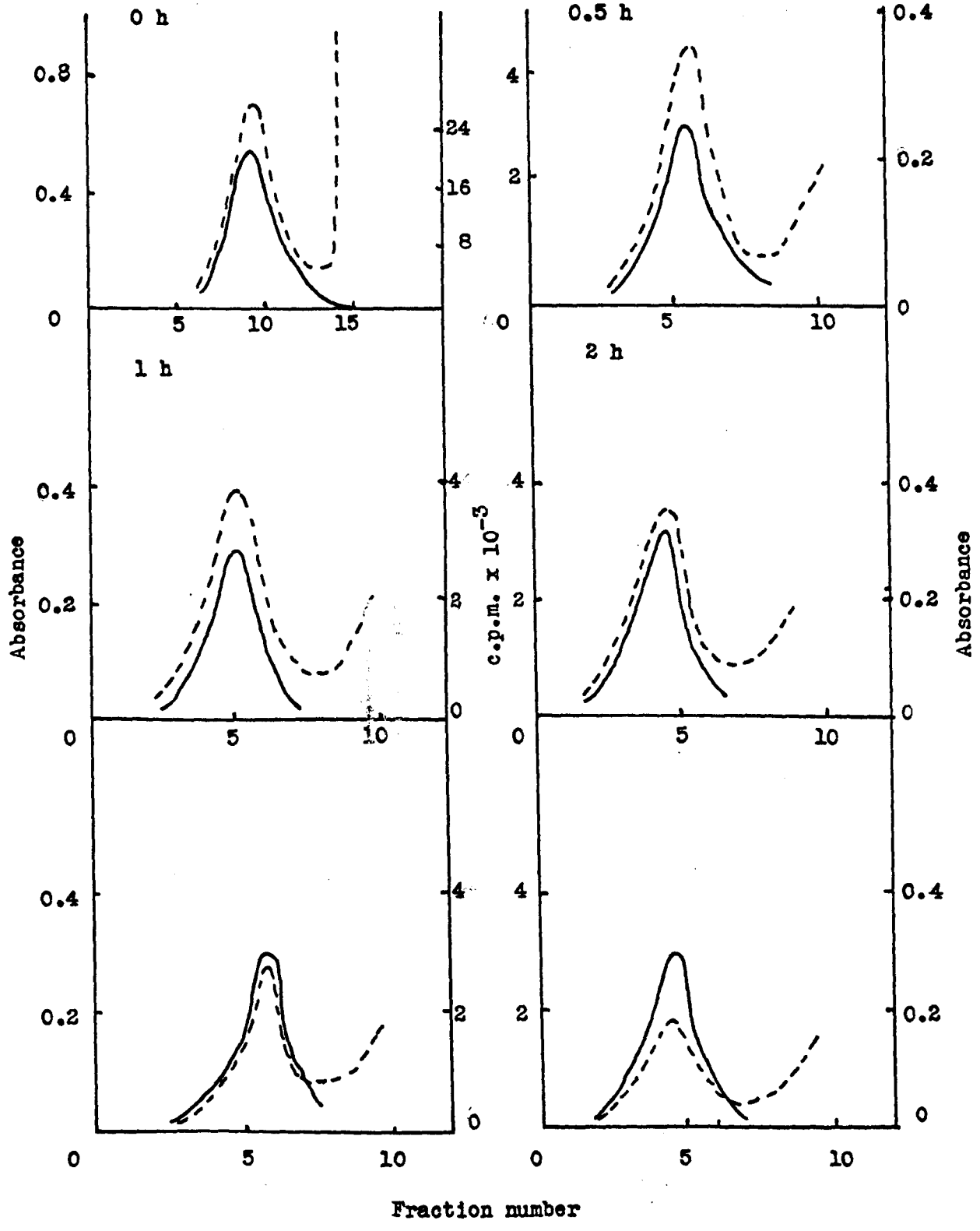
FIGURE 13.

Tritium Exchange.

The results shown here represent a typical tritium exchange experiment. To 4 mg of protein dissolved in 1 ml 0.1M N-ethylmorpholine were added 100 μl of tritiated water (20 mCi/ml). The mixture was left at 25°C for 20 h. The pH of the solution was then adjusted to pH 8.0 using 10% (v/v) acetic acid and the sample was passed through a column of Sephadex G10 (6.0 cm x 2.5 cm). 1 ml fractions were collected and their absorbance at 280 nm and radioactivity were measured (0h graph). The fraction containing protein were pooled and incubated at 25°C. At various time intervals 300 μl of the pooled fractions were removed and passed through a second column of Sephadex G10 (6.0 cm x 10 cm). The effluent from the column was monitored as before, collecting 300 μl fractions in this case.

——— E_{280}

------ C.p.m. x 10^{-3}


0 h
0.5 h
1 h
2 h
Absorbance
c.p.m. x 10^{-3}
Absorbance
Fraction number

$$N = \frac{110.8 \times \varepsilon p}{Co} \times \frac{C}{A}$$

where Co is the original radioactivity of the solution, A is the absorbance and C the radioactivity of a given fraction, 110.8 is the molar concentration of hydrogen in water and εp is the molar extinction coefficient of the protein. Because the Dns groups, when present, absorb strongly at 280 nm, the molar extinction of the protein was measured in each experiment using an aliquot of the original protein solution, after adjusting its pH to 8.0, and measuring the protein concentration using the biuret reaction.

The curve obtained for BPA on substituting the observed values in the above equation is shown in Figure 14. The initial rate of exchange of the tritium atoms is very rapid but as the time of exchange increases it gradually slows down and approaches a point at which no net exchange takes place, leaving a certain number of unexchanged tritium atoms in the BPA.

Having established the shape of the exchange curve for BPA by repeating the experiment several times, the rates of tritium exchange for several samples of Dns-BPA, each with a different molar ratio of Dns groups to BPA, were investigated (Fig. 15). In order to compare the curves obtained for Dns-BPA with that for a completely denatured sample of BPA, the curve produced by a preparation of BPA in which all the disulphide bridges had been broken by performic acid oxidation was also investigated. The results indicated that, although the curves obtained with Dns-BPA differed slightly from that of BPA, they are still very similar, especially when compared with the results produced by a completely denatured protein sample.

FIGURE 14.

Tritium Exchange of BPA.

The tritium exchange rate of BPA was measured as described previously (2.2.4.). Results were obtained similar to those in Fig. 13. From these, the number of unexchanged hydrogen atoms per mole of protein was calculated for the different times of exchange.

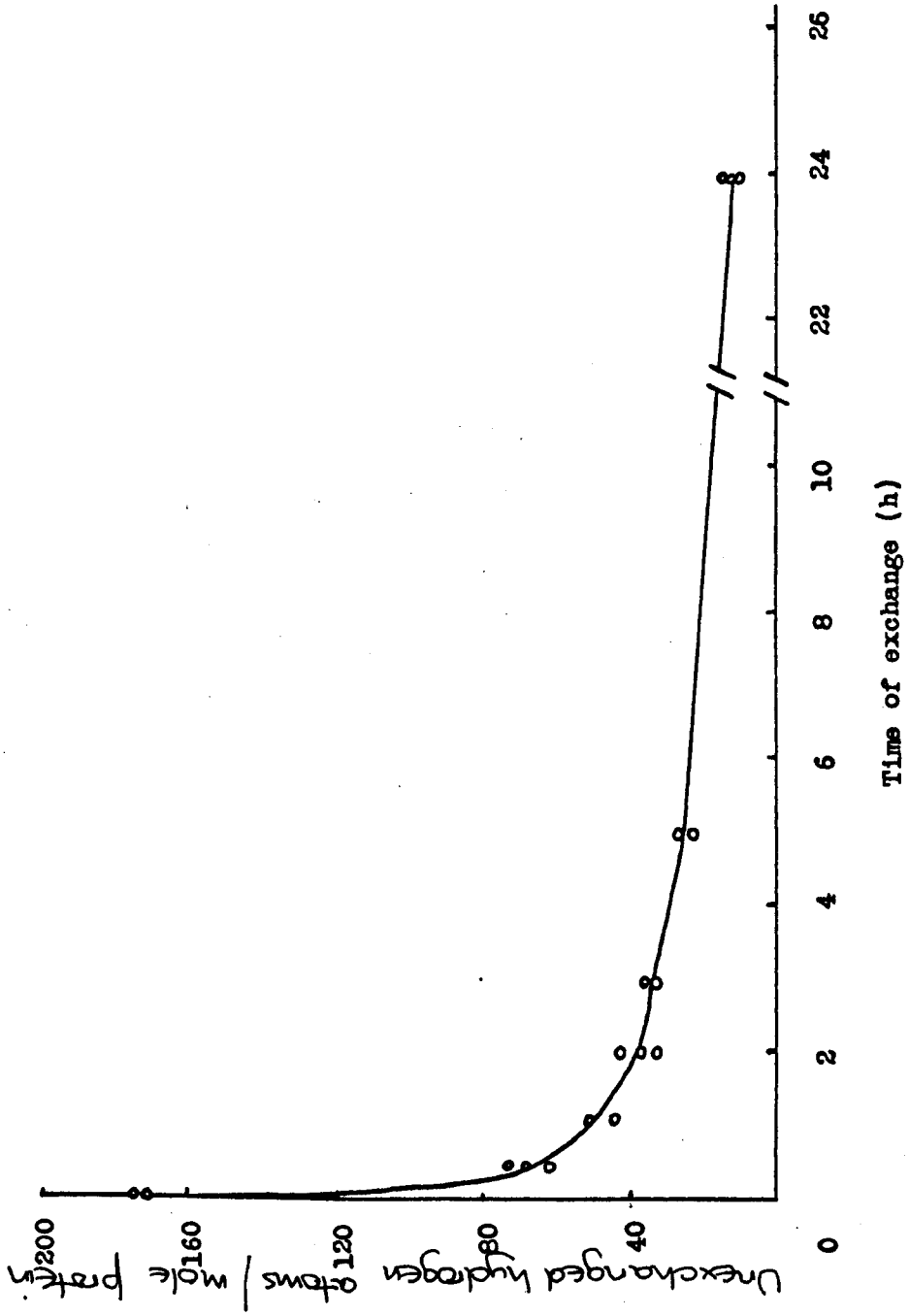

Unexchanged hydrogen atoms / mole protein
0
40
80
120
160
200
0
2
4
6
8
10
22
24
26
Time of exchange (h)

FIGURE 15.

Tritium exchange of Dns-BPA.

Tritium exchange rate measurements were carried out on samples of Dns - BPA containing different molar ratios of Dns groups to protein. Similar measurements were carried out on a sample of BPA in which the disulphide bridges had been reduced and then oxidised using performic acid.

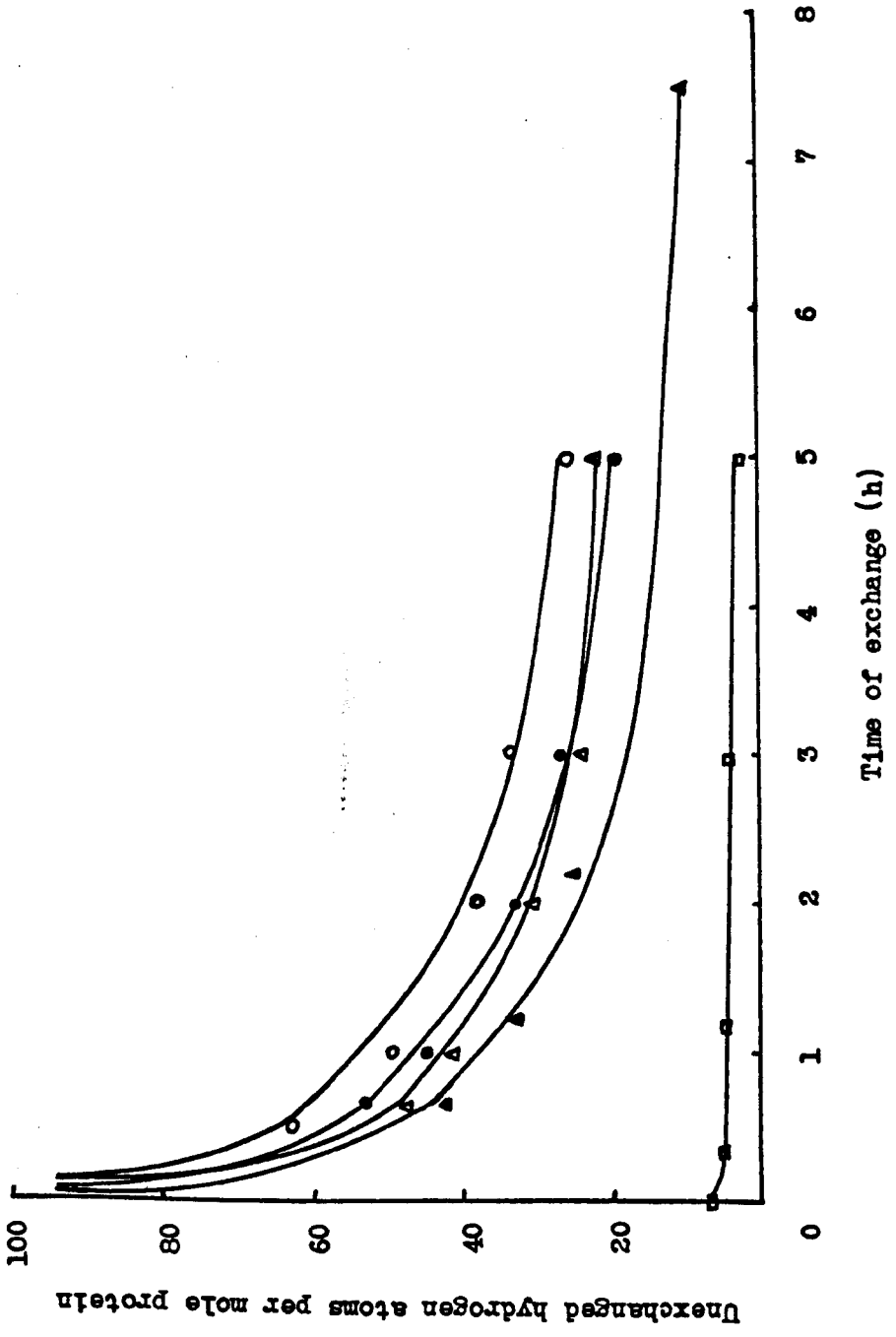
o ——— o BPA

Δ ——— Δ Dns - BPA (5.0 moles Dns groups/mole BPA)

• ——— • Dns - BPA (9.9 moles Dns groups/mole BPA)

▲ ——— ▲ Dns - BPA (17.6 moles Dns groups/mole BPA)

□ ——— □ Performic acid oxidised BPA.


Unexchanged hydrogen atoms per mole protein
100
80
60
40
20
0
1
2
3
4
5
6
7
8
Time of exchange (h)

This suggested that there may be a slight alteration in the structure of BPA when modified with Dns-Cl but that, compared with the denatured sample, Dns-BPA still retained a significant amount of the native structure of BPA. It was unlikely that binding such large amounts of reagent would not affect the shape of the protein and a slight movement of the protein structure was to be expected. Performic acid oxidised BPA was used in the above experiments because of difficulty in dissolving aminoethyl BPA in the 0.1 M NET buffer pH 8.0.

3.2.3. Circular Dichroism.

The circular dichroic spectra of BPA and of several samples of Dns-BPA were measured as described previously (2.2.5.). The samples of Dns-BPA all had different molar ratios of Dns groups to BPA. One other sample which was also studied in this way was a sample of BPA which had been subjected to all the manipulations involved in the preparation of Dns-BPA but without the addition of Dns-Cl. This was carried out to investigate whether the preparative process, in general, and the presence of acetone, in particular, had any effect on the tertiary structure of the protein. The circular dichroism of the samples is expressed in terms of (θ), the molar ellipticity. This is calculated from the measured ellipticity using the equation

$$(\theta) = \frac{\psi}{l\ m}$$

where ψ is the measured ellipticity in degrees, l is the pathlength of sample chamber in centimetres and m is the concentration in decimoles per millilitre.

The results obtained (Fig. 16) show, once more, that the Dns-BPA and the native protein are similar in structure as the spectra obtained have a close resemblance. Moreover, as an indication of the manner in which structural changes would alter the circular dichroic spectra of proteins, it has been shown that denaturation of proteins results in abolition of the trough at 220 nm and reduction, by about half, of the trough at 208 nm (Legrand and Viennet, 1964). These troughs are thought to be due to the α-helical structure present in the protein (Tiffany and Krimm, 1969). It should also be noted that the process of preparing Dns-BPA appeared to cause a slight structural change in the BPA molecule in the absence of Dns-Cl.

In an attempt to clarify the effect of increasing the degree of modification on the circular dichroic spectra, the values for (θ), the molar ellipticity, for the different samples at 210 nm and 220 nm were plotted against the molar ratios of Dns groups to BPA in the samples (Fig. 17). Since the change which occured in the circular dichroic spectrum of the protein on the binding of Dns groups was slight, the same conclusion can be drawn from these experiments as from the tritium exchange studies, i.e. when Dns groups were bound to BPA there was a slight structural change, as would be expected, but the modified protein still retained a sufficiently large amount of its native shape to make any deductions about its structure from the pattern of modification meaningful.

The final step in this study of the tertiary structure of BPA is to locate the residues modified by Dns-Cl in the amino acid sequence of the protein, as it is in this way that information about the shape of the protein can be derived from a study of its chemical modification.

FIGURE 16.

Circular Dichroic Spectra of BPA and Dns - BPA.

The circular dichroic spectra of BPA and several samples of Dns - BPA with different molar ratios of Dns groups to BPA were measured as described previously (2.2.5.). Also measured was a sample of BPA which had been subjected to the same process as required for the preparation of Dns - BPA but in the absence of Dns-Cl. This sample was called mock-reacted BPA. All measurements were carried out at 25°C in 0.1M phosphate buffer pH 8.0. The circular dichroism of the samples is expressed in terms of (θ), the molar ellipticity.

-·-·-· BPA

——— mock-reacted BPA

- - - - - Dns - BPA (5.0 moles Dns groups/mole BPA)

·——· Dns - BPA (9.9 moles Dns groups/mole BPA)

——— Dns - BPA (14.8 moles Dns groups/mole BPA

····· Dns - BPA (17.1 moles Dns groups/mole BPA)

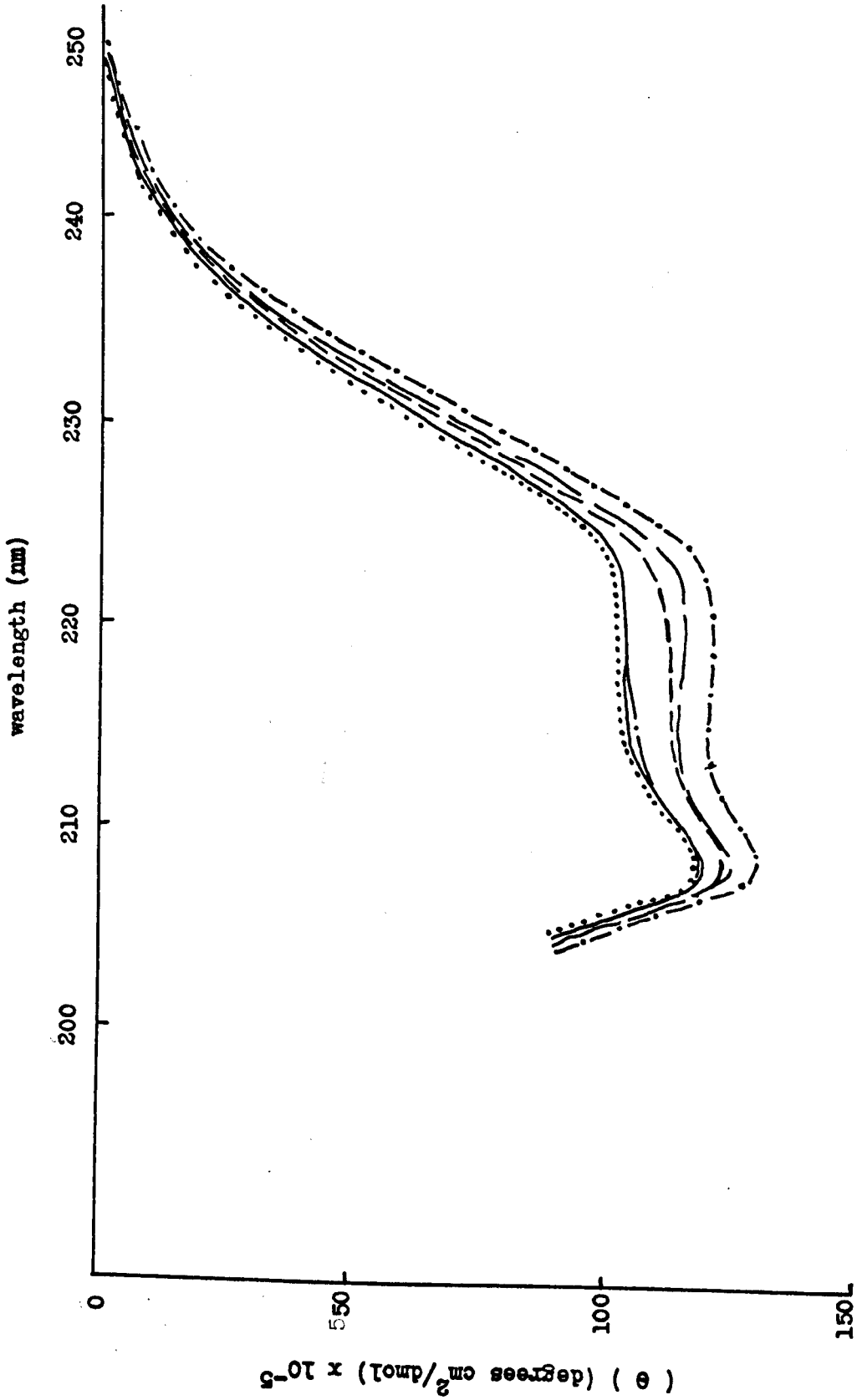

wavelength (nm)
200
210
220
230
240
250
0
50
100
150
(θ) (degrees cm²/dmol) x 10⁻⁵

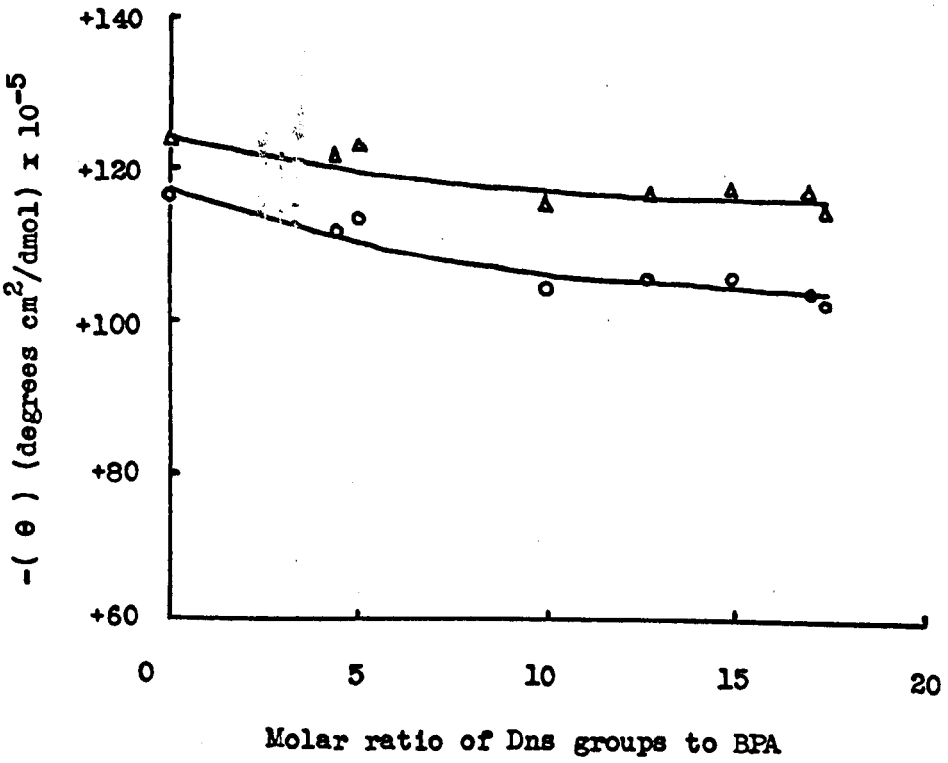
FIGURE 17.

The Relationship between the Molar Ratio of Dns Groups to BPA and Circular Dichroism.

The circular dichroic spectra of several samples of Dns - BPA were measured as described previously (2.2.5.) and the values for the molar ellipticity, (θ), of the samples at 210 nm and 220 nm were compared in terms of the molar ratio of Dns groups to BPA in each case.

o ——o molar ellipticity at 220 nm

Δ ——Δ molar ellipticity at 210 nm


+140
+120
+100
+80
+60
-(θ) (degrees cm²/dmol) x 10⁻⁵
0
5
10
15
20
Molar ratio of Dns groups to BPA

3.3. THE LOCATION OF THE MODIFIED RESIDUES IN THE AMINO ACID SEQUENCE OF Dns-BPA.

The initial step in determining which particular residues in the BPA molecule are modified on reaction with Dns-Cl was to carry out the amino acid analysis of Dns-BPA. It was shown using N^{ϵ}-Dns-lysine that amino acids modified in this way did not elute as a detectable peak in any position during a normal run on the amino acid analyser. As a result of this, if any amino acid in the protein which could react with Dns-Cl gave a lower value on amino acid analysis than expected, it would be indicative of the number of such residues which had been modified. The results of the amino acid analysis of Dns-BPA (Table 6) suggested that about ten lysine residues and six tyrosine residues had Dns groups attached to them. These results agree quite well with the calculated molar ratio of Dns groups to BPA in Dns-BPA which was modified to the maximum degree - 17.5 moles Dns groups per mole BPA. It should also be noted that one residue of aspartic acid, the N-terminal residue of the protein, will also have reacted with Dns-Cl.

The best method of locating the modified residues in the amino acid sequence of Dns-BPA is to break the protein down to small peptides and then to isolate the peptides containing the Dns groups and by studying the sequence of such peptides to fit them into the primary structure of the protein. It was decided, at first, to attempt to use only enzymatic techniques in the cleavage of the peptide chain as use of chemical techniques might lead to alterations in the Dns groups.

Before enzymatic digestion of the Dns-BPA was carried out, the disulphide bridges in the protein were reduced and the cysteine

TABLE 6.

The Amino Acid Analysis of Dns-BPA.

The amino acid acid analysis of Dns-BPA was carried out as described previously (2.2.9.). The average of six analyses is shown, compared with the amino acid analysis of BPA (Table 1.)

AMINO ACID	RESIDUES PER PROTEIN MOLECULE	
	BPA	Dns-BPA
Lysine	58	48
Histidine	17	15
Arginine	23	23
Aspartic acid	54	55
Threonine	32	33
Serine	26	27
Glutamic acid	80	79
Proline	28	28
Glycine	15	16
Alanine	44	46
Half-cystine	35	34
Valine	35	35
Methionine	4	3
Isoleucine	14	14
Leucine	58	58
Tyrosine	19	13
Phenylalanine	26	27

residues were aminoethylated (2.2.7.). Four different proteolytic enzymes were tested in order to determine which was the most suitable for an initial fragmentation of the Dns-BPA. The enzymes used were trypsin, chymotrypsin, thermolysin and pepsin. The conditions of digest, in each case have been described previously (2.2.10.). Of the four proteases pepsin was chosen as the most suitable for two reasons: (1) it was the only one which gave rise to a digestion product which was completely soluble and (2) because it was less specific in the points at which it would cleave the peptide chain than most of the other enzymes used. This would produce smaller peptides, which facilitate exact location of the modified residues, as, in many cases, no further cleavage of the peptide would be required.

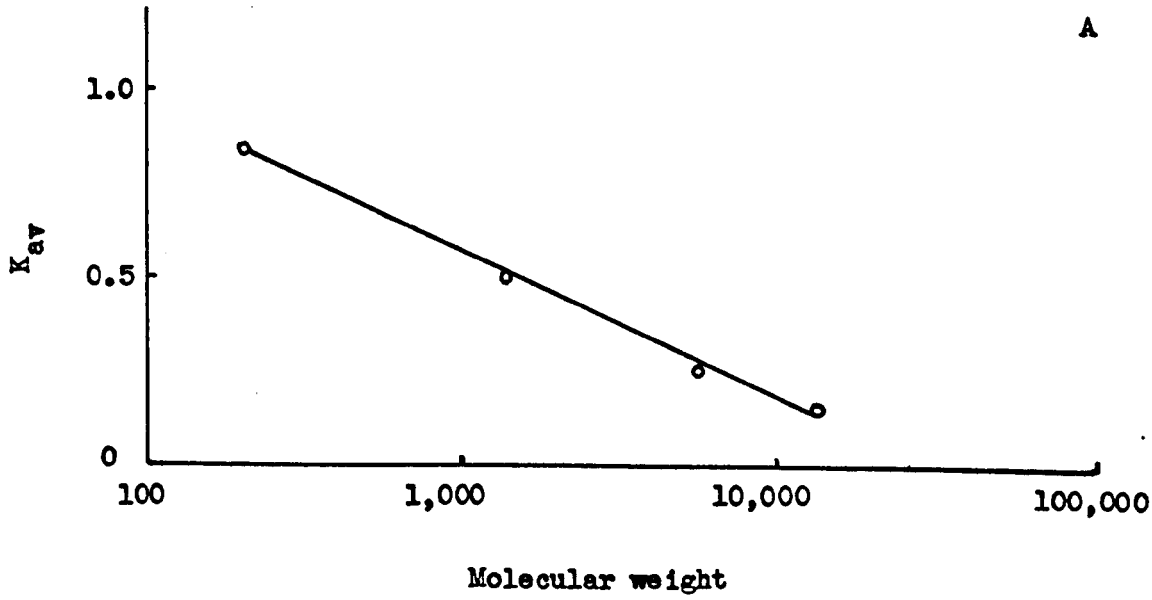
3.3.1. Characterisation of the Peptic Digest of Dns-BPA.

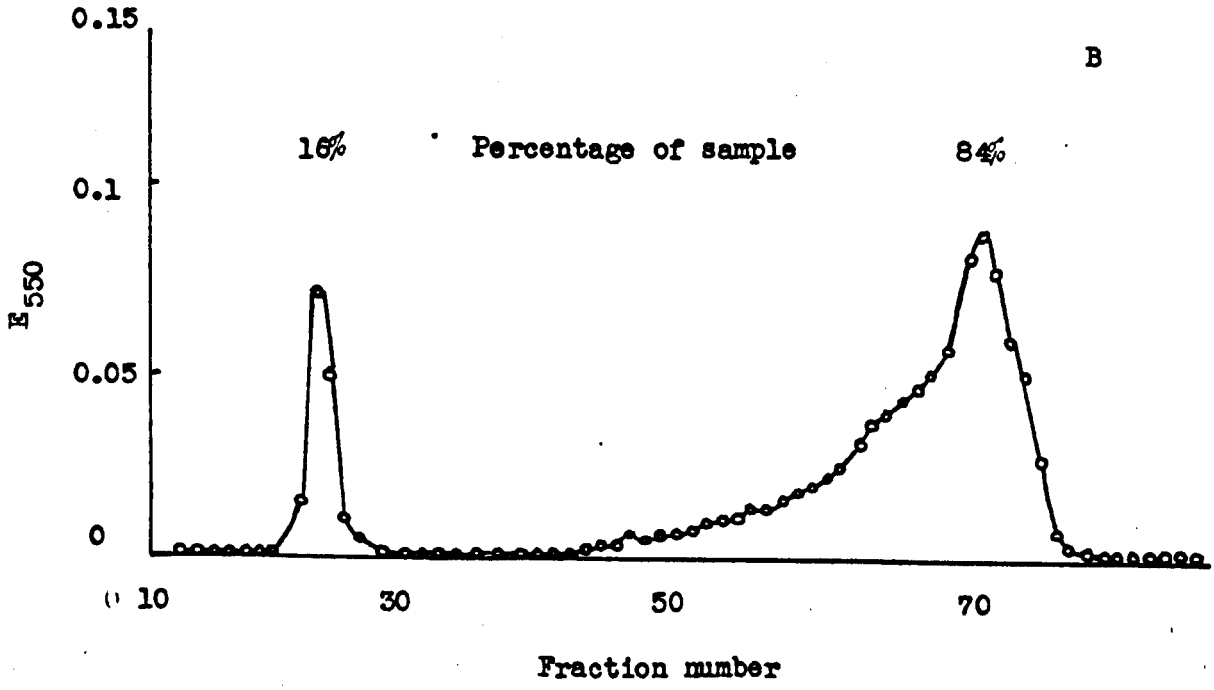
Before proceeding with a fractionation of the peptides produced on the digestion of aminoethyl Dns-BPA with pepsin, it was important to demonstrate that the cleavage occuring was virtually complete and that the fragments obtained were of a suitable size. In order to show this, an approximate molecular weight determination was carried out on the product of digestion using gel chromatography (Fig. 18). A column (115 cm x 1 cm) of Sephadex G50 fine, equilibrated with 5% (v/v) formic acid, was calibrated with proteins of known molecular weight (2.2.11.) and a relationship was established between K_{av}, the partition coefficient between the liquid phase and the gel phase, and molecular weight (Fig. 18A). 20 mg of the peptic digest of aminoethyl Dns-BPA was then chromatographed on the same column. The concentration of protein in the 1 ml fractions collected was estimated

FIGURE 18.

Gel Chromatography of the Peptic Digest of Aminoethyl Dns-BPA.

A sample of Dns - BPA was aminoethylated (2.2.7.) and then subjected to peptic digestion as described earlier (2.2.10.) 20 mg of the digest was then chromatographed on a column (115 cm x 1 cm) of Sephadex G50 fine equilibrated with 5% (v/v) formic acid. 1 ml fractions were collected and assayed for protein concentration using the buiret method (2.2.2.). The column had previously been calibrated with several proteins of known molecular weight (2.2.11.) and the relationship between K_{av}, the partition coefficient between the liquid phase and the gel phase, and molecular weight is shown in Fig. 18A. The results obtained with the peptic digest are shown in Fig. 18B. The first peak elutes in the void volume of the column. The second corresponds to a molecular weight of 680 at its maximum point.


A
1.0
0.5
0
K_{av}
100
1,000
10,000
100,000
Molecular weight


B
0.15
0.1
0.05
0
E_{550}
16%
Percentage of sample
84%
10
30
50
70
Fraction number

using the biuret method (2,2.2,).

The product of peptic digestion was separated into two peaks (Fig. 18B). The first peak eluted in the void volume of the column and accounted for 16% of the total sample. In the calibration of the column, ribonuclease A was just separated from Blue Dextran, the void volume marker, suggesting that material eluting in the void volume has a molecular weight of over 14,000 - 15,000 daltons. Hence the first peak obtained on chromatography of the peptic digest is either uncleaved Dns-BPA or large peptides produced by cleavage at only one or two sites on the molecule. The second peak is a large, assymetric peak which contains 84% of the sample. This peak will contain the small peptides produced by much more extensive cleavage of the protein and does, in fact, correspond to a molecular weight of 680 daltons at its maximum point. The first peak cannot represent a region of the molecule which is not attached by pepsin since, in order to have a molecular weight of over 15,000 daltons it must represent 25% of the sample. This indicates that the complete sequence of amino acids in BPA can be accounted for by the peptides in the second peak. Having demonstrated that peptic digestion is a suitable method of producing short peptides from Dns-BPA, the next stage in the identification of the modified residues is to separate and purify the Dns-peptides.

3.3.2. Peptide Analysis of the Peptic Digest of Dns-BPA.

The first step in the separation and purification of the Dns-peptides produced on the peptic digestion of aminoethyl Dns-BPA was to separate the peptides, using a peptide analyser (2.2.12.).

In this system the peptide mixture is subjected to ion-exchange chromatography on Chromobead P resin eluted with a gradient from 0.1M to 2.0M pyridine-acetate buffer and pH 2.75 to pH 6.5. While 4.5 ml fractions were being collected, a small amount of the eluate was continuously monitored for protein using the ninhydrin method. The fractions were then tested for absorbance at 330 nm which is indicative of the presence of Dns groups (2.2.2.).

The results obtained show (Fig. 19) that, while peptides from the peptic digest are eluted throughout the course of the gradient, all the Dns-peptides, except one, are eluted in the latter half of the gradient.

To ensure that the absorbance observed at 330 nm was due to the Dns groups present in the peptides, the relative fluorescence of fractions was also measured. This was achieved by spotting an aliquot of each on Whatman No. 1 paper and comparing the fluorescence of the spots under an ultraviolet lamp. The relative fluorescence measured in this way corresponded to the absorbance at 330 nm (Fig. 20). The first peak of absorbance at 330 nm was found to have a very blue fluorescence characteristic of Dns-OH and this probably explains why it is eluted at the beginning of the gradient since strongly acidic compounds are not retarded to any large extent, by the resin. The fractions contáining peaks of absorbance at 330 nm were pooled as shown (Fig. 20) and taken for further purification.

The column eluate was monitored for protein and absorbance at 330 nm while the column was being washed with 2M NaOH at the end of a run to ensure that all the applied sample had been eluted in the course of the gradient. As a further check, the total amount of protein eluted from the column was calculated from the results of

FIGURE 19.

Peptide Analysis of the Peptic Digest of Aminoethyl Dns - BPA (I).

3μmol of the peptic digest of aminoethyl Dns-BPA was separated by ion-exchange chromatography on Chromobead P resin. The gradient used to elute the peptides rose from 0.1M to 2.0M pyridine-acetate buffer and from pH 2.75 to pH 6.5. 4.5 ml fractions were collected and a small amount of the eluate was continuously monitored for protein using the ninhydrin method. The protein concentration is directly proportioned to the absorbance at 570 nm of the reaction product. The fractions were then examined for absorbance at 330 nm.

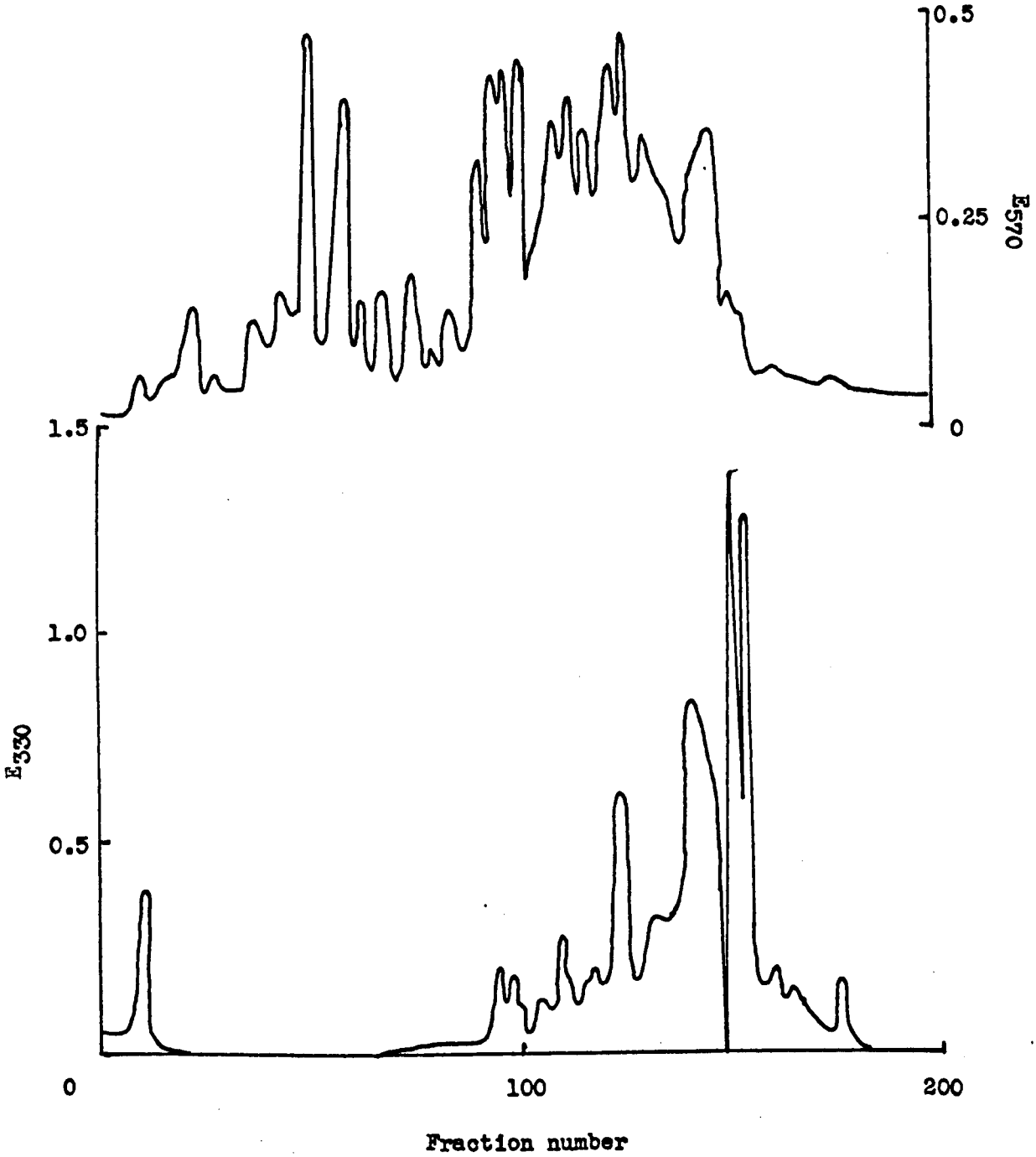
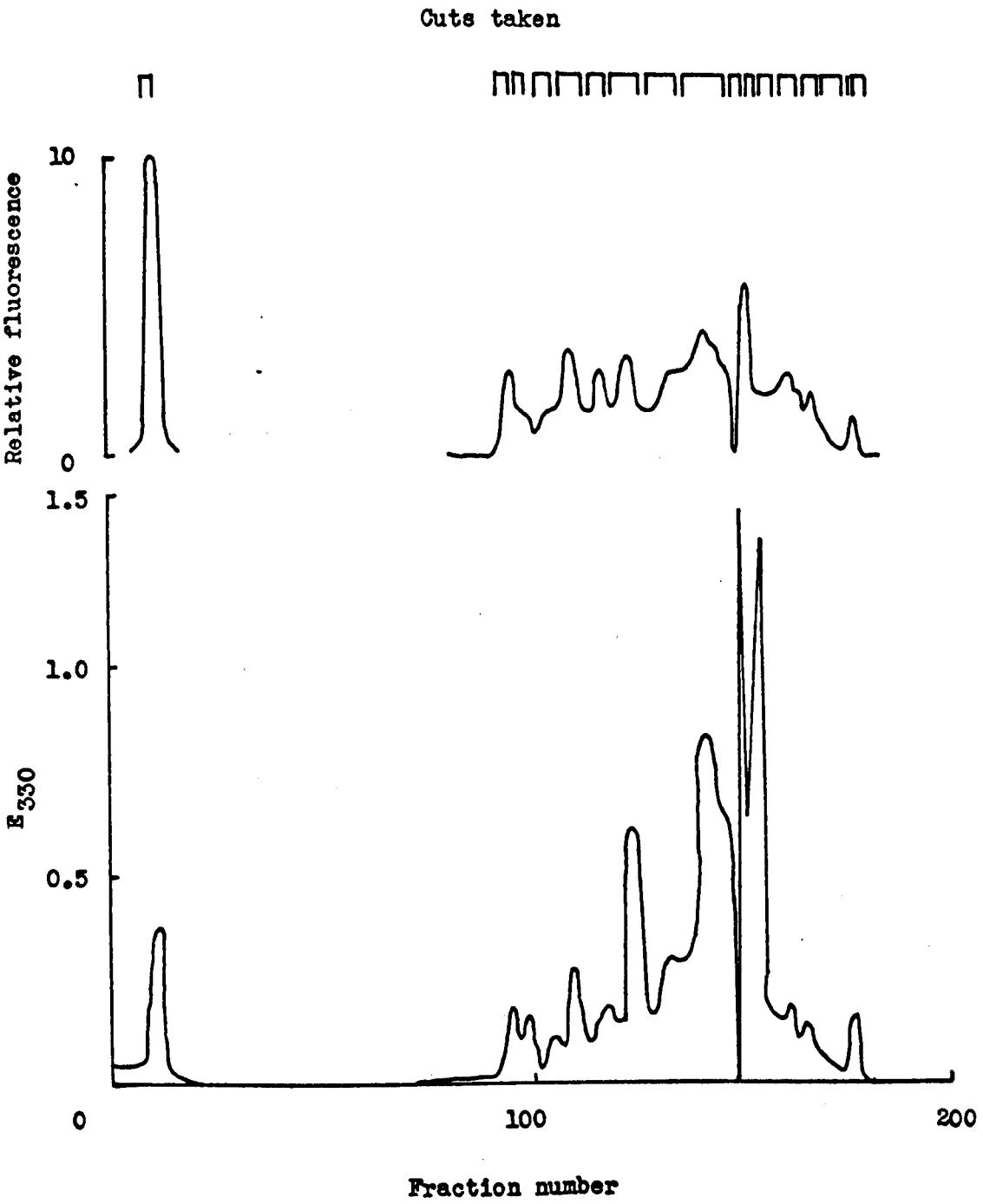

0.5
0.25
0
E570
1.5
1.0
0.5
E330
0
100
200
Fraction number

FIGURE 20.

Peptide Analysis of the Peptic Digest of Aminoethyl Dns - BPA (II).

The fractions collected in the previous experiment (Fig. 19) were also tested for their relative fluorescence. An aliquot of each was spotted on Whatman No.1 paper and the spots were compared under ultraviolet light. The fractions containing Dns - peptides were pooled as shown at the top of the diagram. The absorbance at 330 nm of the fractions, shown in the previous figure (Fig. 19) is included for reference.


Cuts taken
Relative fluorescence
10
0
1.5
1.0
0.5
E_{330}
0
100
200
Fraction number

the ninhydrin reaction on the peptides after hydrolysis. The recovery obtained was over 100% but the discrepancy is probably due to the fact that Dns groups interfere with the ninhydrin reaction (Lagunoff and Ottolenghi, 1966).

An attempt was made to improve the separation of the peptic digest of Dns-BPA on the peptide analyzer by raising the starting concentration and pH of the buffer gradient. In this way, it was hoped to expand the latter half of the original gradient. The modified gradient rose from 0.2M to 2.0M pyridine-acetate buffer and from pH 3.1 to pH 6.5. The results show (Fig. 21) that the resolution of the Dns-peptide peaks had been improved but no new peaks appeared. It was decided, therefore, to use the original system for the peptide analysis of the peptic digest of aminoethyl Dns-BPA as the same Dns-peptide peaks were obtained in fewer fractions.

3.3.3. The Purification of the Dns-Peptides.

Having achieved an initial separation of the Dns-peptides produced by the peptic digestion of amino-ethyl BPA, the pooled fractions from the peptide analyser must now be purified and the Dns-peptides isolated free of all other peptides. It was thought that, since the Dns-peptides differed from all the other peptides in that they had quite a bulky reagent group bound to them, it might be possible to produce a method of purification which would separate only the modified peptides from the remainder. For this reason, small samples of the pooled fractions from the peptide analyser were subjected to a wide range of separation techniques (Table 7). Some of the techniques tested were based on methods used for the separation of peptides while others were based on methods used for Dns-amino acids.

FIGURE 21.

Peptide Analysis of the Peptic Digest of Aminoethyl Dns - BPA (III).

The peptide analysis of the peptic digest of aminoethyl Dns - BPA (2.2.12.) was repeated but in this case the gradient used rose from 0.2M to 2.0M pyridine-acetate buffer and from pH 3.1 to pH 6.5. The eluate from the column was analysed as before (Fig. 19).

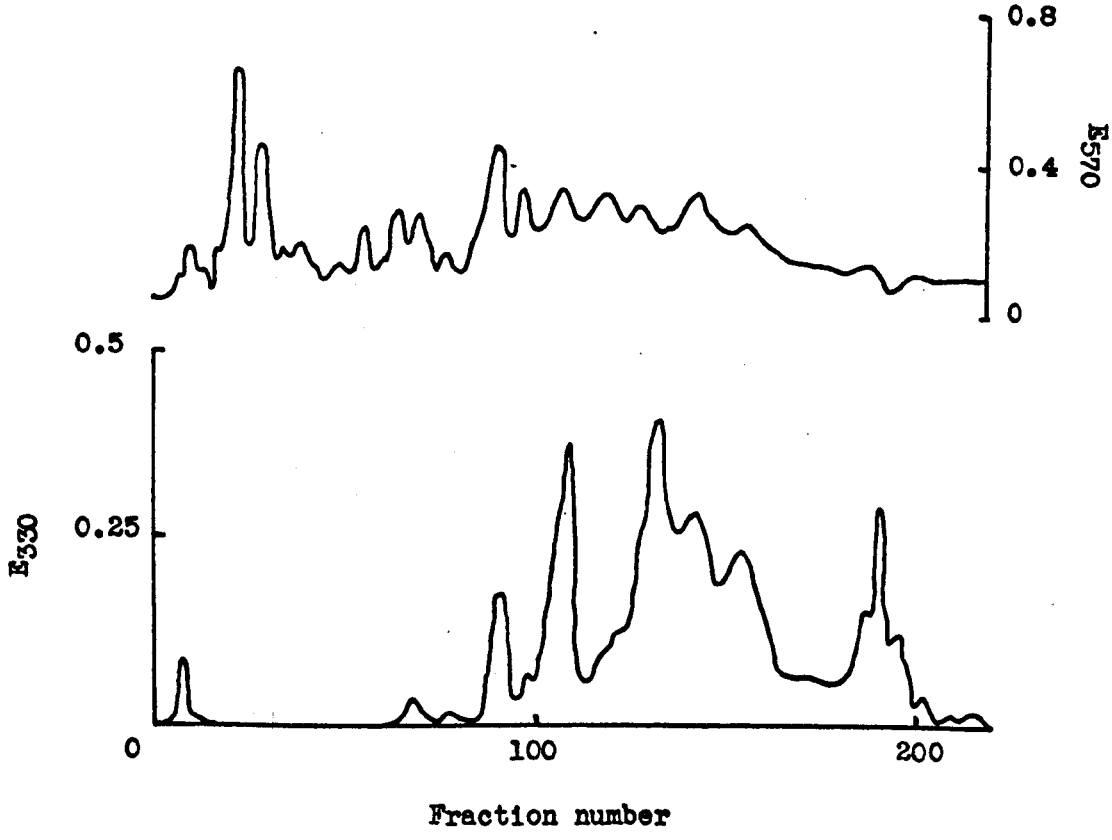

0.8
0.4
0
R_{570}
0.5
0.25
E_{330}
0
100
200
Fraction number

TABLE 7.

Methods for the Isolation of Dns - Peptides.

A number of different techniques were examined in an attempt to produce a method of isolating the peptides containing Dns groups, present in the fractions obtained by separation of the peptic digest of aminoethyl Dns - BPA on an ion-exchange column.

Electrophoresis at pH 1.8	Boyer and Talalay, 1966
Electrophoresis at pH 3.5	Bennett, 1967
Electrophoresis at pH 4.4	Gray and Hartley, 1963
Electrophoresis at pH 6.5	Bennett, 1967
Electrophoresis at pH 12.5	Gray and Hartley, 1963
Chromatography in isobutanol: acetic acid: water (15:4:2)	Zanetta et al., 1970
Chromatography in butan-1-ol: acetic acid: water (4:1:5)	Katz et al., 1959
Extraction into water-saturated ethyl acetate	Percy and Buchwald, 1972

High-voltage paper electrophoresis was tested at five different pH values. After electrophoresis, and chromatography, the Dns-peptides were easily visualised by viewing the paper under an ultra-violet lamp before staining it for peptide material using the cadmium acetate-ninhydrin stain. (2.2.14.). All, except the pH 12.5 method, provided a reasonable separation of the peptides in the mixture. Unfortunately the Dns-peptides did not behave differently from the unmodified peptides and thus they cannot be easily isolated from contaminating peptides using this technique. Electrophoresis at pH 12.5 did not give very good resolution of the peptides in the mixture as the sample did not separate into discrete spots. The two systems of paper chromatography attempted also suffered from this disadvantage. When a solution of one of the sets of pooled fractions from the peptide analyzer was extracted into water-saturated ethyl acetate it was found that fluorescence was present in both phases suggesting that, once more, the Dns-peptides did not have identical properties. Since it was apparent that it would be difficult to devise a simple method of isolating the Dns-peptides it was decided to use repeated preparative electrophoresis at different pH values to purify the modified peptides. After each electrophoresis the fluorescent peptides were located, cut out of the paper and eluted with 5% (v/v) pyridine. The peptides were then checked for purity by subjecting a small amount to electrophoresis at a different pH value. If they proved to be still impure the complete sample was then taken for a further preparative electrophoresis step. The efficiency of elution of the peptides from paper could be tested by drying the eluted paper and looking for any remaining fluorescence under an ultraviolet lamp.

3.3.4. The Sequence and Position of the Dns-Peptides.

Unfortunately, due to losses occuring in the purification methods used for the Dns-peptides, the final yields of such peptides were small.. As a result, it was decided to attempt to locate the peptides in the amino acid sequence of BPA (Brown et al., 1971) on the basis of their amino acid analyses. To test whether this method was feasible or not the published sequence of BPA was investigated and the number of possible tripeptides which contained a lysine residue was noted. The amino acid analyses which these tripeptides would give rise to were compared and it was found that out of the 95 possible analyses 47 would indicate the location of a unique tripeptide. This suggested that location of the peptides in this manner is quite possible for, if the Dns-peptides are larger than tripeptides, more of their analyses will be unique.

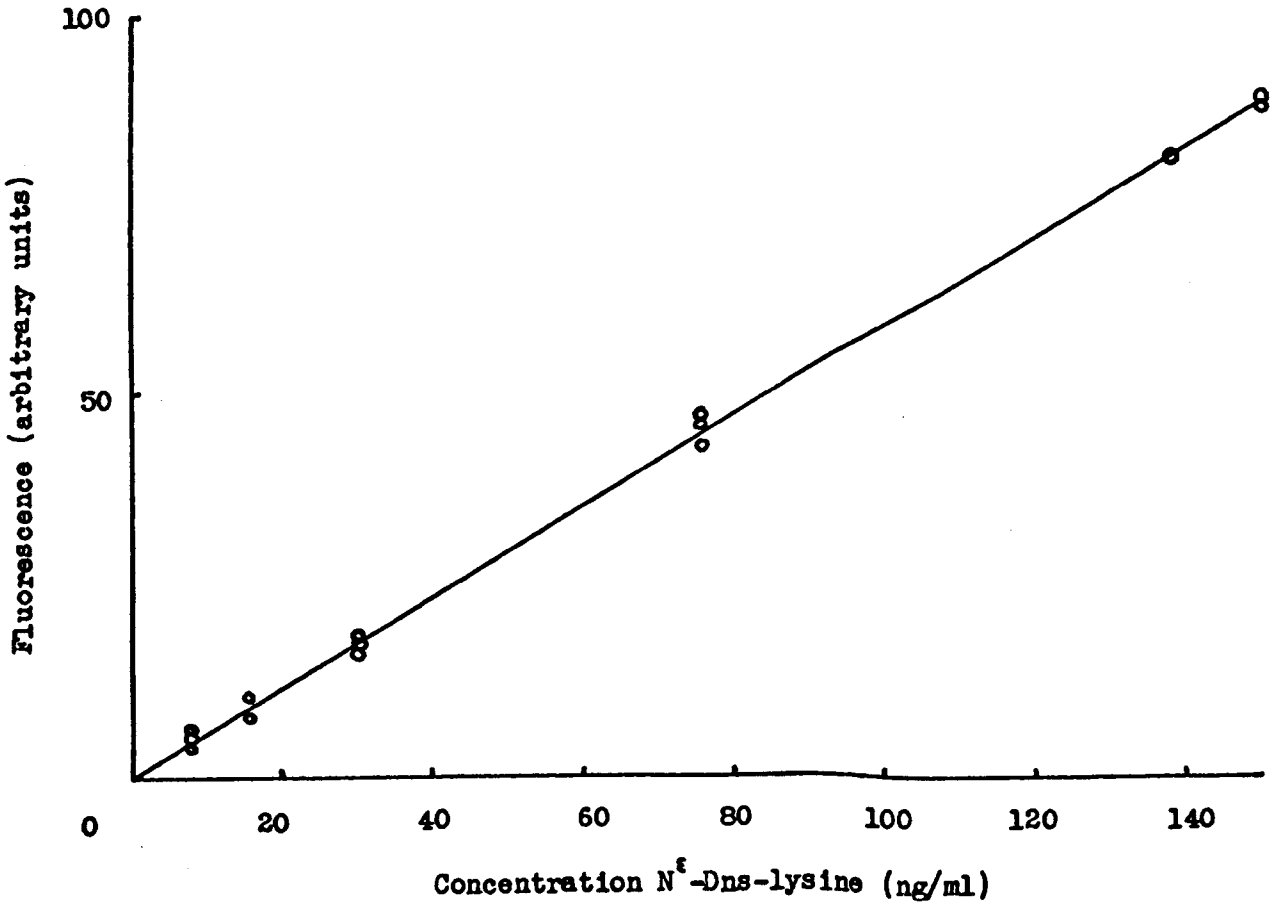
Once the Dns-peptide was obtained in an apparently pure form, an aliquot or, in most cases, all of the sample was hydrolysed for 22 h at 110°C in 100 µl 5.7M HCl. After hydrolysis 5 µl were taken and dried down to be used in the identification of the Dns-amino acid present (2.2.15.). The remainder of the hydrolysate was also evaporated to dryness. Three-quarters of the sample was then used for the amino acid analysis while the remainder was taken for fluorescence measurements to determine the concentration of Dns groups present.

The first step in the fluorescence determinations was to show that the fluorescence of a Dns-amino acid is proportional to its concentration. This was achieved by measuring the fluorescence emission at 510 nm of solutions of different concentrations of N^{ε}-Dns-lysine in 5% (v/v) pyridine excited with light at 340 nm (Fig. 22).

FIGURE 22.

The Relationship Between the Fluorescence and Concentration of N^{ϵ}-Dns - Lysine.

The fluorescence emission at 510 nm of solutions containing different concentrations of N^{ϵ} - Dns - lysine in 5% (v/v) pyridine was measured as described previously (2.2.6.). The wavelength of the exciting light was 340 nm.


100
50
0
Fluorescence (arbitrary units)
20
40
60
80
100
120
140
Concentration N$^{\varepsilon}$-Dns-lysine (ng/ml)

The results showed that the fluorescence emission was proportional to concentration over a suitable range. The concentration of the unknown samples was then calculated by measuring their fluorescence as above against a standard sample of N^{ε}-Dns-lysine. Allowance was made, where applicable, for the fact that the fluorescence of O-Dns-tyrosine is one tenth of that produced by the same concentration of N^{ε}-Dns-lysine (Gros and Labouesse, 1969).

The concentration of Dns groups, determined in this manner was then used to calculate the relative molar amounts of amino acids present in the Dns-peptide, based on the amino acid analysis. The results obtained are shown in Table 8. Meaningful amino acid analyses have been obtained for twenty-three peptides and, of these, six have been located in the amino acid sequence of BPA to within two residues. A further peptide, 12D, can be positioned in one of two unique locations. Of the remaining peptides in Table 8, seven did not contain sufficient residues to permit identification of their position while eight other peptides, mostly those with large amounts of amino acids could not be assigned to a unique location as their analyses did not correspond to any possible sequence in BPA. These latter peptides are probably still impure. From its amino acid analysis, 72B is probably free N^{ε}-Dns-lysine. Twenty-five other peptides were isolated from the peptic digest of Dns-BPA but many of these were not obtained in sufficient amounts for amino acid analysis while in the remaining cases the concentration of Dns groups, calculated from fluorescence measurements did not agree with the amino acid analysis.

As a result of the above method, one third of the Dns groups present in Dns-BPA have been assigned unique locations in the amino acid sequence of BPA.

TABLE 8.

The Amino Acid Analyses and Position in the BPA Molecule of the Dns-Peptides.

Under the heading of "purification" are listed, in order, the pH values at which preparative, high-voltage, paper electrophoresis was carried out in the purification of a given peptide. The concentration of Dns groups was calculated from the fluorescence of a sample relative to a standard solution of N^{ϵ}-Dns - lysine. This concentration is expressed in nanomoles of Dns groups present in the portion of the sample taken for amino acid analysis. The Dns -amino acid present in each peptide was identified by thin-layer chromatography (2.2.15.). The amino acid analyses are expressed in terms of moles of amino acid per mole of Dns groups. The more complex analyses are listed only in terms of the total number of amino acids present per Dns group. In calculation of the analyses allowance was made for the fact that, in a few cases, there may be more than one Dns group per peptide. The position of a modified residue in the amino acid sequence of BPA was deduced from the amino acid analysis and is listed as the number of that residue in the protein sequence.

PEPTIDE	PURIFICATION	MOBILITY **	YIELD (% Peptide Analyser Fraction)	CONCN. Dns GROUPS (nmol/Amino Acid Analysis)	Dns-AMINO ACID	AMINO ACID ANALYSIS	POSITION
2A1	1.8, 6.5, 4.4	0.35	2.6	6.6	N^{ϵ}-Dns-Lys	Asp,Pro	107,109
2A2	1.8, 6.5, 4.4	0.28	1.6	0.24	N^{ϵ}-Dns-Lys	Gly,Glu,Phe,Leu	245
3B	1.8, 6.5, 4.4		1.8	0.43	N^{ϵ}-Dns-Lys	18 amino acids	N.P.
41	1.8, 6.5	0.30	11.2	15.72	N^{ϵ}-Dns-Lys	Thr,Glu,Pro,Val	453
* 82	1.8, 3.5	0.04	15.6	27.90	O-Dns-Tyr	Lys,Asn,Gln,Glu,Ala	346-352
9A2	1.8, 3.5		2.3	1.30	N^{ϵ}-Dns-Lys	36 amino acids	N.P.
9B2	1.8, 3.5	0.13	2.4	1.22	N^{ϵ}-Dns-Lys	Leu	Am.
9B3	1.8, 3.5		3.1	3.24	N^{ϵ}-Dns-Lys	22 amino acids	N.P.
9B4	1.8, 3.5	0.22	2.4	2.72	N^{ϵ}-Dns-Lys	Lys,Thr,2Gln,Ala,Ile,Leu	476
9C1	1.8, 3.5		0.9	1.26	N^{ϵ}-Dns-Lys	18 amino acids	N.P.
9C3	1.8, 3.5	0.44	1.3	0.58	N^{ϵ}-Dns-Lys	Lys,Asp,Ser,2Glu,Leu	304, 306
101	1.8, 3.5	0.09	1.2	0.66	N^{ϵ}-Dns-Lys	Gly	Am.
102	1.8, 3.5	0.11	2.1	2.34	N^{ϵ}-Dns-Lys	Gln	Am.
11A	1.8		5.2	1.32	N^{ϵ}-Dns-Lys	11 amino acids	N.P.
12C	1.8	0.08	14.7	35.02	N^{ϵ}-Dns-Lys	Pro	Am.
12D	1.8	0.66	2.2	2.14	N^{ϵ}-Dns-Lys	Asp,2Glu,Gln,Ala	81, 158
13B3	1.8, 3.5		1.9	2.12	N^{ϵ}-Dns-Lys	47 amino acids	N.P.
14B1	1.8, 3.5	0.36	0.8	0.60	N^{ϵ}-Dns-Lys	Ser,Glu	Am.
14B3	1.8, 3.5	0.10	2.5	3.30	N^{ϵ}-Dns-Lys	2Gly,Ala,Phe	N.P.
$H_2$01	1.8, 3.5	0.06	1.1	0.82	N^{ϵ}-Dns-Lys	Gln,Ala	Am.
$H_2$02B	1.8, 3.5, 1.8	0.10	3.1	2.06	N^{ϵ}-Dns-Lys	Ala	Am.
72B	1.8, 3.5, 1.8		12.6	20.30	N^{ϵ}-Dns-Lys	Lys	
* 5B1	1.8, 3.5	0.42	6.2	5.90	O-Dns-Tyr	Lys,2Asp,Ser,Glu,Pro,Leu	N.P.

* - this sample also contains N -Dns -lysine.

** - measured at pH 6.5 relative to Asp.

N.P. - no sequence of amino acids in BPA will give rise to this amino acid sequence.

Am. - ambiguous. This peptide could have come from one of several locations in the amino acid sequence of BPA.

4. DISCUSSION

A discussion of the results presented in the previous section will be simplified if it is divided into the same three areas outlined earlier (1.6.). These are : (1) a study of the reaction of Dns-Cl with BPA, (2) the effect of the modification on the tertiary structure of BPA and (3) the position of the modified residues in the amino acid sequence of the protein.

4.1. THE REACTION OF Dns-Cl WITH BPA.

The reaction of Dns-Cl with amino acids and with the N-terminal residues of peptides and proteins has been extensively studied (Gros and Labouesse, 1969; Gray, 1964; Lagunoff and Ottolenghi, 1966). The reaction of the same compound with ε-lysine, O-tyrosine and other groups in proteins has also been examined, but to a lesser extent (Weber, 1952; Okabe and Takagi, 1971; Hartley and Massey, 1956). In the study of the chemical modification of proteins using Dns-Cl, all the studies carried out on the reaction of protein functional groups with the reagent have involved only a slight degree of modification, with only one or two Dns groups being bound per protein molecule. So far, no one has studied, in any depth, the factors influencing the reaction of Dns-Cl with proteins. It was because of the lack of information in this area that the first part of this thesis was devoted to an elucidation of the effects of various experimental parameters on the reaction of Dns-Cl with BPA.

A necessary initial step in such a study was to devise a method of determining the extent of the chemical modification occurring (3.1.2.). The first two methods tested were found to be unsatisfactory as both relied on ultraviolet absorbance measurements to determine the

concentrations of protein or Dns-groups, and the absorbances did not remain proportional to the concentrations as the degree of modification increased. A comparison of the three methods used indicated that the molar extinction coefficient of the bound Dns groups varied between 3.78×10^3 and 4.86×10^3 litre $mol^{-1}cm^{-1}$ as the degree of modification increased. Because the Dns group had an absorbance at 280 nm, it was difficult to analyse the effect of increasing the molar ratio of Dns groups to BPA on the absorbances of the protein and the reagent at 280 nm. It appeared that the absorbance of the protein at 280 nm, having been corrected for the absorbance of the Dns group and for the variation in the molar extinction coefficient, was still not proportional to the protein concentration. This suggested that either the molar extinction coefficient of the protein at 280 nm was dependent on the degree of modification, or that the absorbance of the Dns group at 280 nm is not always half of its absorbance at 330 nm.

The anomaly in the absorbance at 280 nm could be attributed to a structural change in the protein due to increasing modification, but this is unlikely as such a change, if it exists, is slight (3.2.). This phenomenon could also be due to interactions between Dns groups and protein chromophores or other Dns groups. Further evidence for this was obtained from a study of the fluorescence of several samples of Dns-BPA (3.1.7.). It was found that at high molar ratios of Dns groups to BPA the fluorescence yield of the modified protein did, in fact, decrease, suggesting some form of energy transfer from the Dns groups to other such groups or to protein chromophores.

The third method of measuring the molar ratio of Dns groups to BPA, involving the use of radioactivity and the biuret method, proved to be quite satisfactory. One other method of carrying out this

measurement, which was not tested in this system, is that of Hartley and Massey (1956). This involves extensive dialysis of the reaction mixture. In this way the Dns-OH, which is produced by the hydrolysis of the excess Dns-Cl, can be collected and its concentration calculated from its ultraviolet absorbance. This method was not used because it makes no allowance for the presence of any Dns-OH molecules which may be strongly adsorbed to the BPA molecule.

Once a method had been established for the determination of the molar ratio of Dns groups to BPA, the effect of different factors on the reaction could then be investigated. The first aspect of the reaction to be studied was the effect of increasing amounts of Dns-Cl in the reaction mixture (3.1.3.). The results showed that, under the conditions used, the degree of modification increased as the amount of Dns-Cl in the reaction mixture was increased, up to about 70 moles Dns-Cl per mole BPA. If the concentration of Dns-Cl in the reaction mixture was increased above this value it had no effect on the molar ratio of Dns groups to BPA which remained at its maximum value of about 17.5. This corresponds to about one fifth of the total number of groups which Dns-Cl could react with in BPA, based on its amino acid analysis (Table 1). Such a result would indicate that for some reason a large majority of the lysine and tyrosine side chains in BPA are prevented from reacting with Dns-Cl.

The second experimental parameter to be investigated in the reaction of Dns-Cl with BPA was the length of time for which the reaction was permitted to take place (3.1.5.). The results show that the first ten or so residues to react with Dns-Cl were modified very rapidly while the remaining functional groups reacted at a slower

rate over the next ten hours (Fig. 10). In one of the few other studies carried out on the rate of reaction of Dns-Cl with proteins, Gros and Labouesse (1969) showed that, with α-chymotrypsin, the modification was complete after 30 min. In their system, however, the reaction was carried out in 4M urea which would lead to exposure to the solvent of all the functional groups in the protein and, as a result, a faster reaction.

One problem caused by the method of preparation of Dns-BPA used in this study is that it was difficult to obtain a zero-time sample in studies of the rate of modification of the protein (1.3.5.). The reason for this is that the reaction is stopped by a centrifugation step which cannot be carried out rapidly. One way of studying the reaction over short time intervals would be to use the method devised by Gros and Labouesse (1969). Using this technique the reaction is studied spectrophotometrically at the wavelength at which there is a maximum difference in absorbancy between Dns-Cl and Dns-BPA. The reaction can be followed, by this method, from as little as 20 s after the addition of the Dns-Cl.

Having studied the effect of the reaction time on the molar ratio of Dns groups to BPA in Dns-BPA, several other factors which could influence the degree of modification of BPA were also investigated (3.1.6.).

One experimental parameter which was studied was pH. The effect of pH on the reaction of Dns-Cl with proteins is due to two factors. The first of these is the pK of the functional group, for it is this which will determine whether at a given pH the group is in the reactive or unreactive form eg. in the case of amino groups the unreactive form is $-NH_3^+$ and the reactive form is $-NH_2$. The other factor which must

be considered/

is the effect of raising the pH on the rate of hydrolysis of Dns-Cl by water since this rises exponentially above pH 9.5 due to catalysis by OH^- groups. As a result of this, if the pK of a functional group lies above 9.5, the rate and, perhaps, the extent of the reaction of that group with Dns-Cl can be increased by increasing the pH to pH9.5. However, if the pH is raised above pH 9.5 there will be no further increase in the degree of modification. Since the pKs of ε-lysine amino groups and tyrosyl hydroxyl groups lie above 9.5 it was decided to examine the effect, on the modification of BPA, of raising the pH of the reaction mixture to 9.1. As expected, a higher ratio of Dns groups to BPA was obtained in the Dns-BPA formed (Table 6). Despite this increase, less than one third of the number of functional groups which could react with Dns-Cl did so. Since the pK of the ε-lysine amino groups in BPA is abnormally low, about pH 9.8 (1.5.3.), the above result suggests that the limitations imposed on the degree of modification of BPA with Dns-Cl are not due, in the main, to the functional groups on the protein being in their protonated forms.

Although Gros and Labouesse (1969) stated that the extent of modification of a protein with Dns-Cl depended solely on the absolute concentrations of the reagents and the reactive species, when the effect of altering the concentration of the reaction mixture was studied, it was found that halving the concentration of Dns-Cl and of BPA had little effect on the ratio of Dns groups to BPA in the product. It appeared that lowering the concentrations of reagent and protein caused a slightly higher degree of modification, if anything. This could be due to an increased amount of soluble Dns-Cl being present in the reaction mixture.

One point of interest which emerges from the above experiments is that only one fifth of the total possible number of reactive groups in BPA are modified on reaction with Dns-Cl under the conditions used. On the basis of the results discussed so far, this phenomenon cannot be due to the use of low concentrations of reagents in the reaction mixture as, once a maximum degree of modification is attained, further increases in the concentration of Dns-Cl have no effect (3.1.3). Furthermore, the low molar ratio of Dns groups to BPA in the modified protein cannot be attributed to protonation of the reactive species as increasing the pH of the reaction mixture to a value close to the pK of the fifty-eight ε-lysine groups in the protein does not have a marked effect on the modification of the product (Table 5). Another possible cause which can be ruled out is the effect of the solubility of the reagent, as halving the concentrations of the compounds in the reaction mixture barely alters the degree of modification. Preferential adsorption of the reagent by regions of the protein has also been shown to influence the pattern of chemical modification, but it cannot be the cause of the low degree of modification in this case, as the use of high concentrations of reagent should overcome this effect. Elimination of the above possibilities suggests that the most probable reason for the low molar ratio of Dns groups to BPA in Dns-BPA is some form of steric effect, in which the Dns-Cl cannot react with all the reactive groups on the protein because the majority of them are not freely accessible.

In an attempt to demonstrate this steric effect it was decided to react Dns-Cl with BPA under denaturing conditions. If the reactivity of the groups on the protein is dependent on the shape of the protein, carrying out the reaction under denaturing conditions

should lead to the modification of all possible groups. When the reaction was carried out in the presence of denaturing agents (Table 5) it was found that the degree of modification obtained was, in fact, less than that obtained using the native protein. A similar result was observed by Goldfarb (1969) in his studies of the reaction of trinitrobenzene-sulphonic acid with human serum albumin. He suggested that the anomaly was due to exclusion of the reagent from the protein by the urea. A second method in which the structure of the protein was disrupted before reaction with Dns-Cl was by breaking the disulphide bonds using performic acid oxidation (Table 5). In this case there was also no increase in the degree of modification of the product. This confirms the fact that disulphide bonds are not essential for maintenance of some degree of tertiary structure in protein (Anfinsen, 1972) as the performic acid oxidised BPA is probably not denatured to a great degree. The final method which was used in an attempt to modify all the reactive groups in BPA was that of Gros and Labouesse (1969). In this experiment almost all the reactive groups do react with Dns-Cl (Table 5). The high degree of modification could be due to the use of urea at a pH of 9.4 although, when tested separately, such conditions had little effect. On the other hand, the alteration in the extent of the reaction could be due to the presence of dimethylformamide in the reaction mixture. Because there are so many parameters involved, it is very difficult to pinpoint the exact cause of the greatly increased molar ratio of Dns groups to BPA in Dns-BPA produced by this method. However, the fact that a large number of the reactive groups in BPA can be modified under these conditions does tend to substantiate the theory that the limiting

factor, which determines the degree of modification obtained, is the steric effect. At present, there is some evidence that many of the reactive side-chains in BPA are buried in the interior of the molecule. Salaman and Williamson (1971) suggested, on the basis of isoelectric focusing studies, that ten basic groups are in the interior of the protein, while Arvidsson (1972) proposed a model for BPA in which positively and negatively charged groups on the protein were involved in the formation of salt bonds within the protein.

A study of the reaction of Dns-Cl with BPA has, already, led to some information about the structure of the protein. More detailed information will be obtained once the residues which can react with Dns-Cl have been located in the amino acid sequence of the BPA but, before this can be of any value, it must be shown that the modification of BPA to the normal extent does not significantly alter the structure of the protein.

4.2. STRUCTURAL CHANGES ON THE FORMATION OF Dns-BPA

It was decided to use two different techniques to investigate whether modifying BPA with Dns-Cl caused a significant change in the protein's structure or not. The two most suitable methods for such a study were deemed to be tritium exchange and circular dichroism (3.2.). In both cases the results obtained with BPA were compared with those obtained from a series of samples of Dns-BPA, each of which was modified to a different extent.

In the set of experiments using tritium exchange, a sample of denatured BPA was also studied to provide a means of determining how close the structure of Dns-BPA was to the native protein (3.2.2.). Further support for the conclusion that the tritium exchange curves (Fig. 15) indicate that BPA and Dns-BPA are very similar can be obtained from a study of other hydrogen isotope exchange experiments. Benson and Hallaway (1970)have shown that the difference in the number of unexchanged hydrogen atoms per mole of protein between BPA in solution at pH 6.2 and in solution at pH 6.4 is about twelve. No detectable structural change occurs in BPA between these pH values (Leonard et al., 1962; Harmsen et al., 1971). The difference between Dns-BPA and BPA is, at most, fifteen unexchanged hydrogen atoms per mole of protein which, in view of the above results, suggests that Dns-BPA and BPA are not significantly different in terms of structure.

Unfortunately, in the case of the circular dichroism experiments, BPA has not been studied to the same extent as it has with tritium exchange. Because of this any deductions about the structural similarity of BPA and Dns-BPA have to be based on the effect of

denaturation on the circular dichroism of proteins (3.2.3.).

Thus the results of two different techniques of detecting structural changes caused by the modification of BPA by Dns-Cl indicate that there is no significant difference between the tertiary structures of BPA and Dns-BPA. This finding also shows that in the course of the modification no abnormal formation of oligomers occurs. This might have given rise to erroneous results in the study of the factors affecting the reaction of Dns-Cl with BPA.

Since there is little difference between the structures of the native and modified protein, deductions can now be made about the shape of BPA, based on the position in the amino acid sequence of the residues which have reacted with Dns-Cl.

4.3. THE LOCATION OF THE MODIFIED RESIDUES IN THE AMINO ACID SEQUENCE OF Dns-BPA

In order, first of all, to determine which residues of BPA had been modified, an amino acid analysis of Dns-BPA was carried out (3.3.). The results obtained agreed well with the calculated molar ratio of Dns groups to BPA in the modified protein. However, if the extent of hydrolysis of the Dns-amino acids which occurs in the preparation of the protein for amino acid analysis (Gros and Labouesse, 1969) is taken into consideration, the agreement is not so good. Gros and Labouesse (1969) found that after 18h of hydrolysis in 5.7M HCl at 110° the recoveries of N^{ε}-Dns-lysine and O-Dns-tyrosine were 80% and 60% respectively. The results for N^{ε}-Dns-lysine were confirmed by carrying out the hydrolysis of the compound under the conditions used in this work (2.2.9.). Corrected for hydrolysis, the results suggest that about twelve lysine residues and ten tyrosine residues have been modified. This is slightly higher than the molar ratio of Dns groups to BPA calculated from the assay system (3.1.2.) which is 17.5. Despite this, the results of the amino acid analysis of Dns-BPA do provide an approximate idea of the distribution of the Dns groups between the lysine and tyrosine residues.

In order to locate the modified residues in the amino acid sequence of the BPA, the protein has to be broken down into small peptides. The peptides containing a Dns group can then be isolated and identified. The peptic digestion of aminoethyl Dns-BPA was selected as the best method of carrying this out (3.3.) and the products of digestion were shown to be of a suitable size (3.3.1.).

The initial separation of the enzymic digest was performed

using an ion-exchange column and a peptide analyser. The Dns-peptides were eluted from the column during the second half of the gradient (Fig. 19). This is to be expected as peptides containing aromatic residues are known to be delayed to some extent in the system used.

An attempt was then made to find a suitable purification method for the Dns-peptides fractionated on the peptide analyser. Since the Dns-peptides were different from all the other peptides present in that they had a Dns group bound to them, it was hoped that a method could be found which would select only the Dns-peptides from the mixture. A large number of different techniques were tested (Table 7) but no system was found in which the Dns-peptides behaved any differently than all the other peptides. This probably is due to the fact that the Dns-peptides are of sufficient size that they will retain many of the properties of normal peptides, and the Dns group attached to them will not be able to overwhelm the peptide-like behaviour of the Dns-peptides. As a result of this, the Dns-peptides were purified using the normal practice for peptides of preparative paper electrophoresis at several different pH values.

Once the Dns-peptides had been purified, a sample of each was taken for amino acid analysis. The position of each peptide in the amino acid sequence of HPA was deduced from its analysis. This has been previously shown to be possible (3.3.4.), depending on the size of the peptide. A sample of the peptide after hydrolysis was used for the identification of the Dns-amino acid present in the peptide, while another aliquot was used to determine the concentration of Dns groups in each sample (Table 8). On the basis of this information, several of the Dns-peptides could be allocated unique

positions in the amino acid sequence of the protein elucidated by Brown et al. (1971). In allocating the positions of the Dns-peptides, one region for which the sequence has not yet been determined (residues no. 260-284) was not considered and it is possible that some of the peptides for which a position could not be found arise from this area. Another problem in the location of the Dns-peptides is that the sequence drawn up by Brown et al. (1971) is still in a preliminary form and, as a result, errors could exist in it. This has been shown to be the case by the work of King and Spencer (1972) who have completely sequenced two regions of the BPA molecule. However, the differences that they found did not permit any further allocation of the Dns-peptides analysed so far. The work of both groups mentioned above is at such a stage that the complete sequence of the BPA molecule should be elucidated in the near future, permitting a more rigorous identification of the modified residues.

The main problem in the location of the Dns-peptides in the amino acid sequence of BPA is that very low yields were obtained on the purification of the peptides. In many cases this does not permit a detailed analysis of the peptides. The cause of the low yields is not clear. The completeness of the elution of the peptides from paper can be checked by drying the paper after elution and testing for residual fluorescence. When this was carried out, it appeared that the elution procedure was comparatively efficient. One reason for the low yields could be that the Dns-peptides adhere to glass. This has been shown to occur with other proteins (Lagunoff and Ottolenghi, 1966). In an attempt to test for this 100 nmol of N^{ε}-Dns-lysine

was subjected to the conditions used for hydrolysis of the Dns-peptides, evaporated to dryness, redissolved in 5% (v/v) pyridine, removed into a second glass tube, diluted and taken for measurement of its concentration using the fluorescence method outlined earlier (3.3.4.). This is the same process that the Dns-peptides undergo. In the case of N^{ε}-Dns-lysine it was found that no loss of material had occurred. Although this might suggest that the Dns-peptides also suffer no losses as a result of this process, such an assumption cannot be taken as correct, since the peptide attached to the Dns group may alter its properties.

Several steps could be taken to overcome the losses which result from purification of the Dns-peptides. One method would be to scale up the whole process, but because of the limits of the peptide analyser and the preparative paper electrophoresis, this would entail running many duplicate samples and then pooling them. A second method would be to alter the fractionation procedure in order to reduce the number of peptides present in the digest before the separation and purification steps. This could be achieved, for example by carrying out a cyanogen bromide cleavage of Dns-BPA. This would give five fractions (King and Spencer, 1970) which could then be separated before further enzymic cleavage. A third, and probably the best method of reducing losses on purification would be to design a system in which the Dns-peptides could be selectively removed from the digest of the modified protein. One such system would be the use of immobilised antibodies to Dns groups to purify Dns-peptides from the other peptides. It is this latter technique which really merits consideration in an attempt to improve the study of the tertiary structure of proteins using

chemical modification with the reagent Dns-Cl.

Despite the low yields obtained on the purification of Dns-peptides, six of them were located uniquely in the amino acid sequence of BPA (Table 8). In some cases the location of the modified amino acid could only be determined to within two residues. This occurred where two lysine residues were separated by a single amino acid and it was uncertain which of the two was modified. One other peptide could be located in one of two unique positions in the amino acid sequence of the protein.

The peptides listed in Table 8 as being ambiguous all had at least five and, in some cases, as many as twenty-one possible positions. If the ambiguous peptides are considered, it is found that, of the seven, all but one could be due to overlap of the positions of modification already identified. The remaining peptide, no. 101, must correspond to another site of modification of BPA. There are, in fact, only five possible locations for such a modification, given the amino acid analysis of the peptide. These are residues no. 3, 9, 17, 19 and 48.

One other interesting fact to emerge from Table 8 is that very few O-Dns-Tyrosine peptides have been analysed. When this is compared with the numbers of ε-lysine and O-tyrosine groups which have reacted with Dns-Cl (3.3.), it suggests that the O-Dns-tyrosine peptides are more susceptible to losses on purification.

It should be noted that the six peptides which have been located unambiguously in the amino-acid sequence of BPA do, in fact, account for seven of the modified residues in the protein, since peptide 82 contains both an O-Dns-tyrosine and an N^{ε}-Dns-lysine group.

One final point which should be noted concerning the location

of the Dns-peptides in the amino acid sequence of BPA is that it may prove impossible to identify all 17-18 modified residues, as some of the Dns-Cl may have reacted with the imidazole groups of histidine residues. Such Dns-amino acids cannot be isolated as they are very susceptible to hydrolysis in strong acid (Gray, 1967). However, the results of the amino acid analysis of Dns-BPA (3.3.) indicate that very few, if any, histidine residues are modified by Dns-Cl, as the number of Dns groups,estimated from amino acid analysis is greater than the value obtained from the assay system (3.1.2.).

4.4. THE TERTIARY STRUCTURE OF BPA

Having identified some of the residues which are modified when Dns-Cl reacts with BPA, the final step is to determine how much information about the tertiary structure of BPA can be derived from these results. If it is assumed that the modification of a residue with Dns-Cl depends on its degree of exposure to the solvent, then the results in Table 8 suggest that there are, so far, about 8 areas of the peptide chain of BPA which can be said to be exposed, in some way, in the tertiary structure of the protein. These areas are spread throughout the peptide chain. It was felt that, in the models for the tertiary structure of BPA proposed at present (Figs. 1-3), the lengths of the peptide backbone which are most likely to be exposed are those which are involved in linking the "pseudosub-units" (1.6.). It was hoped that the functional groups in such regions would be readily modified by Dns-Cl. However, a comparison of the results obtained (Table 8) with the structure of the models showed that none of the modified residues was found in a region of the chain corresponding to the sections linking the "pseudosub-units".

The main piece of structural information which can be derived from the results obtained in this study is the surprisingly low reactivity of the functional groups in the protein towards Dns-Cl. It has been shown earlier (4.1.) that this phenomenon is probably caused by a steric effect which renders many of the reactive groups in the protein inaccessible to the reagent used. Heterogeneity in the reactivity of lysine and tyrosine side-chains has also been demonstrated by other types of chemical modification of BPA. Riordan *et al.*, (1965) found that, when BPA was reacted with

N-acetylimidazole, only four of the tyrosine residues were modified. In studies with other proteins it was found that the number of tyrosine residues reacting with N-acetylimidazole corresponded closely to the number of such residues thought to be exposed at the surface of the protein on the basis of spectrophotometric titrations (Crammer and Neuberger, 1943).

In studies of the heterogeneous nature of lysine residues in serum albumin, Goldfarb (1970) reported the presence, in human serum albumin, of lysine groups having three distinct velocity constants for the reaction with trinitrobenzenesulphonic acid. The results obtained by Jonas and Weber (1970) on the partial modification of BPA with dicarboxylic anhydrides suggested that about 20% of the lysine residues in the protein were buried.

One problem in using chemical modification to investigate the extent to which reactive groups are exposed in a protein is that the number of groups which are classified as being on the surface of the protein can vary depending on the reagent used. Boyd et al., (1972) found that, using N-acetylsuccinimide, 50% of the lysine residues in BPA could be acetylated, whereas, using N-acetylimidazole only 30% of the lysine residues reacted. On the other hand, using a different reagent, Habeeb (1966) found that virtually all the lysine residues in BPA could be acetylated. He also showed that all the lysine residues in the protein could also be succinylated, nitroguanylated, guanylated and amidinated, indicating that the accessibility of reactive groups in a protein must be defined in terms of the reagent used to study them.

One other interesting fact which emerged from the work of Habeeb (1966) was that the effect of modification on the tertiary structure of the protein depends on the reagent used. He found that amidination and

guanidination produced no significant structural change, while nitroguanidination produced a moderate change and succinylation and acetylation caused a large alteration in the shape of the protein.

The one advantage that the present study has over most of the other investigations of chemical modification is that an attempt is being made to locate the reactive residues in the amino acid sequence of the protein. This will give much more information about the structure of the protein. At the moment, completion of this part of the study is dependent on the improvement of the purification methods for the Dns-peptides.

Once the required information has been obtained and a more detailed picture of the tertiary structure of BPA has emerged, the system outlined in this work can be adapted to study several other aspects of the shape of the protein. One example of this is that, by altering the degree of modification, the order in which the residues react with Dns-Cl can be established. A second application of the chemical modification of BPA with Dns-Cl is in the identification of the binding sites for small molecules on BPA. This can be studied by binding a given molecule to the protein, modifying the BPA and then determining whether binding of the small molecule has masked any of the residues which react with Dns-Cl. A similar application would be in the study of the regions of BPA which are involved in the dimerisation of the protein. It is also hoped that, since the modification has been extensively studied in BPA, the system can also be applied to an investigation of the tertiary structure of other proteins.

In conclusion, therefore, it may be stated that the work carried out in the course of this thesis has demonstrated several points:

(1) the reaction of Dns-Cl with a protein to produce a large degree of modification has been well characterised. (2) The best conditions for the production of Dns-BPA have been established. (3) The extensive modification of BPA has been shown not to significantly affect the structure of the protein. (4) It has been discovered that the normal methods of peptide purification are probably not the best way of isolating the Dns-peptides produced on digestion of Dns-BPA. (5) The only problem preventing a large amount of information about the tertiary structure of BPA being obtained is the design of a purification method for the Dns-peptides. Study of this last point will lead to completion of the system for investigating the structure of BPA using chemical modification with Dns-Cl.

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SUMMARY

The main aim of this study was an attempt to produce a simple method of investigating the tertiary structure of bovine plasma albumin (BPA). It was hoped that the techniques devised would be applicable to the study of other proteins of a similar size.

There were two main reasons for using BPA in this study. Firstly, it is easily obtainable in a relatively pure form. Secondly, the tertiary structure of this protein is of interest because it binds an unusually large and structurally very diverse range of compounds. This binding is largely determined by the structure of the protein.

The basic idea behind this research was to bind a reactive molecule to certain residues in the protein, enzymatically digest the modified protein and separate the peptides to which the molecule is bound. Amino acid analysis of the peptides would then locate the modified residue in the primary sequence of the protein, which is already virtually complete. If a sufficiently large modifying reagent was used, it was hoped that only the reactive groups exposed on the surface of the protein would be able to react. When the reacted groups were then positioned in the amino acid sequence of BPA they would indicate which regions of the peptide chain lay at, or near, the surface of the protein. This, in turn, would permit deductions to be made about the shape of the protein.

It was decided to use the fluorescent reagent 1-dimethylamino-naphthalene -5- sulphonyl chloride (Dns-Cl) in this study as, for several reasons, it appeared to be very suitable.

The initial step in this study was to investigate the extent of, and the factors which influence, the reaction of Dns-Cl with

BPA. Before this could be carried out, it was essential to devise a method of determining the molar ratio of Dns groups to BPA in the modified protein. Three different assay techniques were tested and a suitable system was selected.

When the reaction of Dns-Cl with BPA was studied it was found that under normal conditions 17-18 Dns groups were bound to each BPA molecule. On the basis of the amino acid analysis, this would suggest that only about one-fifth of the reactive groups in the protein have been modified. Alteration of several experimental parameters did not greatly increase the degree of modification obtained. If, as is suggested, the major restriction on the modification of groups in the protein is a steric effect, it should prove possible to modify all the reactive groups in BPA if the tertiary structure of the molecule is disrupted. This was also investigated and shown to be possible.

Having studied the reaction of Dns-Cl with BPA, the next step was to show that the structure of the modified BPA was not significantly different from that of the native protein. This is essential if it is intended to relate the pattern of modification to the tertiary structure of the original protein. The structures of the modified and native proteins were investigated using circular dichroism and tritium exchange. In both cases slight structural changes were found on modification, but these were not significant compared with other well-characterised changes in protein structure.

The final stage in this study is to locate the residues, which have been modified, in the amino acid sequence of BPA. Several proteolytic enzymes were examined and it was found that pepsin was most suitable for the degradation of Dns-BPA. The mixture of Dns-

peptides produced by this digestion was initially separated using a peptide analyser. A number of fractionation procedures were tested for use in the further purification of the Dns-peptides. As a result of this investigation it was decided to use high-voltage paper electrophoresis. Once the Dns-peptides had been purified, they were subjected to amino acid analysis, the modified residue was identified and, where possible, the amino acid which had reacted with Dns-Cl was located in the amino acid sequence of the protein. In this way, eight of the modified residues were identified and it appeared that the exposed regions of the peptide chain occurred at regular intervals in the sequence. However, the location of the remaining modified residues, and the more detailed information which would result from this, could not be deduced due to low yields on the purification of the Dns-peptides. Until this problem has been overcome, the main result of this research is the information that has been obtained concerning the unusually high number of reactive groups which are not exposed on the surface of the protein in its native form and a comprehensive survey of the reaction of high concentrations of Dns-Cl with HPA.

EPILOGUE

Ah, yes, I wrote the 'Purple Cow' -
I'm sorry, now, I wrote it!
But I can tell you, anyhow,
I'll kill you if you quote it!

Frank Gelett Burgess

NH2-Asp-Thr-His-LYS-Ser-Glu-Ile-Ala-His-Arg-Phe-LYS-Asp-Leu-Gly-Glu-Glu-His-Phe-LYS-Gly- Asp-Glu-His-Val-LYS-

2 30 40 50

Leu-Val-Asx-Glx-Leu-Thr-Glu-Phe-Ala-LYS- Thr-Cys-Val-Ala-Asp-Glu-Ser-His-Ala-Gly-Cys-Glu-LYS- Ala-Asp-

60 70

Glu-LYS- Ser-Leu-His-Thr-Leu-Phe-Gly-Asp-Glu-Leu-Cys-LYS-Val-Ala-Ser-Leu-Arg-Glu-Thr-TYR-(Gly,Asp)-Met-

80 90 100

Ala-(Cys,Cys,Glu,Asp)-LYS-Glu-(Glu,Gln,Pro)-Arg-Asn-Glu-Cys-Phe-Leu-Ser-His-LYS-Asp-Asp-Ser-Pro-Asp-Leu-

110 120

Pro-LYS-(Leu,Pro,Asp)-LYS- Leu-LYS-Pro-Asp-(Pro,Asn)-Thr-Leu-Cys-Asp-Glu-Phe-LYS-(Asx,Glx)-Ala-LYS-

130 140 150

LYS-Phe-Trp-Gly-LYS-TYR-Leu-TYR-Glu-Ile-Ala-Arg-Arg-His-Pro-TYR- TYR-Ala-Asn-LYS-TYR-Ala-Gly-Val-Phe-Glx-

160 170

Cys-(Glx,Glx,Glx,Cys,Ala,Asx)-LYS-Gly-Ala-Cys-Leu-Leu-Pro-LYS-Ile-Glu-Thr-Met-Arg-Glu-LYS-Val-Leu-Thr-Ser-

180 190 200

Ser-Ala-Arg-Gln-(Cys,Asx,Glx,Phe)-LYS- Leu-Arg-Cys-Ala-Ser-Ile-Gln-LYS- Leu-Val-Thr-Asp-Leu-Thr-LYS-

210 220

Val-His-LYS-Pro-LYS- TYR-Thr-Arg-LYS-Val-Pro-Gln-Val-Ser-Thr-Pro-Thr-Leu-Val-Glu-Val-Ser-Arg- His-Leu-

230 240

Val-Asp-Glu-Pro-Gln-Ile-Leu-Asn-LYS- Ile-LYS-Glu-Asp-Cys-(Asp,Gln)-Phe-Glu-LYS-Leu-Gly-Glu-TYR- Glu-

250 260 270

TYR-Glu-(Cys,Glu,LYS)-Ala-Thr-Leu-Glu-Glu-(Cys,Cys,Ala)-LYS- (Ala,Ser,Thr,Asp,Asp,Thr,Ser,Glx,Glx,

280 290

Glx,Glx,Pro,Ala,Ala,Leu,TYR,Cys,Cys,Cys,His,LYS)-Val-(Phe,Asp)-LYS- Leu-LYS- Ser-Arg- TYR-Ile-

300 310

Cys-Asx-(Asx,Glx,Asx,Ser,Ile,Ser,Thr)-LYS- Leu-LYS-Glu-Cys-LYS-(Pro,Cys,Asp)-Leu-Leu-Glu-LYS-Ser-His-

320 330 340

Cys-Ile-Ala-Glu-Val-Glu-LYS-Asp-Ile-Pro-Glu-Asp-Glu-Pro-Pro-Leu-Thr-Ala-Asp-Phe-Ala-Glu-Asp-LYS-Asp-Val-Cys-LYS-

350 360 370

Asn-TYR-Gln-Glu-Ala-LYS-Asp-Ala-Phe- Leu-Glu-Cys-Ala-Asp-Asp-Arg-Ala-(Asp,Leu,Ala)-LYS- Leu-Ser-Gln-LYS-

380 390

Phe-Pro-LYS-Ala-Glu-Phe-Val-Glu-Val-Thr-LYS- Ser-Leu-Gly-LYS-Val-(Thr,Gly)-Arg-Cys-Cys-Thr-LYS-Pro-Glu-Ser-

400 410 420

Glu-Arg-(Leu,Asp,Glu,TYR,Cys,Met,Leu,Leu,Asp,Ile,Pro,Ser,Leu)-Arg-Arg-Pro-Cys-Phe-Ser-Ala-Leu-Thr-Pro-Asp-Glu-

430 440 450

Thr-TYR-Val-Pro-LYS-Ala-Phe-Asp-Glu-LYS-Leu-Phe- Ser-(Asp,Asp,Thr,Thr,Glu,Glu,Ala,Leu)-LYS-Leu-Cys-Val-Leu-

460 470

His-Glu-LYS- Thr-Pro-Val-Glu-Ser-LYS- LYS-Val-Thr-LYS-Cys-Cys-Thr-Glu-Ser-Leu-Val-Asn-Arg- Ile-Gln-LYS-

480 490 500

LYS-LYS-Gln-(Ala,Thr)-Leu-Val-Glu-Leu-Leu-LYS-His-LYS-Pro-LYS-Ala-Thr-Glu-Glu-Gln-Leu-LYS- LYS-Thr-Val-Met-

510 520

Glx-Asx-Phe- (Glu,Ala,Cys,Cys,Gln)-Phe-Ala-Val-Glu-Gly-Pro-LYS-Leu-Val-Val-Ser-Thr-Gln-Thr-Ala-Leu-Ala-COOH

APPENDIX : The Amino Acid Sequence of EPA (Brown et al., 1971)

Lysine and tyrosine residues are shown in capitals for clarity.