THE NUCLEOPROTEIN OF CALF THYMUS GLANDS

THE REACTION OF MANNOSE WITH GLUCOSAMINE HYDROCHLORIDE

A Thesis

submitted by

KENNETH MURRAY

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Synopsis

Part One

Preparations of nucleoprotein having reproducible characteristics were obtained from calf thymus glands by a mild extraction procedure. A preliminary examination of the dissociation of the complex was made and its physico-chemical behaviour was investigated in relation to that of its nucleic acid component.

The nucleoprotein was highly aggregated in solution at low ionic strength, but at high ionic strength a weight-average molecular weight lower than that of nucleic acid itself provided additional evidence for the dissociation of the nucleoprotein.

Spectrophotometric studies showed that the nucleoprotein was denatured by heat and by alkali in a similar manner to nucleic acid, although denaturation by heat was slightly retarded. The spectrophotometric titration behaviour of the two substances was almost identical, but their potentiometric titration curves differed significantly.

Nucleoprotein solutions exhibited the features of nucleic acid devolving from its unique helical structure, indicating that this structure is retained by the nucleic acid within the complex.

Part Two

A general reaction has been discovered between amino sugars and aldoses for which the essential requirements are a free amino group and an aldehyde group.

Three compounds (A, B and C) were separated by ion-exchange chromatography from a mixture obtained by heating D-mannose with D-glucosamine hydrochloride. Compound B was identified as 5-hydroxymethyl furfuraldehyde (HMF) from its ultra-violet absorption spectrum, chromatographic behaviour, and distribution coefficients between two different solvent systems, and was characterised as its 2:4-dinitrophenylhydrazone. Compound A was very unstable and was hydrolysed by cold water to mannose (characterised as its p-nitranilide) and glucosamine (characterised as its carbobenzoxy derivative). On heating in aqueous solution, the hydrolysis was accompanied by degradation to HMF (characterised in the same way as compound B), and the formation of melanoidins. The HMF isolated from the reaction mixture was shown to arise from compound A and C^{14}-HMF was obtained when the condensation was effected with a mixture containing C^{14}-mannose and glucosamine hydrochloride, showing that the HMF originated in the mannose moiety of compound A. From these and other experiments, the structure N-mannosyl-glucosamine was assigned to compound A. Compound C was stable in aqueous solution, and hydrolysis with

acid gave equimolecular quantities of mannose and glucosamine hydrochloride, but was not accompanied by a browning reaction. On the basis of periodate oxidation experiments, its behaviour in the Elson-Morgan reaction, and a number of other colour tests, compound C was provisionally assigned the structure 6-O-α-D-mannosyl-2-amino-2-deoxy-D-glucose.

Certain aldehydes were found to behave as bases on a sulphonated polystyrene resin. This interesting discovery may provide the basis of a new method for the separation and analysis of aldehyde mixtures.

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Contents

PART ONE

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GENERAL INTRODUCTION

Deoxyribonucleoproteins (DNP) are the principal constituent of the chromosomes of all living cells[1] and are consequently of great biological importance. The thymus glands of calves, pancreatic tissues, fish sperm, and yeast cells are particularly rich in DNP and these are the main sources, though by no means the only ones, of materials which have been studied. Chemically DNP is a complex of deoxyribonucleic acid (DNA) and protein and it is desirable to consider these two components briefly before discussing DNP itself.

In recent years DNA has been the focus of much research, although its existence has long been known[2] and its basic structure was established over twenty years ago[3,4]. As a result of cytological and genetical experiments the importance of DNA in heredity[5] and the identification of DNA with transforming principles[6,7] is now widely recognised. The chemical structure of DNA has been examined in detail[8] and the application of X-ray crystallography has allowed the formulation of theories concerning its fine structures[9,10] which are supported by other physico-chemical evidence (loc. cit.).

These advances have been made possible by the development of mild methods for extracting DNA with a minimum of

deformation from its original state. As long ago as 1899, Bang[11] obtained thymus DNP by extraction with water and precipitated it with salt and this is still the basis of many methods currently in use. The refinements have been mainly in speed of operation, extraction at lower temperatures, and in using various agents to inhibit enzymic degradation. The extraction of DNP is almost always the preliminary stage in the preparation of DNA and protein, and its solubility is usually markedly dependent upon sodium chloride concentration[12]. Mirsky and Pollister[13] dissolved thymus DNP in molar sodium chloride in which the protein is largely, if not entirely, dissociated from the DNA and the protein may then be removed in a number of ways (loc. cit.) leaving a solution from which the DNA is recovered by precipitation with ethanol.

The methods of organic chemistry have shown DNA to consist of purine and pyrimidine bases, phosphoric acid, and 2-deoxy-D-ribose[14,3]. Hydrolysis of DNA (both chemical and enzymic) has shown the stages of its degradation to be:

DNA ⟶ nucleotides ⟶ nucleosides + phosphoric acid

sugar + purine and pyrimidine bases ↵

The purine bases are adenine and guanine and the pyrimidine bases are thymine and cytosine (some DNA samples also contain small amounts of 5-methyl cytosine or 5-hydroxy-methyl cytosine[15]) and the sugar is 2-deoxy-D-ribofuranose[3]. In the nucleosides the

bases are linked β-glycosidically to 2-deoxy-D-ribofuranose and the nucleotides are phosphate esters of the nucleosides. The esterification is on the primary hydroxyl group of the sugar (C_5), but the hydrolysis products of DNA include some nucleoside-3-5-diphosphates[16,17] which indicates that the C_3 atom, as well as the C_5 atom of the sugar is involved in the nucleic acid molecule. On this evidence, DNA was formulated as a polynucleotide chain[18] (Figure 1) and although it was originally thought to be a tetranucleotide[3], molecular weight measurements have shown it to be highly polymerised[19]. At one time it was thought that all the bases were present in equal amounts[3] but more modern chromatographic methods of analysis have been applied to DNA samples from several sources and have disproved this conception. In addition, these analyses have furnished the important information that in DNA samples, irrespective of their source, the sum of purine bases is equal to the sum of pyrimidine bases and the molar ratio of adenine to thymine, and that of guanine to cytosine is unity[20].

Although physical methods were employed over thirty years ago to study the reaction of thymus DNA with proteins and amino acids[21], it is only during the last decade that physico-chemical measurements made on DNA samples extracted by mild methods have supplemented the information yielded by organic chemistry.

Potentiometric titrations were carried out on neutral solutions of the sodium salts of DNA and the curves obtained on titration to pH 2.5 and pH 12 differed from those obtained on back titration, with alkali and acid respectively, to neutrality[22,23,24,25]. These differences were attributed by Gulland and co-workers to the presence of hydrogen bonds between titratable groups of DNA (amino groups and keto groups of the bases), the bonds being irreversibly broken by acid or alkali[22]. The ultra-violet absorption of DNA solutions (due to the purine and pyrimidine components) was found to increase markedly when the solution was titrated to pH 2.5 or pH 12 and was only partly restored to its original value on back-titration[26]. This permanent change in absorption, or of the spectrophotometric titration curve can be interpreted in terms of hydrogen bond rupture provided that this causes other structural changes[27,28].

Viscosity measurements have been made on DNA solutions and these indicate a high molecular weight[19,20]. Its behaviour is non-Newtonian and the marked dependence of viscosity upon the rate of sheer was typical of a long, fibrous type of molecule[30], a property which was also reflected in its streaming birefringence[29]. The action of acid or alkali on DNA caused a marked fall in viscosity and the disappearance of streaming birefringence[31] and these data, in conjunction with titration evidence tended to suggest that the hydrogen bonds were inter-molecular rather than intra-

molecular[32].

Measurements of the molecular weight of DNA samples have been made from sedimentation and diffusion experiments but difficulties were encountered in assessing the concentration dependence of these properties[33]. The application of the theory of light scattering to molecular weight determinations[34,35,36,37] and the parallel development of experimental methods[38,39] have provided a useful new technique in macromolecular studies. From light scattering data the molecular weight of macromolecules can be measured[40] and information also obtained concerning molecular shape. Using this method, Doty and Bunce obtained molecular weights of 4×10^6 [41] and similar values were obtained by other workers[33,42]. The values recently obtained on newer DNA preparations are around 6×10^6 [43,44], although the discovery of DNA fractionation by Chargaff[45] and other workers[46] indicates that a spread of values is to be expected, and some larger molecular weights have been reported[33,42].

Probably the most important physical evidence for formulating the DNA molecule has come from X-ray crystallographic studies. Samples of DNA from a number of different organisms all gave very similar X-ray diagrams[10] and on the basis of these and similar experiments a model has been con-

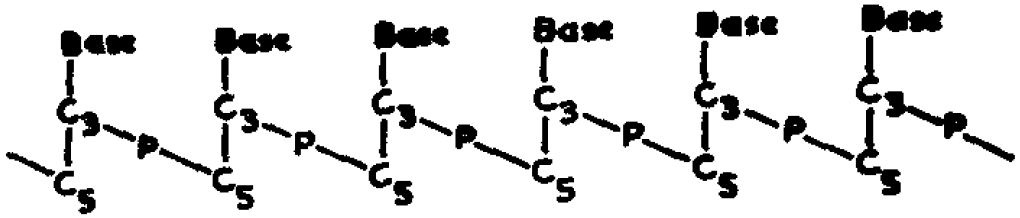


Figure 1. The DNA model used by Brown and Todd[18]

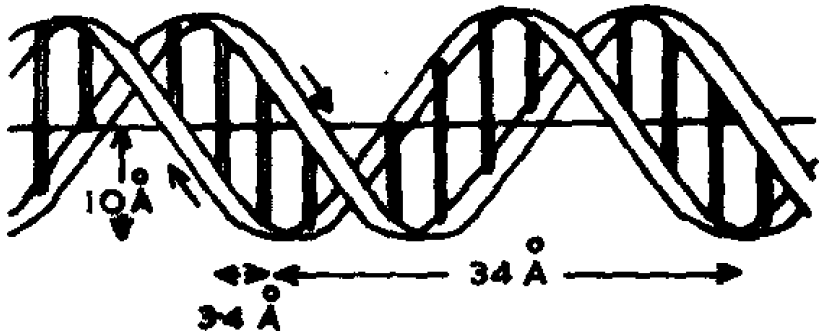


Figure 2. The DNA model of Watson and Crick[9]

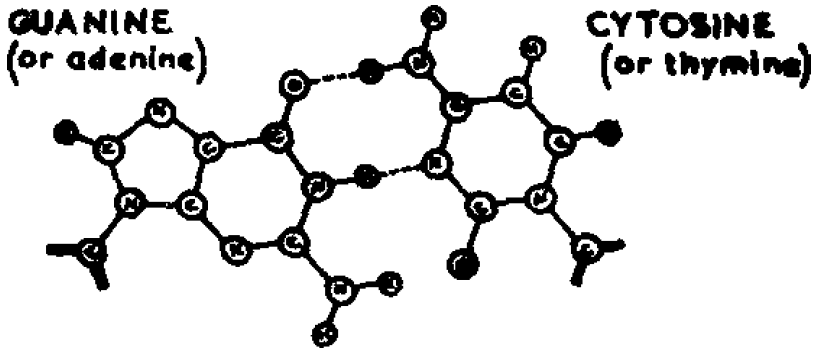


Figure 3. Hydrogen bonding (indicated by the broken line) between pairs of bases from opposite strands of the helix[48].

structed, which has a helical form, typical of water soluble polymers, and with which the chemical evidence is consistent[9,10]. This model comprises two polynucleotide chains intertwined helically around a common axis, but running in the opposite sense, and the base molecules project inside the helix perpendicular to its fibre axis: it is represented diagrammatically in Figure 2. The rigidity of the structure is maintained by hydrogen bonds between the pairs of bases inside the helix; the phosphate groups project out from the helix and are therefore able to bind cations. The groups involved in the hydrogen bonds are shown in Figure 3[48], and the steric requirements of the model will only permit hydrogen bonding between a purine and a pyrimidine: a third hydrogen bond between guanine and cytosine (between the amino group on C_2 of guanine and the carbonyl on C_2 of cytosine) has quite recently been suggested by Pauling[47]. An important and unique feature of this structure is that if the nucleotide sequence of one of the helical strands is fixed this automatically fixes the sequence in the other helix. The biological ramifications of this structure have been emphasised[48] for it suggests the basis for a process of self-replication.

The rupture of the intra-molecular hydrogen bonds would lead to a collapse of the highly ordered structure, and if sufficient hydrogen bonds in sequence are broken this does in fact occur giving, by analogy with proteins, a denatured molecule. The

process of denaturation has been studied by the application of potentiometric titration[49,50] experiments, and viscosity and ultra-violet absorption measurements[27,28], and more recently light-scattering methods have also been utilised[51]. These experiments have shown that if DNA solutions are heated, exposed to acid or alkaline pH, or irradiated with ultrasonic waves or γ-rays, the molecule can be denatured, and the extent of denaturation can be assessed[50,52]. Denaturation by dilution has also been reported[53].

The protein moiety of the DNP complex has received less attention than the DNA and most of the work has been analytical. Although other proteins are combined with DNA in the DNP complex[13,54,55], the main ones are usually either histone, which is characterised by a high arginine and lysine content, or protamine which is almost an extended polyarginine molecule of quite low molecular weight[56]. Protamines have been extracted from the sperm of several species of fish[57], but the proteins associated with DNA in thymus glands are mainly histone[54].

The protein of DNP was extracted by Hammarsten[21] by precipitating with salt and ethanol from a solution of DNP. It may also be recovered by solvent extraction[54] or by acidifying a solution of DNP[58] and Butler and co-workers have compared the

sedimentation properties of materials extracted by salt precipitation and by acid treatment. From their results they suggested that the greater heterogeneity of the material obtained by salt precipitation might have arisen from degradation by cathepsins which would be avoided in acid solution and they therefore recommended the preparation of histones by acid treatment.

Thymus histone has been fractionated by Crampton, Moore and Stein[59] using ion-exchange resin column chromatography and at least five different proteins were obtained. These fractions were chromatographically homogeneous and complete amino-acid analyses were made on three of the major ones (again by an ion-exchange chromatography method[60]) and show them all to have a very high lysine content and a smaller and more variable arginine content. It is noteworthy that about a half of the total histone was irreversibly absorbed on the column and this therefore probably represents more basic components. Many other analyses have been reported from time to time[57] and larger variations are found than one would attribute to experimental errors, but it is unwise to interpret these in terms of different histones of DNP when their histories are dissimilar. None of the methods used is likely to have been so elegant as those developed by Crampton and his colleagues. On the basis of some of the other analytical figures,

molecular weights of 14-20 x 10^3 have been estimated[60].

Ultracentrifugation and electrophoretic examinations of thymus histone have been made and the material has always proved to be heterogeneous[57]. Sedimentation has given two components and these, when examined again by prolonged electrophoresis and ultracentrifugation, have been found to be capable of further resolution[61,62]. Physical measurements, other than these, have not been attempted and on so ill-defined material it is doubtful if they would be of value.

As the source of many DNA and histone preparations, DNP has been well known since work in this field began to intensify about sixty years ago. Reference has already been made to Bang's extraction[11] of DNP from thymus glands with water and essentially the same method was used later by Hammarsten[19]. Many years later Mirsky and Pollister[12] found that DNP was insoluble in sodium chloride solutions at concentrations between 0.02M and 0.5M. From this observation arose the main extraction methods in current use. In one of them (Mirsky and Pollister[13]) the DNP (after washing) was dissolved in M sodium chloride solution and precipitated by dilution to 0.14M and this process was repeated several times to effect purification of the DNP. Stern and Davis[63] made an approach from the opposite side of the solubility minimum

and extracted the washed tissue with water and precipitated DNP by adding sodium chloride to a molarity of 0.14. When this precipitate was dissolved in water a more homogeneous product (electrophoretically) was obtained. The preliminary maceration and washing of the tissues was carried out in 0.14M saline and, to reduce the degradation of DNP during extraction, the procedure was carried out at low temperature, usually about 2°C. Modifications of these methods involve the use of depolymerase inhibitors such as arsenates and citrates in the extractants[57(p.149)]. The two general methods outlined above have both been criticised, the former because DNP is known to dissociate in strong salt solutions so that the process in all probability gives an artefact, and the latter because extensive purification may give a selection of smaller fractions[57]. Butler and his collaborators have used an aqueous solution of the washed tissue but found that there was a great tendency to gelling and aggregation[57, 64].

One of the earliest pieces of evidence for the dissociation of DNP in strong salt solutions was the increase in viscosity and eventual precipitation of protein which occurred when salt was added to aqueous solutions of DNP[65]. This behaviour had however been foreshadowed in the work of Miescher on mixtures of salt, nucleic acid, and protamine, which led him to suggest a dynamic equilibrium in this system[66]. Physico-chemical studies have

subsequently been made on solutions of DNP in sodium chloride at high ionic strengths (often 1.0M) and these have established beyond doubt the dissociation of DNP into DNA and protein, although the extent of dissociation appears to be variable and governed by the concentration of sodium chloride. A DNP from rat-liver has been reported, however, which does not dissociate in strong salt solution[57]. The sedimentation experiments made by Stern[67] showed changes in the DNA/protein ratio at different sodium chloride concentrations and dissociation into two components was observed in the ultracentrifuge by Butler and Gilbert[57]. Frick showed that the electrophoretic behaviour was complex[68] and a more precise study was made by Fleming and Jordan[69] who concluded, from the boundaries obtained at both high and low ionic strengths, together with analyses of fractions taken from the various boundaries, that the dissociation was usually incomplete. Other studies of the dissociation have been made by precipitation[70] or extraction[45] with organic solvents from salt solutions of varying concentration and analysis of the fractions obtained; the results indicate a fractional dissociation of DNP. All this work shows that DNP prepared by methods involving the use of strong salt solutions must be a re-formed DNA-protein complex which probably bears little relation to the native DNP and preparations at low ionic strength are therefore to be preferred, even though

Crampton, Lipshitz and Chargaff[45] have shown preparations of DNP made at both high and low ionic strengths to have similar viscosities, ultraviolet absorption and analyses.

A study of the properties of DNP prepared by Stern's method[63] has been made by Steiner[42] and after several dissolutions and reprecipitations to purify the material he obtained a molecular weight of 5.8×10^6 in water or dilute phosphate buffer which is surprisingly low in view of the molecular weight of DNA and does suggest that heavier particles may have sedimented out during the high-speed centrifugation employed in the purification process[57]. Ultracentrifuge and viscosity measurements were also made; the material gave a rather broad sedimentation peak and the viscosity was much lower than that of DNA and was sheer independent. The preparation of DNP made by Butler et al.[57,64] gave a rapidly sedimenting gel component which was followed by a slower boundary moving at a similar speed to that of Steiner's material.

At present, little is known of the detailed structure of DNP. Chemical analysis has shown some samples of DNP to comprise approximately equal weights of DNA and protein[54] and the dissociation behaviour of DNP implies that these two components are held together, principally at least, by ionic linkages. It can be inferred from the spectrophotometric studies of Shack and Thompsett[26] that the helical

structure of DNA is present in the DNP complex, as one would anticipate. X-ray crystallography has also provided evidence which indicates the helical form of DNA in DNP. Studies on fish sperm[71] suggest that a fully-extended polyarginine chain is wound round the shallower of the two grooves of the DNA helix, and such a structure would have a diameter only slightly greater than that of DNA.

The properties of DNA consequent upon its unique helical structure should be present in the original DNP and the object of the present studies was to establish a mild extraction method which would yield a reproducible sample of undissociated DNP, and then to study the modification imposed by the presence of the protein on the properties of DNA through a comparison of the physico-chemical behaviour of samples of DNP and DNA obtained from an identical source. Information of this nature could provide a useful starting point for studies on DNA-protein interactions, which are of interest in connection with biological replication, by indicating properties which might profitably be employed to follow the course of the interaction.

Chapter I. Preparation and Characterisation of Materials

Samples of DNP, DNA, and histones were prepared from a number of different batches of calf thymus glands and several chemical tests and analyses were made to examine the reproducibility of the samples.

The thymus glands were thoroughly washed in a cold saline solution prior to the extraction of DNP by a method very similar to that described by Butler and his collaborators[64]. From solutions of the DNP, DNA was obtained after precipitating the majority of the protein with an anionic detergent[72,73] and removing final traces by solvent extraction[74], and histones were prepared by acid treatment in the way used by Stedman and Stedman[75].

In addition to the qualitative colorimetric tests the properties used as criteria of reproducibility were, for DNP, ratio by weight of nitrogen to phosphorus, ratio by weight of arginine to phosphorus, absorption at 259 mμ (extinction coefficient per mole of phosphorus, ϵ_P), and ratio of absorption at 259 mμ to that at 235 mμ; for DNA, ratio by weight of nitrogen to phosphorus, absorption at 259 mμ (ϵ_P), and ratio of absorption at 259 mμ to that at 235 mμ; and for histones, arginine content, qualitative analysis of amino acids, absorption at 275 mμ (extinction coefficient per mole of nitrogen, ϵ_N), and ionophoretic behaviour over a range of pH values.

Experimental

a) Preliminary Tissue Washing

Calf thymus glands, taken from the animal within thirty minutes of its death, were transported to the laboratory in melting ice and, after cutting away extraneous connective tissues, the material was macerated in a solution of sodium chloride (0.14M) and sodium citrate (0.01M) at 3°C. In the first two extractions, about 280 g. of thymus glands (equivalent to about 64 g., dry weight) were macerated with 500 ml. of cold sodium chloride-sodium citrate solution (referred to as the saline solution), but in the three later extractions the quantity used was reduced to 20 g. per 500 ml. of saline solution and a total of 80 g. of thymus glands was used. The homogenate was centrifuged at 2,000 r.p.m. for 20 minutes at 2°C in a refrigerated centrifuge and the supernatant liquid was discarded: the residue was again macerated in the cold saline solution and centrifuged. The residue was macerated in the cold saline solution for a third time and the slurry was strained through four thicknesses of butter muslin and again centrifuged.

The process of maceration and centrifugation was then repeated thrice more, by which time the suspension sedimented in two fairly distinct layers; a lower, matted, fibrous layer, and an upper, thin, creamy layer. The upper layer was separated by lightly scraping with a nickel spatula and was washed, as before,

with cold saline solution and the lower, fibrous layer was discarded. The saline suspension of the upper layer was centrifuged and the residue was finally rinsed very lightly (no maceration) with cold distilled water and centrifuged: the upper aqueous layer was discarded and the residue constituted the washed tissue.

b) DNP Preparation

In the preparation of DNP from the first two batches of thymus glands, half of the washed tissue was macerated in 1 ℓ. of cold distilled water and allowed to stand for 18 hours at 3°C before centrifuging at 2,000 r.p.m. for 30 minutes. The supernatant liquid was dialysed against distilled water for 2 days at 3°C. and freeze dried, and the lower, gel-like layer was macerated with more cold water (1 ℓ.) and again centrifuged to yield two layers. On the first batch of material this process was repeated several times to furnish a series of supernatant solutions, and after eight macerations the small quantity of gel-like residue which remained on centrifuging was discarded. After application of the Dische and Sakaguchi colour tests (loc. cit.), the supernatant solutions were dialysed against distilled water in the cold and freeze-dried and were designated DNP I/A to H. Aqueous solutions of selected samples were prepared for analysis and were clarified by centrifuging at about 2,000 r.p.m. The second batch of material was treated in a similar manner except that the residual gel was discarded after four extractions of the washed tissue with cold water, and that a

small portion of the washed tissue was dissolved in 1.0M sodium chloride solution and the DNP recovered by diluting with water and spooling of the precipitate on a glass rod in the way described by Mirsky and Pollister[13]. After repeating this process five times, the DNP was dissolved in cold distilled water, dialysed as were the other preparations, and freeze-dried. These samples were numbered in succession DNP II/M,N,S, and T, and that prepared by the Mirsky and Pollister method was DNP II/P.

In the third extraction, the process was modified by using a much smaller quantity of thymus glands and the homogenate of the washed tissue was clarified by centrifugation at 13,000 r.p.m. in a vacuum centrifuge for $2\frac{1}{2}$ hours. Only one extraction of the washed tissue was made, but this was done in three parts giving the samples DNP III/X,Y, and Z, and the residual gel was then discarded.

Some of the washed tissue of extraction IV was dissolved in water, some in 0.0007M phosphate buffer (pH 6.8), and some in 0.05M sodium chloride solution. All these solutions were prepared by macerating the washed tissue in the appropriate solvent and allowing to stand at 3°C. for 18 hours before clarifying by high speed centrifugation (13,000 r.p.m.). The procedure used in preparing a solution of DNP V was the same as for DNP IV, but only one solvent was employed and that was 0.001M sodium chloride solution.

Solutions of DNP in 1.0M sodium chloride were obtained either by dissolving the washed tissue directly in this stronger electrolyte, when a clear solution was obtained and centrifugation was unnecessary, or by adding the required volume of 5.0M sodium chloride to solutions of DNP in water or in 0.001M sodium chloride.

c) DNA Preparation

Solutions of DNP in 1.5M sodium chloride were prepared by macerating the washed tissue in cold distilled water and adding the required volume of 5.0M sodium chloride solution. This solution was allowed to reach room temperature and was then treated with an aqueous sodium dodecyl sulphate solution to make the final sodium dodecyl sulphate concentration 0.4% (w/v)[73]. A white precipitate formed and after standing at room temperature for a few hours and at 3°C. for about 18 hours the precipitate was centrifuged out. The supernatant solution gave a red colour with Sakaguchi reagent[76] and was shaken with a 10% (v/v) solution of octan-1-ol in chloroform[74], the two liquid phases being separated by centrifugation. Solvent extraction was repeated in this way until the aqueous phase no longer gave a faint pink colour with the Sakaguchi reagent and a total of six to eight extractions was usually necessary.

The de-proteinised solution was poured into twice its volume of ethanol when DNA was precipitated as long fibres which were collected by spooling onto a glass rod. The precipitate was

then washed with 70% (v/v) ethanol, ethanol, and ether, and was dissolved in distilled water, dialysed against distilled water at 3°C. for 2 days, and finally freeze-dried.

d) Histone Preparation

From the first batch of thymus glands, histones were prepared by acidifying solutions of DNP in both 1.5M sodium chloride (Histone I A) and water (Histone I B) to a normality of about 0.3 with hydrochloric acid[75]. In each case a heavy white precipitate was produced which was removed by centrifugation to leave a clear solution. To these solutions of the histone hydrochloride, five volumes of acetone were added and the precipitate was filtered at the pump, dissolved in water, and freeze-dried after dialysis against cold water for 2 days.

Only aqueous solutions of DNP were used in subequent preparations and the solution was divided into two parts in extraction II, one of which was acidified with hydrochloric acid, and one with sulphuric acid. Sulphuric acid was used in all the later extractions.

e) Analysis of Materials

Analyses of the materials from the first two extractions were carried out on freeze-dried samples, but in later experiments the solutions were normally analysed before freeze-drying. The freeze-dried materials were dissolved in water, unless a description to the contrary is given, and the solutions were

clarified by centrifuging at 2,000 r.p.m. for half-an-hour.

The qualitative colorimetric tests used were the Dische reaction[77] for DNA, the Sakaguchi reaction[76] for histones, and both of these for DNP.

Phosphorus determinations were carried out by the method of Jones et al.[78], the intensity of the blue colours produced after adding ammonium molybdate reagent being measured with a portable colorimeter (Evans Electroselenium Limited) using a No. 626 filter.

Nitrogen was determined in all cases by the hypochlorite titrimetric method of Kolthoff and Stenger[79,80] after digestion of the sample with a modified Kjeldahl mixture[80].

Brandl and Kassel's modification[81] of the Sakaguchi reaction was used to measure the arginine content of DNP solutions and the intensity of the red colours was measured with a portable colorimeter (using a No. 623 filter) exactly two minutes after addition of the reagents.

Ultraviolet absorption measurements were made with a Unicam spectrophotometer using 1 cm. silica cells. Measurements were made on solutions in water and in sodium chloride at various concentrations; the absorption of the solution was always measured against a blank of the corresponding solvent.

For amino acid analysis, the histones were hydrolysed by heating with 20% (w/v) hydrochloric acid at 160°C for 6 hours in a sealed tube[82] (2 mg. of freeze-dried histone and 0.2 ml. of

hydrochloric acid). The hydrolysates were evaporated to dryness, and the acid simultaneously removed, in a vacuum desiccator containing trays of phosphorus pentoxide and sodium hydroxide pellets, and the brown residue was dissolved in water (0.2 ml.). A qualitative amino acid separation was effected by paper ionophoresis in phosphate buffer at pH 7 (10 volts per cm., 14 hours) (loc. cit.) and a more detailed qualitative analysis was obtained by paper chromatography on Whatman No. 1 paper[83]. A one dimensional chromatogram was developed for 40 hours with n-butanol-ethanol-water-acetic acid (4:1:2:1, completely miscible) and was stained with ninhydrin after drying in a current of air for several days. Aliquots of the neutral hydrolysates were used for arginine analysis and colorimetric determinations were made using the Rosenberg[84] modification of the Sakaguchi reaction, the intensity of the red colours being measured on a Unicam spectrophotometer at a wavelength of 535 mμ.

Paper ionophoresis experiments on the various histone samples were carried out on Whatman No. 3 paper using an apparatus similar to that described by Foster[85]. Dilute phosphate buffers at pH 7, 9 and 11[86] and an acetate buffer at pH 5[87] were used and, usually, a potential difference of about 700 volts (a potential gradient of about 10 volts per cm.) was applied across the length of the paper (57 cm.) for 5 to 9 hours. In order to avoid excessive

permanent adsorption of the protein on the paper, which results if the solution is applied to the paper and dried in the customary manner, the paper was first moistened with the buffer solution and the histone solution applied to the base-line immediately prior to electrolysis. After drying, the ionophoretograms were stained with ninhydrin.

Results and Discussion

The aqueous extracts of the washed glandular tissue all gave positive reactions in the Dische and Sakaguchi tests, which are used as characteristics of DNA and proteins respectively, and were thereby classed as DNP solutions. The Dische test depends on the formation of a blue colour when 2-deoxy-ribose is heated with an acetic acid solution of diphenylamine in the presence of a small amount of sulphuric acid, and the Sakaguchi test utilises the development of a red colour by compounds containing a guanidyl group with alkaline solutions of α-naphthol and sodium hypochlorite.

When the aqueous extracts of washed tissue (i.e. aqueous preparations of DNP) were centrifuged a considerable quantity of a gel of variable consistency was deposited and the gel was partly dissolved on re-extraction with cold water. Since the various DNP fractions obtained from the first two extractions had similar analyses (Table I, p. 24), they would appear to be obtained as a result of the gradual dissolution of a reasonably homogeneous DNP

rather than the progressive extraction of differing components as has been observed in the extraction of sea urchin DNP[55]. It therefore appeared unnecessary to continue the preparation of a large number of samples from one batch of washed tissue and the process was modified in extraction III so that a smaller quantity of the washed tissue was prepared and its aqueous extract was centrifuged at a very high speed which resulted in the sedimentation of the residual gel to a firm pellet, thus facilitating the decantation of the clear supernatant solution. In this way a clear solution of DNP was quickly and easily obtained.

Dilute electrolytes replaced water as the solvent when solutions were required for physical measurements. Those used in extraction IV were 0.05M sodium chloride solution and 0.0007M potassium phosphate buffer, pH 6.8[88], but the DNP solutions obtained were too dilute for accurate measurements on them to be made. After preliminary experiments with some other freeze-dried DNP samples to determine the highest concentration of sodium chloride which could conveniently be used, the DNP from extraction V was dissolved in 0.001M sodium chloride solution.

The use of sodium chloride solutions for histone preparation was avoided after the first extraction (Histone I A) for it has been shown that this can produce a more heterogeneous sample of histone than when aqueous solutions are used[58]. Both sulphuric and hydrochloric acids were separately used in extraction II for the preparation

Table I

Material	Qualitative Colour Test		N/P	Arg/P	Arginine N% total N	$\epsilon_{N_{275}}$	$\epsilon_{P_{259}}$	$\epsilon_{259}/\epsilon_{235}$
	Dische	Sakaguchi						
DNP I G	+	+	4.2				7090	1.66
DNP I H	+	+					7160	1.56
DNP II M	+	+	3.7	0.5			7150	1.68
DNP II N	+	+	3.9	0.6				1.63
DNP II N_{M+P} (II P)	+	+	3.7	0.6			7160	1.67
DNP II S	+	+	3.8	0.6				1.76
DNP II T	+	+		0.6				1.60
DNP III X	+	+	3.7	0.6				1.84
DNP III Z	+	+	3.8					
DNP IV	+	+	3.9				7520	1.69
DNP V	+	+	3.8				7250	1.78
DNA I	+	-					7240	2.42
DNA II	+	+					6800	
DNA IV	+	-					7270	2.36
DNA V	+	-	1.8				6650	2.16
Histone I A	-	+			21.7	26		
Histone I B	-	+			22.0	29		

Table I continued

Material	Qualitative Colour Test		N/P	Arg/P	Arginine N% Total N	$\epsilon_{N_{275}}$	$\epsilon_{P_{259}}$	$\epsilon_{259}/\epsilon_{235}$
	Dische	Sakaguchi						
Histone II HCl	-	+			22.4			
Histone II H_2SO_4	-	+			21.9			
Histone III	-	+			23,0	27		0.29
Histone IV	-	+			20.8	27		
Histone V	-	+			22.1	27		0.30

Properties of Materials from five independent extractions

of histone salts. A higher yield was obtained when sulphuric acid was used, probably because the sulphate is less hygroscopic than the hydrochloride, and sulphuric acid was consequently used routinely.

The first three extractions served to establish a standard preparative procedure for DNP and histone (that used for DNA was already well known[72]) and this is summarised in the form of a flow sheet in figure 4. The materials obtained from extractions IV and V were employed for physical experiments and although small reference samples of each substance were freeze-dried, physical measurements and analyses were carried out on the freshly prepared solutions.

The analytical results obtained on the various samples of DNP, DNA, and histone are listed in Table I and they indicate that, by these criteria, fairly reproducible materials were being obtained.

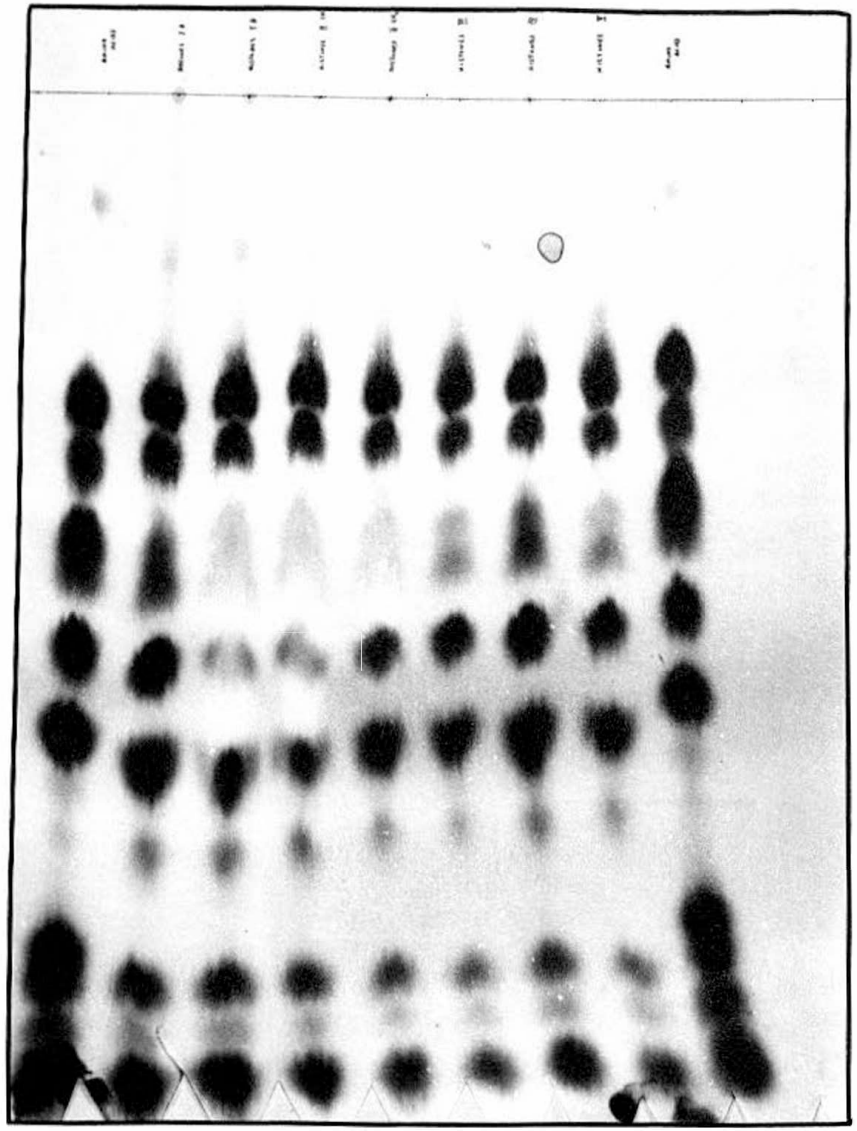
The ratio by weight of nitrogen to phosphorus of 3.8 ± 0.1 obtained on all but the first samples of DNP is in agreement with values quoted in several publications (e.g. refs. 26, 45, 88, 89 and 90). Although values for the ϵ_P at 259 mμ (ϵ_P 259) of around 6600 have been reported for DNP samples[91] obtained from calf thymus glands by the aqueous extraction method of Crampton et al.[89], values of 7400 were obtained for DNP from avian tubercle bacilli[90] and this is in closer agreement with values obtained (7000 to 7500)

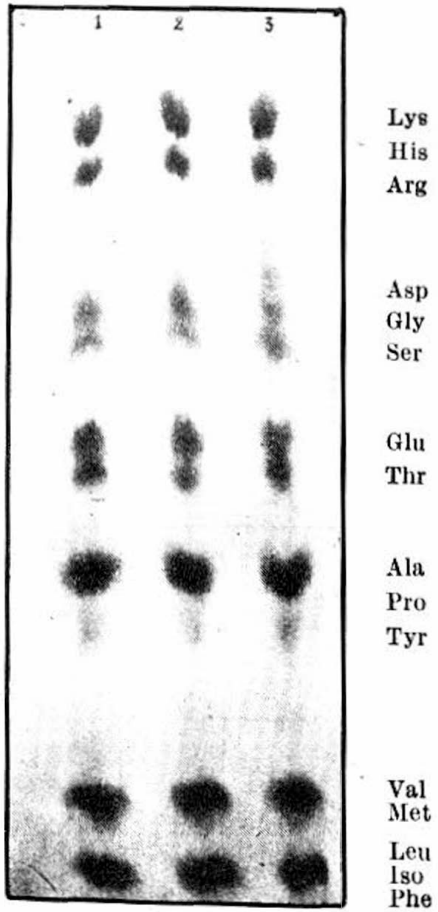
on the later DNP preparations described here. Also Doty and Zubay[88] quote an extinction coefficient at 259 mμ for a 1% (w/v) solution of calf thymus DNP of 106 ± 5 and conversion of this to ϵ_P gives a value of 7350 ± 350. The ratio of the absorption at 259 mμ to that at 235 mμ has an average value of about 1.7 and variation from this seldom exceeds 7%. In view of the different values of this ratio for DNA (about 2.2) and histones (0.3), the property can usefully be used as a characteristic of DNP and it arises in consequence of the superimposition of the absorption curve of histones with a high absorption in the region 230 to 240 mμ (see figure 10) on that of DNA which has a much lower absorption in this region. The absorption curves of DNP and DNA are reproduced in figure 9 (Chapter IV). Within the very rough limits of the method of estimation, the ratio by weight of arginine to phosphorus of the DNP samples is consistent, but the more accurate arginine determinations on the histone hydrolysates (loc. cit.) are a much better indication of the reproducibility of the samples.

The ϵ_P 259 values obtained for DNA samples II and V (6800 and 6650 respectively) are in good agreement with the findings of other workers[28,92,93] and the data of Lawley[28] afford the calculation of the ratio of the absorption of DNA at 260 mμ to that at 230 mμ. A value of 2.36 is obtained in comparison with 2.27 and 2.16 for the ratios of the absorption at 259 mμ to that at 235 mμ

obtained for DNA samples II and V respectively. DNA V had a ratio by weight of nitrogen to phosphorus of 1.8 which is the value quoted by Lawley[28], and very similar to that obtained (1.67) by Shaek and Thompsett[26]. Samples I and IV of DNA were shown by light scattering and potentiometric titration experiments to be degraded and denatured respectively, and this is the reason for their higher ϵ_P 259 values. Molecular weights calculated from light scattering data from DNA IV and V are 6×10^6 which is in good agreement with the value now generally accepted[43,44].

Ultraviolet absorption measurements on several of the histone samples showed a consistent ϵ_N 275 value of 27, but there appear to be no similar data quoted in the literature for comparison. The absorption curve is shown in figure 10 (Chapter IV). Arginine values (arginine nitrogen as the percentage of total nitrogen) obtained on the histone hydrolysates are also consistent and the average value of 22.0 compares well with that of 21.5 found by Crampton et al.[59] and 23.3 which is calculated from the data published by Vendrely and his colleagues[83]. Paper ionophoresis of all the histone hydrolysates showed that they contained neutral amino acids and relatively large quantities of lysine and arginine. The hydrolysates were also analysed by chromatography on Whatman No. 1 paper, and although it is customary to make two dimensional paper chromatograms of amino acid mixtures in order to obtain the





Chromatograms of nucleohistones extracted in *M* sodium chloride from calf thymus, liver and kidney. Hydrolysates containing identical quantities of deoxyribonucleic acid were used in all three cases. Whatman paper No. 1. Solvent: 4 parts, butanol; 1 part, ethanol; 1 part, acetic acid; 2 parts, water

Figure 5. Chromatogram of histone hydrolysates, with amino acid controls corresponding with those shown in the photograph on the right, which is reproduced from the publication of Vendrely et al.[83]

best resolution[82], a one dimensional method was chosen in order to make a direct comparison of all the samples on one chromatogram. Figure 5 shows the chromatogram obtained, and controls comprising all the amino acids found by Crampton et al. in their analysis of histone hydrolysates by ion-exchange chromatography[59] are situated at either end of the row of the seven histone samples examined. The figure shows that, although some differences do occur, the amino acid content of the various hydrolysates is similar. Included in figure 5 is the chromatogram obtained by Vendreley and his collaborators[83] from the hydrolysate of calf thymus DNP (as distinct from the separated histones) and this is very similar to those of the histone hydrolysates. The various amino acid spots of histone hydrolysates were identified by developing a second chromatogram carrying separate amino acid reference spots, but the key which is incorporated in Vendreley's diagram permits a rapid assessment of the various components from the chromatograms illustrated in figure 5. The major constituent amino acids of the histones are lysine, arginine, glutamic acid, alanine, valine, and leucine, and smaller amounts of glycine, serine, tyrosine and phenylalanine are present.

Serum proteins have been efficiently resolved by paper ionophoresis[94,95], but the attempts made to separate the histone samples by this means were not successful. Using buffers at

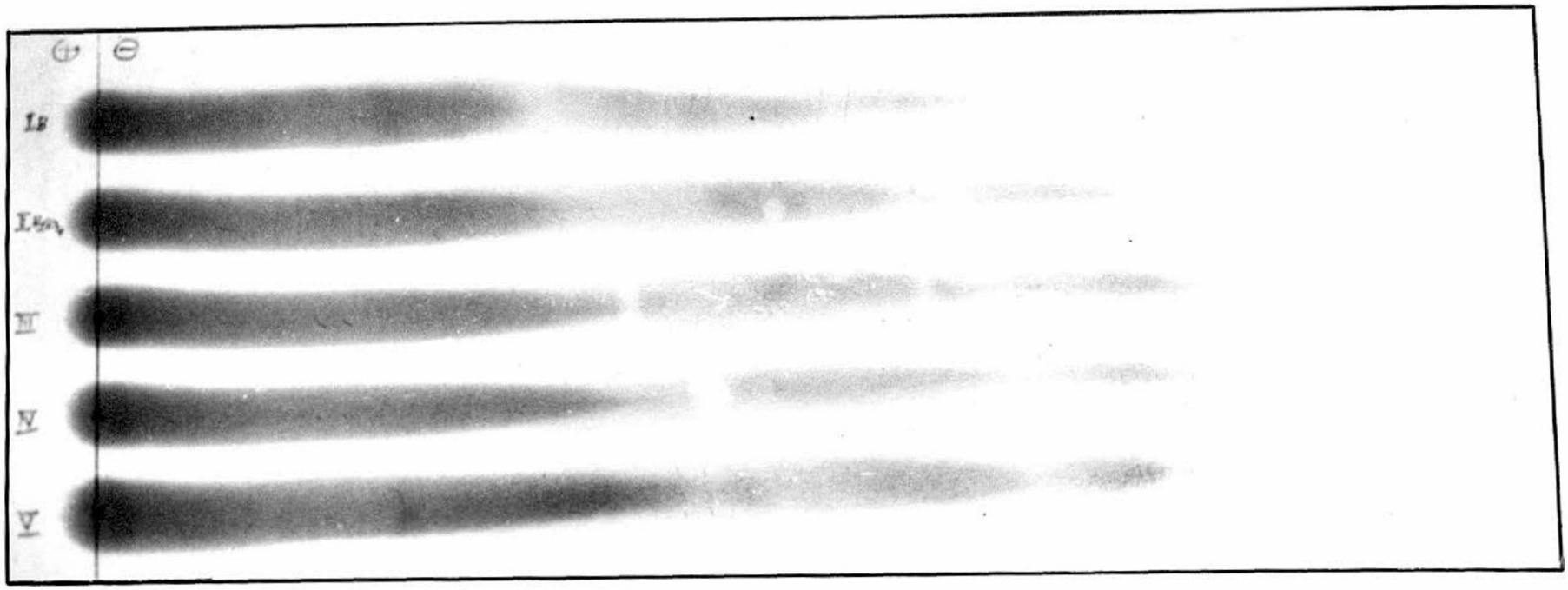


Figure 6. Ionophoretogram, stained with bromphenol blue, of histone samples (from five independent extractions) in acetate buffer, pH 5

pH 5, 7, 9, and 11, the histone samples all gave similar patterns indicating the presence of at least three main components, two of which are very basic and migrate towards the anode at pH 11, but heavy streaking made further interpretation impossible. A typical ionophoretogram is illustrated in figure 6, and the streaking is probably due to strong adsorption on the paper of the heavily changed histone molecules, rather than overloading of the paper with protein. The complexity of the histone obtained from calf thymus glands has been emphasised by chromatographic separations made by Crampton, Moore and Stein[59], and by Luck and co-workers[96]. Using gradient elution from ion-exchange resins, the former group obtained three major and two minor components, but noted from analyses before and after the experiment that about half of the total histone was irreversibly adsorbed on the resin. By improved elution methods, Luck and his group separated at least twelve components on a column of ion exchange resin.

On the basis of these analyses, the extraction methods described appeared to furnish reasonably reproducible materials on which physico-chemical experiments could be attempted.

Chapter II. The Dissociation of DNP

The protein of calf thymus DNP has been shown to consist principally of histone[54] which is dissociated from the DNA in solutions of high ionic strength[57]. Dilution of the dissociated system to a low ionic strength results in the recombination of the histone and DNA and precipitation of the re-formed complex. Such behaviour has normally been regarded as an implication that the main forces binding the protein and DNA are electrostatic, presumably between the basic groups of the protein and the phosphate groups of the DNA. The state of ionisation of these basic groups is markedly dependent on pH and thus under appropriate conditions a particular basic group may no longer be involved in an ionic bond with DNA. Dissociation of the DNP complex might therefore be dependent on pH as well as on ionic strength.

Electrophoresis[57,67,69] and distribution between immiscible solvents[45] have been employed in studies of the effect of ionic strength on the dissociation of DNP and a stepwise release of differing protein fractions was indicated. The dissociation and recombination of protein and DNA could conveniently be followed by determination of a particular functional group as it is released and re-absorbed during the process. Measurements of this sort

would provide information concerning the recombination process and also illustrate differences between the native complex and recombined artefact. An attempt was made to investigate this possibility by the determination of the guanidyl group of arginine before and during dissociation, and after recombination. The effect of pH on the dissociation of DNP has also been studied by means of paper ionophoresis experiments.

Theoretical

a) Dissociation in Sodium Chloride Solutions

Suppose that a well defined structure involving a specific sequence of basic groups in linkage with DNA phosphate groups exists in DNP and that the whole protein moiety comprises m particular basic groups (e.g. ϵ-amino groups of lysine), of which n are involved in linkages with DNA. Then m and n may, or may not, be equal. If an analytical method is available for this basic group, the number of such groups remaining free in the original DNP (i.e. m-n) could be determined and dissociation of the protein from the DNA by the addition of sodium chloride followed by a second determination would give the value of m, and hence n by difference.

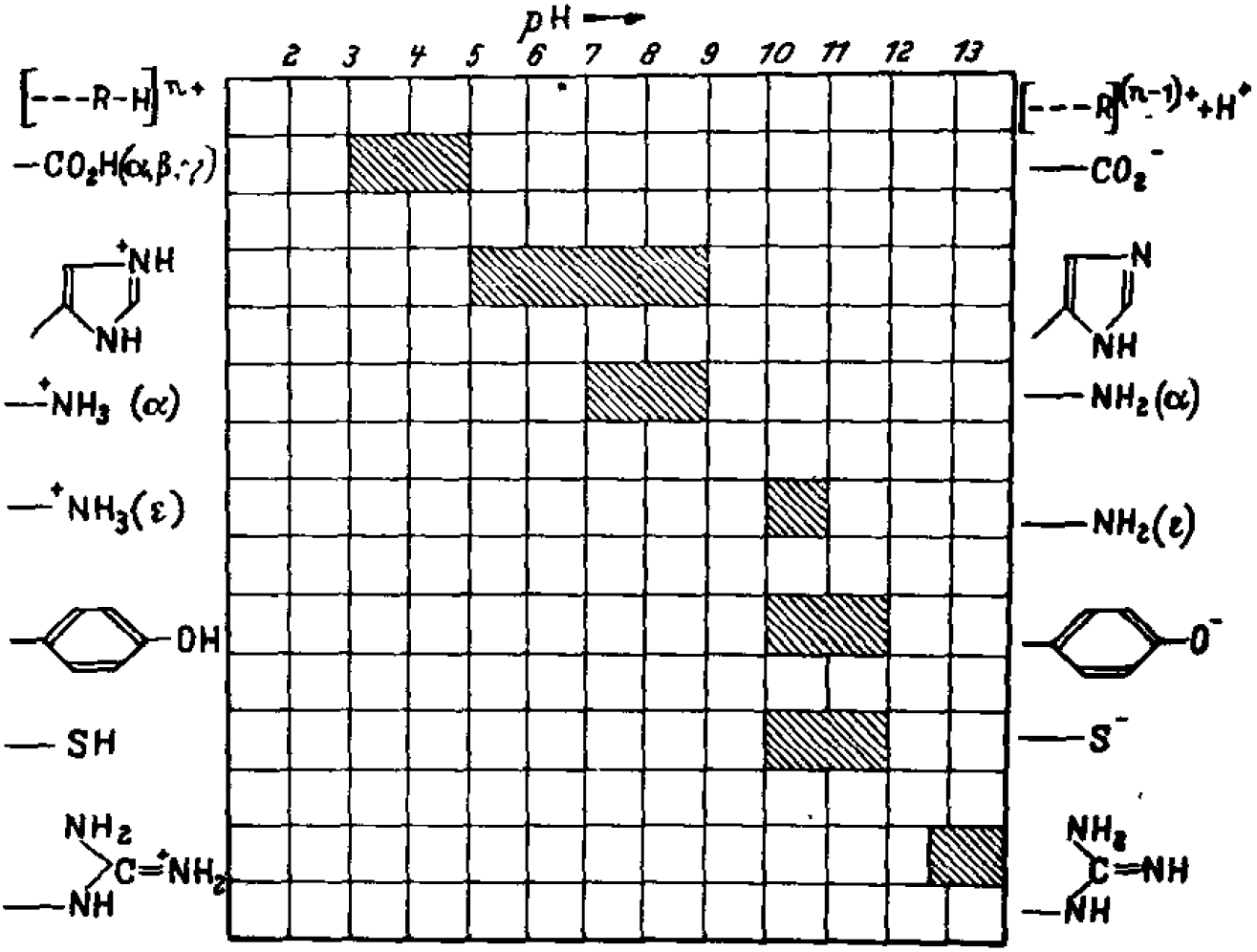
Dilution of the dissociated complex solution to a sodium chloride molarity of 0.14 would effect the recombination of protein and DNA and precipitation of the DNP thus formed. That the original, or native, structure will be re-formed is highly improbable,

for the recombination is here regarded as arising from the electrostatic attraction between DNA phosphate and protein basic groups, and should therefore be random. The sequence of groups bound will almost certainly change and some of the basic groups originally bound by DNA phosphate groups may be sterically prevented from making such a linkage in the recombined DNA-protein complex, and the value of n for a particular group would then be different in the reconstituted DNP from that in the original DNP. A difference of this sort would be observed by measuring again the number of the particular basic group under examination which remained free (i.e. m-n). If a series of dissociations and recombinations is carried out and the value of m-n measured after each recombination, a random recombination process would be reflected in a set of random values for m-n.

Provided that suitable analytical methods are available, experiments of this sort could be useful in studying the dissociation of DNP at varying ionic strengths, and to illustrate differences between the original and recombined DNP which may not be found by physico-chemical methods.

b) The Effect of pH on Dissociation in Solutions of Low Ionic Strength

The dependence on pH of the ionisation of the various functional groups of amino acids is illustrated in a diagram by Springall[97] which is reproduced in figure 7. The de-ionisation,



Indicates pH range within which the dissociation

$$[---R-H]^{n+} \longrightarrow [---R]^{(n-1)+} + H^+$$

occurs as pH increases

Figure 7 . *Dissociation constants of the various* ------ **R — H** ***groups***

or dissociation, of the histone basic groups which would be involved in linkage with DNA may be represented by the equation

$$-NH_3^+ \longrightarrow -NH_2 + H^+$$

and when this reaction is complete the basic group loses its positive charge and hence its means of attraction to DNA phosphate groups. The basic groups involved in histone-DNA ionic linkages would be the iminazole of histidine, the α-amino of N-terminal peptide chains, the ε-amino of lysine, and the guanidyl of arginine. These groups dissociate over the pH ranges 5 to 9, 7 to 9, 10 to 11 and 12.6 to 14, respectively (see Figure 7).

As the pH of a DNP solution is raised there will be a stepwise de-ionisation of the basic groups and less basic proteins would be dissociated before the histones. Histones containing, inter alia, lysine but not arginine would be released from linkage with DNA before those containing arginine. By virtue of its negatively charged carboxyl end groups, it should be possible to move the dissociated protein away from the DNA by the application of an electric field. The analyses of Toennies and Bakay[54] show that the protein of DNP is not entirely histone (non-histone protein constitutes 16% of the total protein) and the non-histone protein might be expected to dissociate completely from the DNA above pH 9. Crampton, Moore and Stein[59] have published quantitative

Table II

Amino Acid	Histone Fraction A			Histone Fraction B			Histone Fraction C		
	Proportion by weight	Molar proportion	Mols %	Proportion by weight	Molar proportion	Mols %	Proportion by weight	Molar proportion	Mols %
Aspartic acid	1.45	1.09	2.0	3.36	2.53	5.5	2.13	1.60	3.5
Glutamic acid	2.72	1.88	3.5	5.66	3.91	8.5	3.92	2.70	5.9
Glycine	2.72	3.63	6.7	2.88	3.84	8.4	2.64	3.52	7.6
Alanine	11.3	12.7	23.4	4.82	5.42	11.8	8.26	9.30	20.0
Valine	3.16	2.70	5.0	3.60	3.08	6.7	2.62	2.24	4.9
Leucine	3.14	2.40	4.4	5.07	3.87	8.4	3.78	2.89	6.3
Isoleucine	0.72	0.55	1.0	2.76	2.11	4.6	1.48	1.13	2.5
Serine	3.77	3.60	6.6	3.48	3.32	7.2	3.34	3.18	6.9
Threonine	3.45	2.90	5.3	2.79	2.34	5.1	3.25	2.73	5.9
Proline	5.52	4.80	8.8	2.48	2.16	4.7	4.07	3.54	5.1
Phenylalanine	5.30	3.21	5.9	1.05	0.64	1.4	1.07	0.65	1.4
Tyrosine	0.63	0.35	0.6	2.65	1.47	3.2	1.73	0.96	2.1
Histidine	-	-	-	1.79	1.16	2.5	0.76	0.49	1.1
Lysine	19.62	13.4	24.6	8.84	6.05	13.2	12.91	8.86	19.2
Arginine	2.09	1.20	2.2	6.04	3.48	7.6	3.83	2.20	4.8

The Relative Proportions of Amino Acids in Histone Fractions Calculated from the Data of Crampton, Moore and Stein[59].

amino acid analyses of their histone fractions A, B and C obtained by ion-exchange chromatography, and from these data the molar proportions of each amino acid in the respective fractions can be calculated and these are listed in Table II. Although none of these histone fractions is arginine-free, the molar ratio of arginine to lysine is only about 1:10 in fraction A and this protein might therefore be removable from DNA by electrolysis at pH 11 when lysine and all the less basic groups would be deionised, whereas fraction B with the higher arginine content would almost certainly not be separable. Only at pH values of 13 and higher would the very basic proteins (i.e. fraction B, and those eluted from ion-exchange resins only by concentrated guanidinium chloride solutions[96]) be dissociated from the DNA.

A series of ionophoresis experiments could provide a convenient means of investigating a dissociation of DNP in solutions of low ionic strength as the pH of the solution is raised.

Experimental

a) *Arginine Determinations on DNP Solutions*

An aqueous solution of freeze-dried DNP (II N) was prepared and clarified by centrifugation at 2,000 r.p.m. for 20 minutes. An aliquot of the solution was diluted with water for arginine determination by the method of Brand and Kassel, as described on p. 20. A second aliquot was similarly diluted, but with sodium chloride

to give a molarity of 1.0, and for determinations on this solution, sodium chloride was added to the reagents to the same molarity. These measurements were done in triplicate and similar measurements were made on four other DNP samples (I D, II M, II S and II T) and also on a solution of DNP (II P) prepared by the Mirsky and Pollister procedure (p. 17). Phosphorus determinations were carried out on all the DNP solutions using the method described by Jones, Lee and Peacocke (p. 20).

b) Ionophoresis Experiments

Solutions of histones (III) and of DNP (III X) in the appropriate buffer solution were separately spotted onto strips of Whatman No. 3 paper (10 cm. x 57 cm.) which had been moistened with the same buffer solution. Phosphate buffers at pH 9 and 11[86] were used and a similar experiment was prepared using 0.1N sodium hydroxide solution (pH 13). The papers were electrolysed in the phosphate buffers for 7 hours using a potential difference of 700 volts (about 10 volts/cm.), and in the sodium hydroxide solution for 12 hours using a potential difference of 300 volts (about 4 volts/cm.). After electrolysis, the papers were dried, heated in an oven at 110°C for 10 to 15 minutes, and stained with bromophenol blue by immersing in an ethanolic solution of the dye (0.1% w/v) and mercuric chloride (10% w/v) for 10 minutes, followed by washing in running water[94].

The paper which had been electrolysed in sodium hydroxide solution was stained heavily with red mercuric oxide: this was dissolved out by gently agitating the paper in aqueous potassium bromide solution (20% w/v). The paper was then washed briefly in water, dried, heated as before, and again stained with bromphenol blue.

Ionophoresis of the histone solution in phosphate buffer at pH 7[86] was carried out, four spots being applied to the paper at intervals of one inch along the base line. After electrophoresis, the paper was dried, heated at 110°C for 15 minutes and cut into four strips each carrying one of the spots. One of the strips was stained with ninhydrin and the other three with bromphenol blue. Two of the latter were gently agitated in potassium bromide solution (20% w/v), until the blue stain disappeared, and then washed lightly with water, dried, heated as before, and stained once again, one of them for a second time with bromphenol blue, and the other with ninhydrin.

Solutions of histone (III) and DNP (III X) in 0.4M sodium chloride were separately spotted onto a strip of suitably moistened No. 3 paper and electrolysed in phosphate buffer (at pH 7) which was also 0.4M in sodium chloride. The electrolysis was prolonged for 18 hours using a low potential difference (150 volts, i.e. about 2 volts/cm.) and the paper was then dried, heated and stained with

bromphenol blue.

Results and Discussion

Various analyses of histone hydrolysates (figure 5, refs. 59 and 83) have shown lysine and arginine to be their principal basic amino acid components. Since there is no colorimetric analytical method for lysine (which occurs in greater quantity than arginine) an attempt was made to determine the number of arginine residues on solutions of DNP before and during dissociation, and after recombination, using a quantitative adaptation[81] of the Sakaguchi reaction. Measurements were made on six DNP samples (including a recombined artefact prepared by the Mirsky and Pollister method) in water and in 1.0M sodium

Table III

DNP Sample	I D	II N(1)	II N(2)	II N(3)	II M	II S	II T	II P
Arg./P in water	0.67	0.46	0.63	0.54	0.45	0.57	0.63	0.52
Arg./P in 1.0M NaCl	0.75	0.65	0.58	0.74	0.62	0.54	0.58	0.60

chloride solution, and the measurements on one of the samples were done in triplicate (II N). The results, expressed as ratio by weight of arginine to phosphorus, are shown in table III. Based on the analyses by Toennies and Bakay[54] of DNP and the arginine analyses of histone hydrolysates (Table I, also refs. 59 and 83) the

theoretical ratio by weight of arginine to phosphorus for DNP is 1.16, and this value should be obtained on dissociated DNP solutions unless arginine groups are involved in inter-peptide linkages.

The results obtained do not indicate the differences anticipated between solutions of DNP in water and 1.0M sodium chloride, and the method of analysis appears to be neither sensitive nor accurate when applied to these solutions of large molecules, for the ratios of arginine to phosphorus obtained in sodium chloride solution are only about half the theoretical value. An insensitive analytical method would not necessarily be useless, but the absence of significant differences between values in water and 1.0M sodium chloride suggests that, either the functional group being assayed reacts even though it is linked to the DNA phosphate group, or the analytical reagents employed cause the dissociation of the DNP complex. The latter event is very probable for the reagent is highly alkaline and the ionophoretic study of the effect of pH on dissociation at low ionic strength (loc. cit.) shows that the DNP complex is dissociated in 0.1N sodium hydroxide solution.

A much milder method of determination is needed for a functional group assay study of DNP dissociation at increasing ionic strengths and it is possible that experiments using the

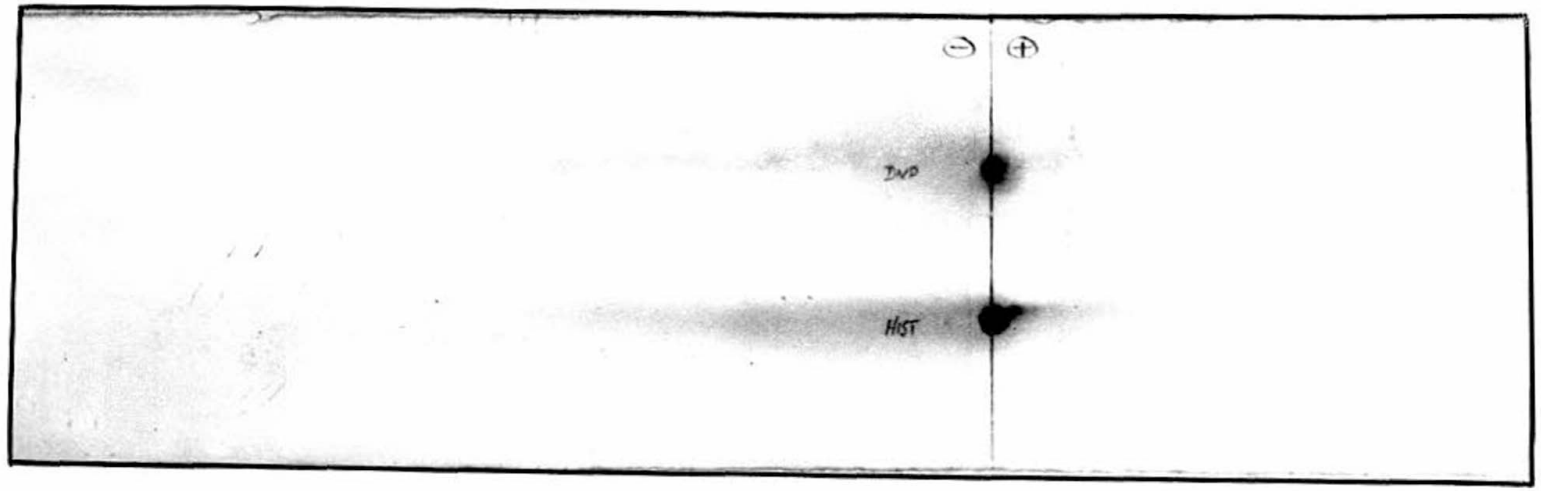


Figure 8. Ionophoretogram of DNP and Histone in 0.1N sodium hydroxide

reaction of dinitrofluorobenzene[98] with solutions of DNP in water and in 1.0M sodium chloride could be advantageously adapted to this problem. Hydrolysis of the N-dinitrophenyl proteins followed by chromatographic analysis would show whether or not there was a difference in end-amino groups of the protein in association with, and dissociated from, the DNA.

The ionophoresis experiments, in which parallel samples of histone and DNP were electrolysed in buffer solutions at pH 9 and 11, and in 0.1N sodium hydroxide solution, did not exhibit a stepwise dissociation of the protein from the DNP complex corresponding with the dissociation of the basic groups of the protein. Under conditions where all the basic groups other than the guanidyl group of arginine would be de-ionised (i.e. at pH 11) there was no evidence of dissociation of the DNP complex and only at a very alkaline pH (i.e. in 0.1N sodium hydroxide solution)where the guanidyl group would begin to dissociate (see figure 7) was a migration of protein away from the DNA observed. This ionophoretogram is illustrated in figure 8. The efficiency of ionophoresis in demonstrating a dissociation of DNP was illustrated by the experiment with 0.1N sodium hydroxide solution, and by an experiment using a high ionic strength buffer. Histone and DNP samples were found to produce a parallel migration of protein on ionophoresis in 0.4M sodium chloride solution buffered at pH 7 with potassium phosphate.

From these experiments it can be concluded that dissociation of the DNP complex does not occur in solutions of low ionic strength unless the solution is very alkaline (i.e. about pH 13).

(An interesting possibility of carrying out two staining tests on one ionophoretogram arose from these experiments. When staining the paper after ionophoresis in 0.1N sodium hydroxide solution with bromphenol blue, a heavy precipitate of red mercuric oxide was formed on the paper. This could be removed by dissolution in a concentrated aqueous solution of potassium bromide and, after washing, drying, and re-heating, the paper may be re-stained with bromphenol blue, or another reagent. In this way it is possible to use two different staining procedures on one ionophoretogram. A comparison of ninhydrin and bromphenol blue stains was made on a histone ionophoretogram (p. 35) in this manner and both gave the same pattern. The pattern obtained by the re-staining procedure with both ninhydrin and bromphenol blue was identical with the pattern produced by these reagents in the usual way).

Chapter III. Potentiometric Titrations

The anomalous potentiometric titration behaviour of DNA observed by Gulland, Jordan and Taylor[22] has been confirmed and intensively investigated by several other workers [23,24,25,99,100] and may be employed in quantitative studies of denaturation[50,101], for the hysteresis of the curves decreasses and finally disappears as a sample of DNA is denatured. Similar experiments on DNP have not been reported and attempts were made to carry out potentiometric titrations on this material for comparison with titrations of DNA.

Solutions of DNA at a concentration of about 0.15 mg. phosphorus per ml. in 0.05M sodium chloride are conveniently used for small-scale potentiometric titrations[99]. When one of the DNA samples was titrated at this concentration, with acid, titration curves exhibiting the well known hysteresis were obtained. Solutions of DNP in 0.05M sodium chloride are, however, extremely dilute (0.002 mg. phosphorus/ml.) and in 0.001M sodium chloride the concentration of DNP (0.02 mg. phosphorus/ml.) was still very much lower than the desired level for titration experiments. A solution of DNA was titrated at this low concentration in 0.001M sodium chloride solution and, although the results were much less accurate than previously, an hysteresis of the titration

curves was observed. Titration curves are conventionally plotted as the number of equivalents of acid bound by the DNA (per 4 g. atoms of phosphorus) against pH. The amount of acid bound is the difference between the volumes of acid required to titrate equal volumes of the solution and solvent to a given pH. When dilute solutions are titrated the volume of acid bound is small, and is obtained as the difference of two large quantities in the region where large additions of acid produce only a small change of pH, and cannot, therefore, be obtained with accuracy.

Attempts were then made to titrate DNP in 0.001M sodium chloride. A large initial uptake of acid occurred very slowly above pH 5, probably due to the protein, and on back titration the absorption of alkali was very slow above pH 4.5. Because of this slow equilibration of pH, a titration of DNP could not be completed in less than six hours, during which time a considerable volume of water evaporated from the solution and condensed on the stem of the electrode, the "Agla" syringe, and the bang of the vessel, thereby making true volume corrections impossible. This slow rate of reaction together with the inherent errors in the titration of dilute solutions made the construction of accurate and reproducible titration curves for DNP impossible. The curves obtained did, however, show an hysteresis similar to that of the DNA solution titrated at a comparable concentration,

and though they are unsuitable for quantitative studies they indicate that the hydrogen bonded structure of DNA, to which the titration hysteresis is attributed, is also a feature of DNP solutions.

The low solubility of DNP in dilute sodium chloride solutions thus precluded accurate comparative potentiometric titrations of DNP and DNA. Hysteresis similar to that of the potentiometric titration curves of DNA has, however, been observed in the spectrophotometric titration of DNA and DNP[26], and greater accuracy is achieved in these experiments using very dilute solutions than with concentrated ones. A comparative study of DNA and DNP was possible by this method and is described in the following chapter.

Chapter IV. Ultraviolet Absorption Studies

The purine and pyrimidine components of DNA are responsible for its ultraviolet absorption, but the absorptivity of DNA, measured as the extinction coefficient per mole of phosphorus (ϵ_p)[102], is much less than the value calculated by summation of the absorptivities of its constituent nucleotides[103]. Several spectrophotometric studies[27,28,53,104,105] have shown that an irreversible increase in ϵ_p is effected by several factors which do not degrade the molecule, but cause permanent changes in viscosity[31,52] and potentiometric titration behaviour[101], thus indicating an alteration of the macromolecular structure. By analogy with protein chemistry, this change is termed "denaturation" and its important feature is the disappearance of intramolecular hydrogen bonds which is illustrated by titration studies[106]. A variation of ϵ_p values for DNA between 6000 and 8000 has been observed[103], but more consistent values of about 6600 have been obtained with fresh preparations from a number of sources[92], and higher values are probably an indication of denaturation, if only to a limited extent.

Permanent increases in the ϵ_p value are produced when DNA solutions are heated[27,28,53], treated with acid or alkali[26,27,28,105], subjected to ultrasonic irradiation[27]

or irradiated with γ-rays[52]. Plots of absorption against pH are of particular interest because a considerable difference between the forward and back titration curves is observed, and Shaek and Thompsett have shown that these data can be plotted in a manner which furnishes hysteresis curves comparable with those from potentiometric titrations[26]. This hysteresis is greatly diminished when the solutions are previously heated or treated with acid or alkali.

The absorption of DNP comprises contributions from its DNA and protein components, but the protein absorption is very small compared with that of DNA (Table I and ref. 103) so that the essential features of the absorption curves of DNA and DNP are similar, both having a maximum of 259 mμ with a minimum at 230 mμ in the case of DNA and 235 mμ in that of DNP (figure 9 and refs. 26 and 90). In studies of the dissociation of DNP, Crampton, Lipshitz and Chargaff obtained a considerable variation of ϵ_P values, but the majority were between 6800 and 7300[89]. A specimen of DNP prepared from avian tubercle bacilli had an ϵ_P of 7400[90], and the data of Doty and Zubay[88] for calf thymus DNP give an ϵ_P of 7350 ± 350. The ϵ_P values listed in table I (p. 24) for the later preparations of DNP (7100-7500) are in agreement with these two latter findings and thus the ϵ_P value of DNP is 8 to 10% higher than that of DNA, which is a

difference greater than one can attribute to experimental error. An increase in ϵ_P of this magnitude is greater than would result from the algebraic addition of the absorptions of DNA and histone (using data from table I and the analyses of Toennies and Bakay[54] this would be less than 1%) and would therefore appear to be a consequence of DNA-protein interaction. The interactions of serum proteins and lysozyme with DNA have been studied spectrophotometrically[103,107] and in these instances a depression of the absorption is indicated.

Treatment of DNP solutions with alkali produces a permanent increase in absorption, and spectrophotometric titration curves of DNP solutions are similar to those of DNA[26]. These observations suggest that acid, heat, and irradiation will also have a denaturing effect on DNP similar to that on DNA, but studies of such processes have not yet been reported.

At a particular pH the denaturing action of acid and alkali on DNA is instantaneous[26], but that of heat and γ-rays is dependent on the dosage[52,53], i.e. the duration of application of the effect as well as its intensity. The protein of the DNP complex could provide some protection against denaturation which would be observed in relative studies of the denaturation of DNA and DNP. The work described in this chapter was concerned with the investigation of such an effect by comparative spectrophoto-

metric titration experiments with DNA and DNP solutions, and by measuring the relative rates of increase in absorption of such solutions on heating. A spectrophotometric study of the interaction of DNA with histone was also carried out.

Experimental

a) The Effect of Heat on the Absorption of DNA and DNP Solutions

Solutions of DNA and DNP in 0.001M and in 1.0M sodium chloride were prepared and their phosphorus content was determined colorimetrically (p. 20). After suitable dilution, the ultraviolet absorption curve of each solution was obtained (p. 20). Aliquots (5 ml.) of each of the four solutions were heated in boiling water for 15 minutes in stoppered tubes, and after cooling they were diluted as before and the absorption curves measured once again. The absorption curves are shown in figure 9.

In order to compare the relative rates of change of absorption of the four solutions on heating, sufficient of each stock solution was appropriately diluted to give about 60 ml. of solution, the absorption of which could be conveniently measured without further dilution. Twelve aliquots (4 ml.) of each solution were transferred to thin-walled test tubes which were stoppered with glass marbles and placed in a bath of boiling water. At noted time intervals one tube of each solution was withdrawn and cooled in an ice bath for about a minute. When the experiment was complete

(a period of 15 minutes heating was covered) the absorption of each solution at 259 mμ was measured against the appropriate solvent blank, and plots of ϵ_p against time were constructed for the four solutions, and are illustrated in figure 12.

b) Spectrophotometric Titrations

Solutions of DNA and DNP in both 0.001M and 1.0M sodium chloride were used and the phosphorus content of each solution was determined (p. 20). Aliquots of these solutions were so diluted (with the appropriate sodium chloride solution) as to have a convenient absorption at 259 mμ (the phosphorus content of such solutions was about 0.002 mg./ml.) and the solutions were titrated with sodium hydroxide solutions (initially 0.1N, followed by 1.0N) to pH 12.5 and then back titrated to neutrality with hydrochloric acid solutions (initially 1.0N, followed by 0.1N). About 30 ml. of the diluted solution, accurately measured, were used for each titration. During the titration, aliquots of about 4 ml. were withdrawn for measurement of the absorption (at 259 mμ only), and were then returned to the titration vessel. A Cambridge portable pH meter with "alkacid" glass electrode was used for pH measurement and it was standardised before use with phthalate and borate buffers (pH 4.00 and 9.18, respectively) and checked with the same buffers after each titration. The titration to pH 12.5 and back to neutrality was repeated on each titrated solution to

provide the corresponding data for alkali-treated materials.

A solution of DNA in 0.001M sodium chloride was titrated to pH 2 and back to neutrality, the titration again being followed spectrophotometrically. The acid-treated material, which this solution then represented, was subsequently titrated to pH 12.5 and back to neutrality, and a titration to pH 2 of an alkali-treated solution of DNA was also carried out, the titrations again being followed spectrophotometrically.

The spectrophotometric titration in the alkaline region was repeated on solutions of DNA and DNP in 0.001M and 1.0M sodium chloride after heating for 15 minutes at 100°C, the heat treatment having been applied to the solutions before dilution for titration. An additional titration of a solution of DNA after heating in 0.001M sodium chloride was done in which the solution for titration was diluted with 1.0M (instead of 0.001M) sodium chloride solution. The titration curves are reproduced in figures 13 to 17.

Two experiments were carried out in which more concentrated solutions of DNP were titrated and samples withdrawn at suitable pH intervals and diluted for absorption measurements, but this procedure proved to be far less accurate than the titration of diluted solutions.

c) Interaction of DNA with Histone

The concentration of solutions of DNA and histone in

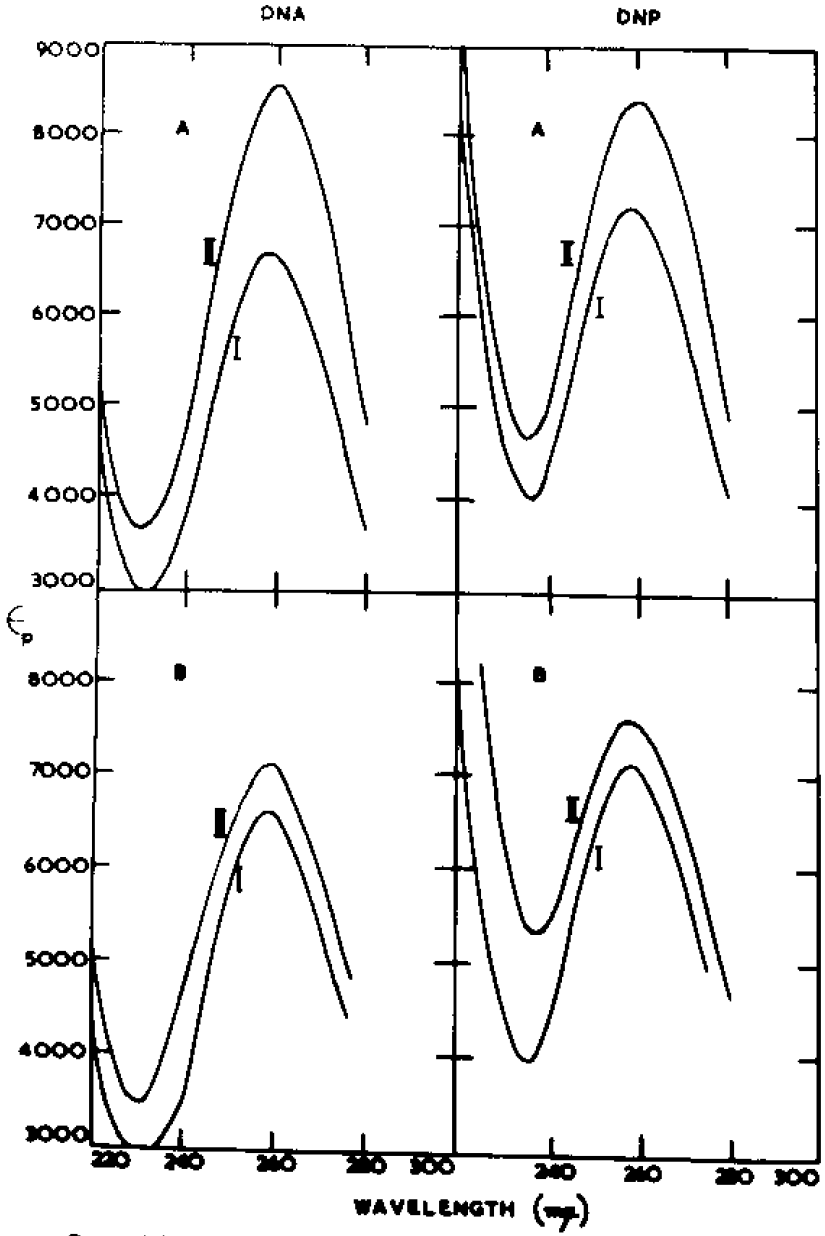


Figure 9. Absorption curves of DNA and DNP in A, 0.001M sodium chloride and B, 1.0M sodium chloride. Curve I, before heating, and curve II, after heating.

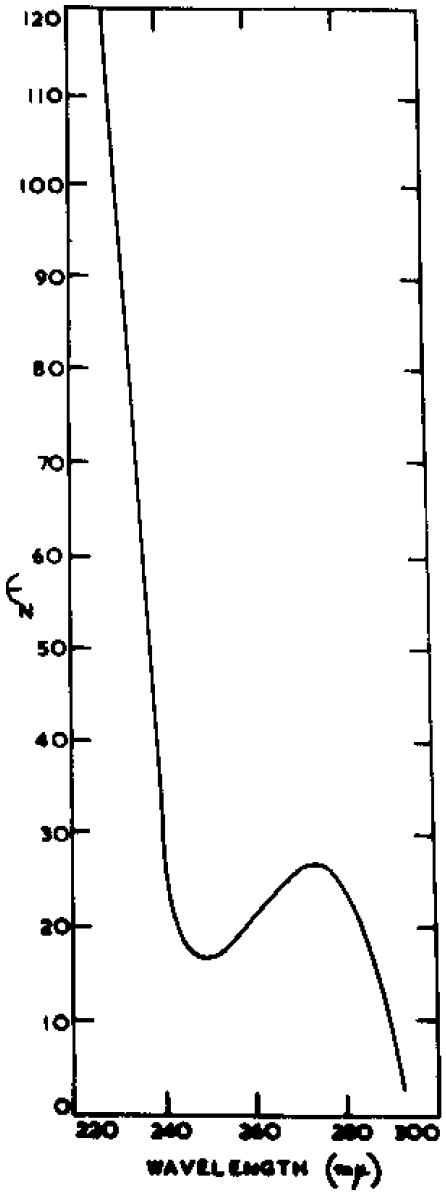


Figure 10. Absorption curve of histone

0.001M sodium chloride was determined by phosphorus analysis (p. 20) in the case of DNA and from the intensity of absorption at 275 mμ (figure 10) in the case of histone. An aliquot of the DNA solution was diluted to give a directly readable absorption at 259 mμ and 5 ml. of this solution were pipetted into a test tube. The histone solution was diluted so that its concentration by weight was about ten times that of the diluted DNA solution; this required a twenty-fold dilution. Aliquots of 0.05 ml. of this diluted histone solution were successively added to the DNA solution and the absorption of the mixture at 259 mμ was measured after each addition. When 0.4 ml. of the histone solution had been added, the mixture became slightly opalescent and the subsequent addition of histone solution produced an increasing turbidity. An aliquot (0.5 ml.) of the histone solution was diluted with 5 ml. of water and its absorption at 259 mμ was measured and was less than 1% of the absorption of the DNA solution prior to the addition of histone. The pH of the histone solution, before dilution, was determined with a Cambridge portable pH meter. Figure 18 shows the change in absorption as histone is added to the DNA solution.

Results and Discussion

The effect of heat on the absorption curves of solutions of DNA and DNP in both 0.001M and 1.0M sodium chloride is illustrated in figure 9 which shows that the well known increase

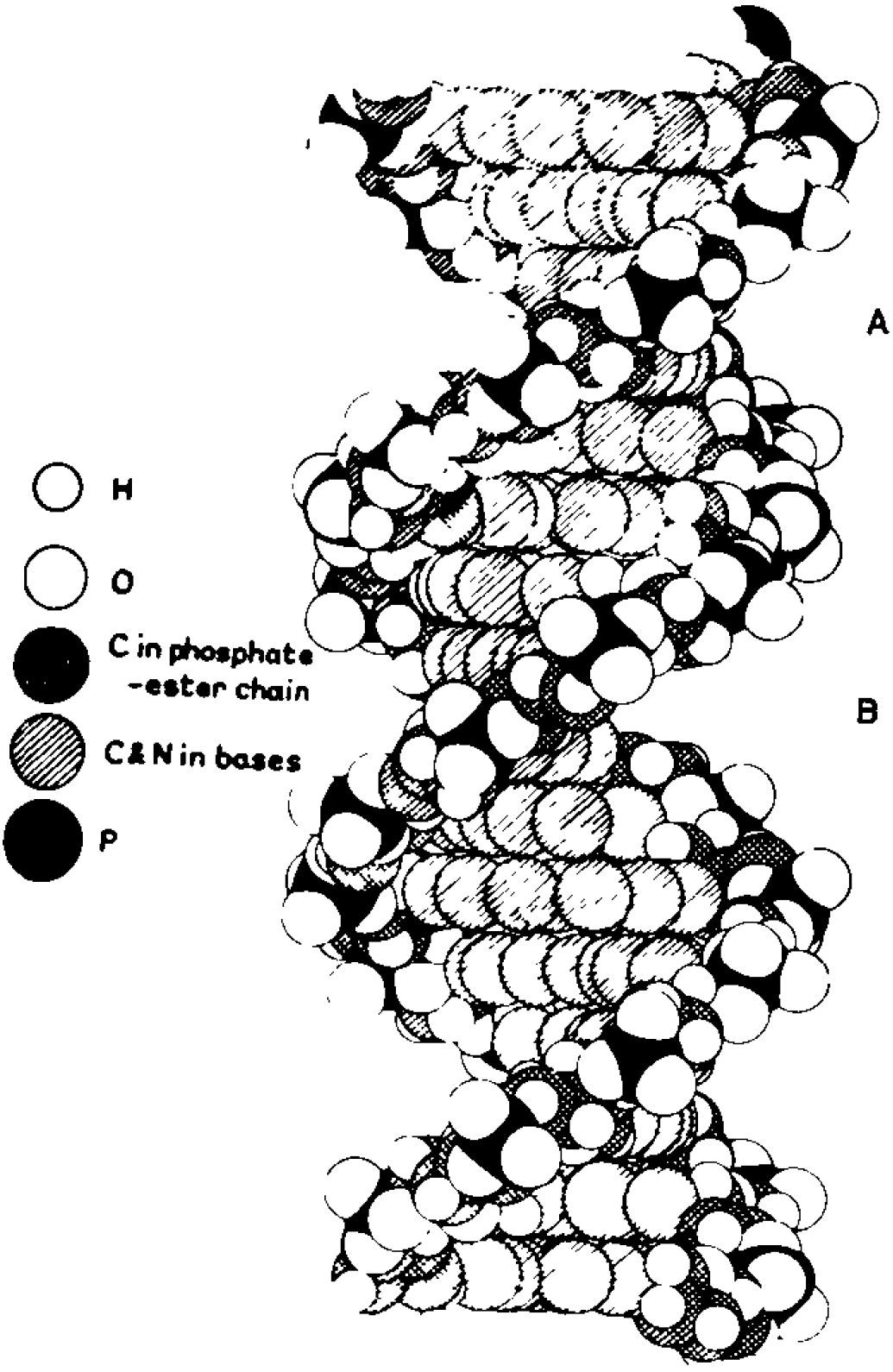


Figure 11. The DNA model of Wilkins et al.[71]

in ϵ_p of DNA is observed with DNP at low ionic strength, and the ϵ_p value after heating is virtually the same for the two substances; 8530 for DNA and 8450 for DNP. At the higher electrolyte concentration the behaviour is different and a relatively small increase occurs, the final ϵ_p values being 7160 for DNA and 7590 for DNP.

Explanations of the rise in ϵ_p of DNA on denaturation have been advanced on the basis of the Watson and Crick structure[9]. A molecular model of a part of this structure has been constructed by Wilkins et al.[71] and is represented in figure 11, from which it can be clearly seen that within the DNA helix the nucleotides are closely stacked above each other in a continuous spiral permitting electronic interaction between the aromatic rings of the purine and pyrimidine bases. An interaction of this type between superimposed layers of aromatic rings in concentrated solutions of certain dyes has been shown to result in a different absorption spectrum from that of dilute solutions where the arrangement is probably random[108]. Overlapping of the π-orbitals from the aromatic rings of partially superimposed bases could, in a similar way, account for the absorption of DNA being lower than that calculated from the nucleotide absorptions. Laland and his colleagues[27] have suggested that the increase in ϵ_p which occurs as the DNA molecule is denatured is the result of a partial break-

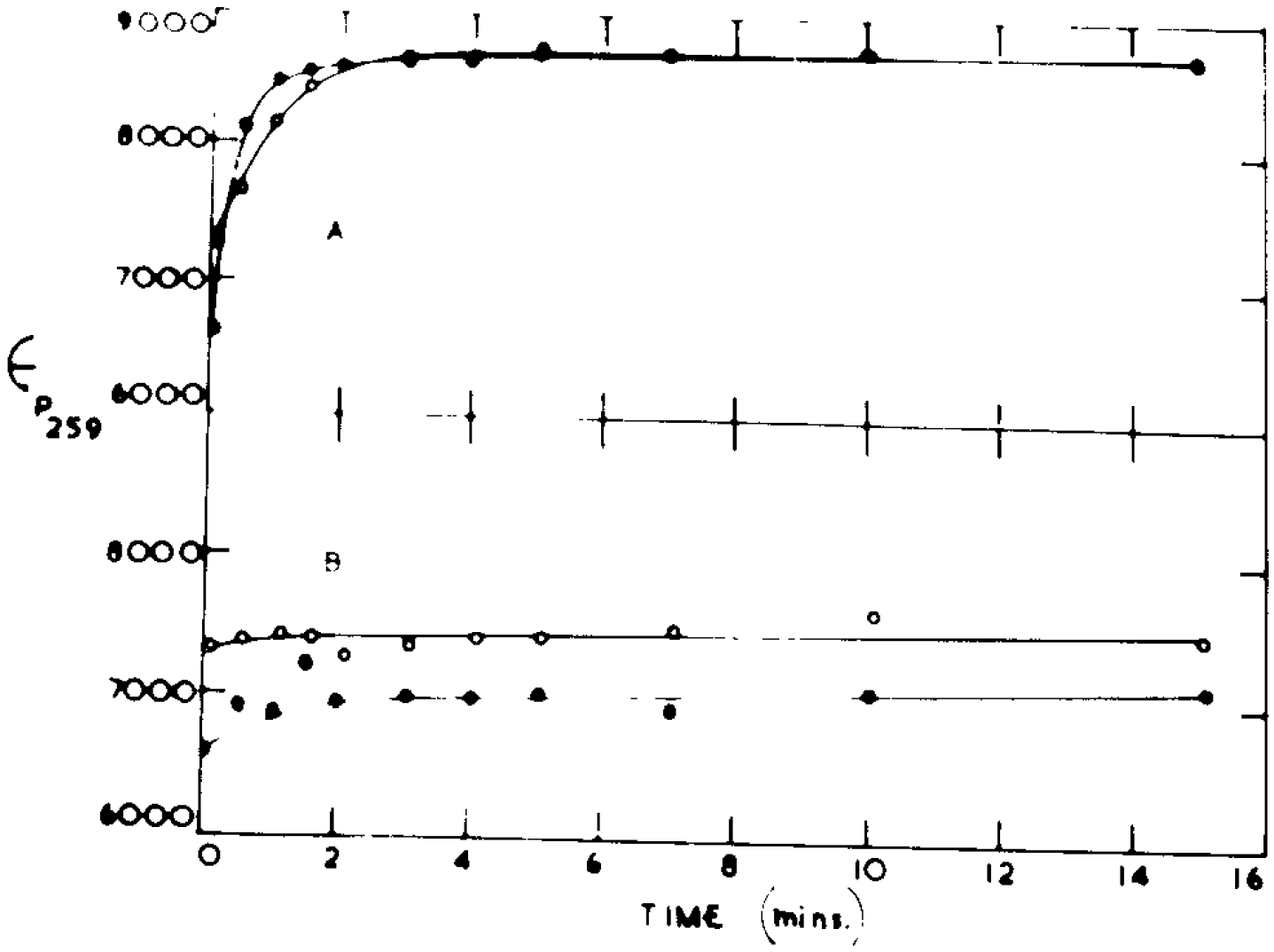


Figure 12. The effect of time of heating on the $\epsilon_{P_{259}}$ value of solutions of DNA (closed circles) and DNP (open circles) in 0.001M sodium chloride (A) and 1.0M sodium chloride (B).

down in these electronic interactions resulting from the rupture of intramolecular hydrogen bonds and the consequent deformation of the helix. In more mundane terms, Lawley explains the change in ϵ_P in terms of an increase in chromophoric area as the orderly stacking of bases in the DNA helix is replaced by a random arrangement in the denatured molecule[28].

The ϵ_P value of about 8500 for the heat-treated solutions of DNA and DNP in 0.001M sodium chloride may thus be termed the ϵ_P of the randomly disordered structure. The different values obtained for solutions in 1.0M sodium chloride will be discussed later with the spectrophotometric titration data.

Data for the relative increase in ϵ_P of DNA and DNP on heating are presented in figure 12 which is a plot of ϵ_P against time of heating at 100°C. Thomas[53] has shown (spectrophotometrically) that at 70°C denaturation of DNA in dilute sodium chloride does not proceed at a measurable rate, at 75°C it occurs slowly, and at 77.5°C the process becomes more rapid. The curves of figure 12 show that denaturation in 0.001M sodium chloride occurs very rapidly at 100°C and the final ϵ_P value for both DNA and DNP is 8700, but only a very slow increase occurs in a strong sodium chloride solution. Under these conditions, the protein appears to afford only a little protection against denaturation of the DNA within the complex. If the protein of the DNP is wound around the shallower

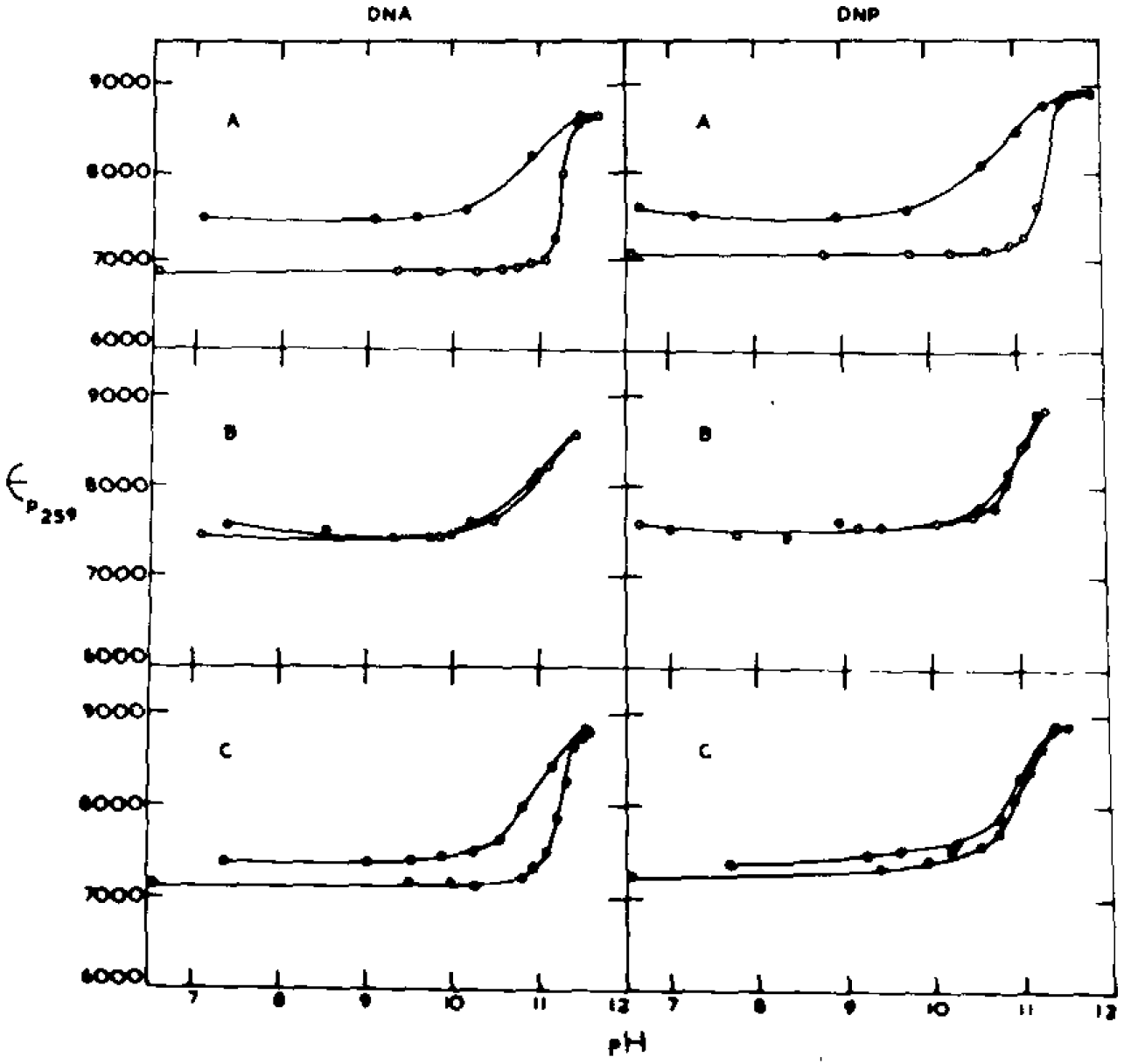


Figure 13. Spectrophotometric titration curves of solutions of DNA and DNP in 1.0M sodium chloride. A, original solutions; B, after alkali treatment; and C, after heat treatment. Open circles, forward titration and closed circles, back titration.

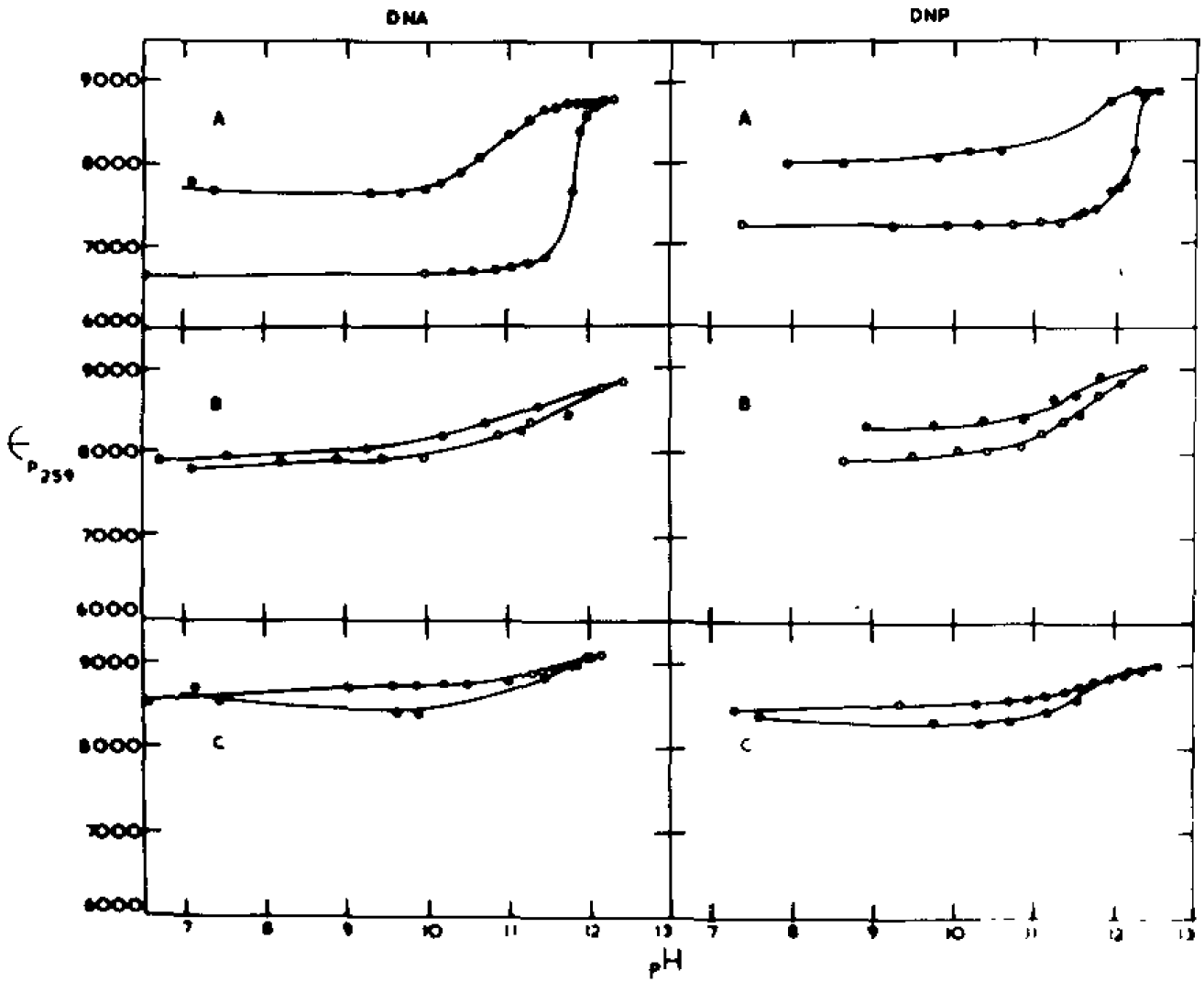


Figure 14. Spectrophotometric titration curves of solutions of DNA and DNP in 0.001M sodium chloride. A, original solutions; B, after alkali treatment; and C, after heat treatment. Open circles, forward titration and closed circles, back titration.

groove of the DNA helix (A in figure 11) and held there by interaction of its basic groups with DNA phosphate groups, as has been suggested in the case of fish-sperm DNP[71], it is probable that it could afford only slight protection against attack of the inter-base hydrogen bonds via the deeper groove (B in figure 11) of the helix.

The experimental results of the spectrophotometric titrations are summarised in Table IV and the titration curves are reproduced in figures 13 to 17. Plots of ϵ_P versus pH were made in preference to those used by Shaek and Thompsett (loc. cit.) for they afford easy and direct comparison of the various experiments, and comparison of these data with those reported by others.

On titration to pH 12.5 and back to neutrality, solutions of DNA and DNP in either 1.0M or 0.001M sodium chloride exhibit an hysteresis (figures 13A and 14A) which is removed, or greatly diminished, in each case by previous heating (figures 13C and 14C) or by the action of alkali (figures 13B and 14B). The titration curve of a DNA solution in dilute sodium chloride to pH 2.5 and back to neutrality shows a similar hysteresis (figure 15A) which is removed if the solution is first titrated to pH 12.5, as shown in figure 15B. When the acid treated solution of DNA was titrated with alkali to pH 12.5 and back titrated to neutrality the hysteresis

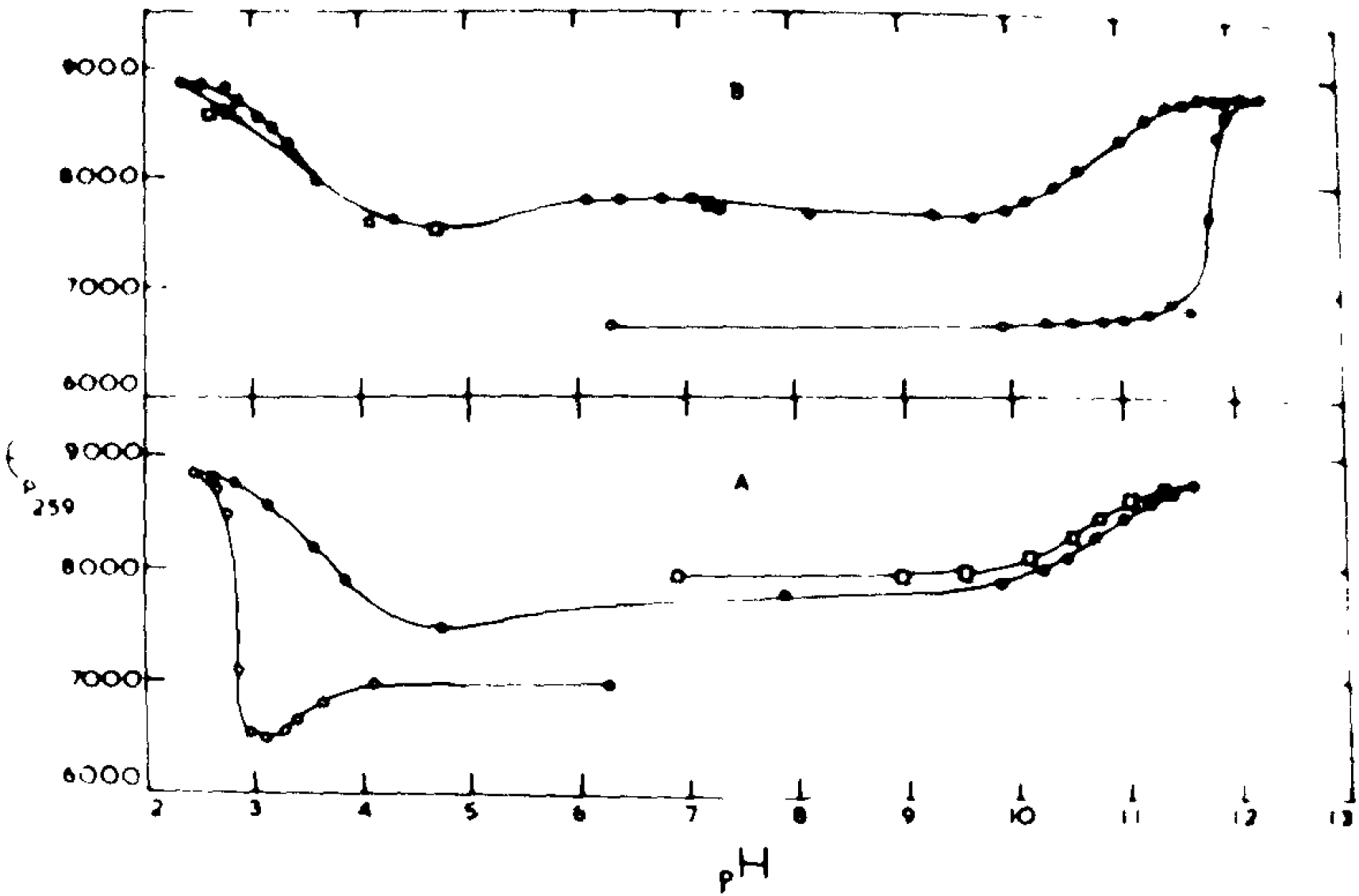


Figure 15. Spectrophotometric titration curves of DNA in 0.001M sodium chloride
A, original solution filtrated first with acid (open circles), then with alkali (closed circles) a back to neutrality (squares). B, original solution titrated first with alkali(open circles) then with acid (closed circles) and back to neutrality (squares).

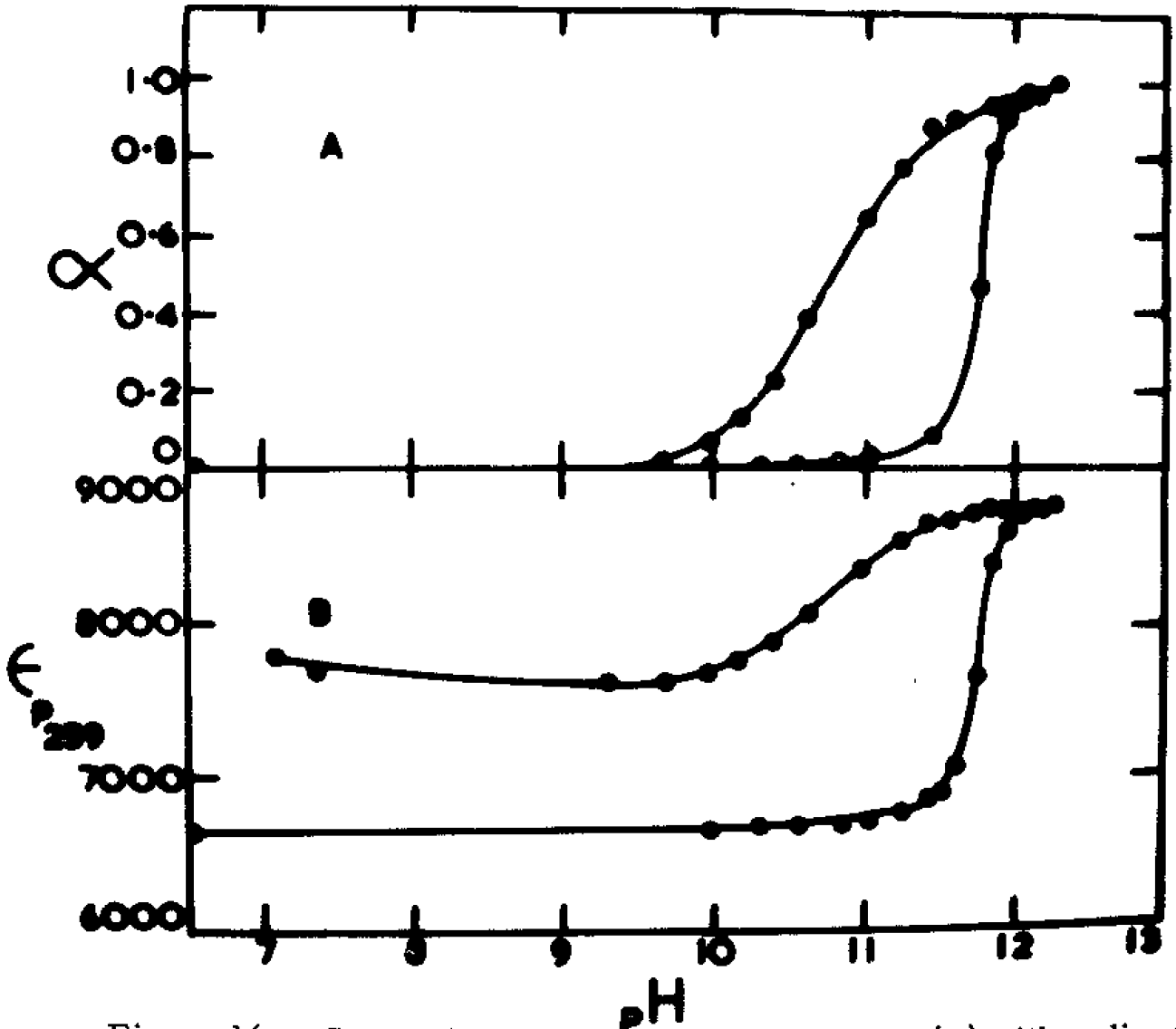


Figure 16. Comparison of a plot of α against pH (A) with a direct spectrophotometric titration curve (B). Open circles, forward titration, and closed circles, back titration.

Table IV

Description	DNA				DNP			
	Initial ϵ_P	Maximum ϵ_P	Final ϵ_P	$\%\Delta\epsilon_P$	Initial ϵ_P	Maximum ϵ_P	Final ϵ_P	$\%\Delta\epsilon_P$
I Heat Treatment of Solutions								
a) Solution in 0.001M NaCl	6650	-	8530	28.3	7200	-	8450	17.4
b) Solution in 1.0M NaCl	6590	-	7160	8.7	7090	-	7590	7.0
II Acid Titration in 0.001M NaCl								
a) Original Solution	6990	8830	7760	11.1	-	-	-	-
b) After alkali treatment	7690	8870	7750	0.8	-	-	-	-
III Alkali Titration in 0.001M NaCl								
a) Original Solution	6660	8800	7660	15.0	7240	8870	8000	10.4
b) After alkali treatment	7790	8850	7900	1.3	8000	9040	8340	4.2
c) After acid treatment	7760	8760	7970	2.7	-	-	-	-
d) After heat treatment	8530	9120	8550	0	8460	9010	8400	0
IV Alkali Titration in 1.0M NaCl								
a) Original Solution	6860	8650	7450	8.6	7100	8910	7590	6.9
b) After alkali treatment	7450	8550	7560	1.5	7580	8870	7540	0
c) After heat treatment	7160	8820	7400	3.3	7250	8910	7400	2.1

The Effect of Heat, Acid, and Alkali on the $\epsilon_{P_{259}}$ of DNA and DNP under Various Conditions
(see also figures 12, 13, 14 and 15)

of the titration curve was no longer observed (figure 15A). These titration curves are all similar in shape to plots of absorption against pH which have been previously recorded for DNA solutions[27,53,105], and the ϵ_P values of solutions of both DNA and DNP in dilute sodium chloride after titration are all in the region of 8000 (Table IV), excepting those denatured by heat which have a somewhat higher value of 8500 . Lawley[28] has reported ϵ_P values of 8000-8500 for DNA solutions denatured by acid, alkali, and heat, and the data of Laland et al.[27] give ϵ_P values of about 8500 for DNA solutions after treatment with alkali or acid. Two interpretations of these data can be made. The action of heat, acid, or alkali may result in complete denaturation to a random state of disorder having a characteristic ϵ_P of about 8000, or denaturation may occur only to a certain degree giving a distorted or deformed helix which, whilst not in a random state of disorder, has a characteristic ϵ_P dependent upon the extent of deformation, and the action of heat would then appear to produce a greater degree of deformation than that of acid or alkali. The similarity of the ϵ_P values of denatured DNA and DNP solutions indicates that the protein does not restrict the state of deformation of the DNA within the DNP complex.

Shack and Thompsett[26] presented their spectrophotometric titration data as a plot of α against pH, α being defined by

the equation $\alpha = \frac{D - D_1}{D_2 - D_1}$. Here, D is the optical density at a given pH and D_1 and D_2 are the optical densities when $\alpha = 0$ and 1 respectively, and are obtained from the asymptotes of the forward and back titration curves. By this means they were able to show that the hysteresis of the spectrophotometric titration curve was almost identical in form with that of potentiometric titration curves. A comparison of Shaek and Thompsett's method and the direct plot of ϵ_p against pH is made in figure 16 using data from the spectrophotometric titration of DNA in 0.001M sodium chloride solution. The DNP solution in 0.001M sodium chloride furnished a titration curve very similar to that of DNA (figure 14A and ref. 26) and it may therefore be inferred that this material would also exhibit the anomalous potentiometric titration behaviour of DNA (cf. chapter III) indicating the hydrogen bonded structure. Spectrophotometric titrations of DNP to an acid pH could not be made for precipitation of the material occurred below pH 5.

The curves for solutions of DNA and DNP in 1.0M sodium chloride show two points of similarity with, and two differences from, their counterparts for solutions in 0.001M sodium chloride. The similarities are the attainment of a maximum ϵ_p of about 8700, and the removal of the originally present hysteresis by treatment with alkali or by heating. The differences are in the ϵ_p value after titration, which is about 7500 compared with 8000

for the solutions of low ionic strength (Table IV), and the lower pH at which the steep increase in absorption occurs. These properties in 1.0M sodium chloride are observed with solutions of both DNA and DNP.

The hysteresis behaviour is analogous with that in the dilute sodium chloride solutions, thus the presence of the hydrogen bonded structure for both materials is again indicated. Removal of the hysteresis from the curves by treatment with alkali or by heating is of importance when considered in conjunction with the relatively low ϵ_P value after titration. By analogy with the potentiometric titration curves, the disappearance of this hysteresis signifies the rupture of the intramolecular hydrogen bonds and a denatured molecule would then be expected, but the ϵ_P value of 7500 compared with 8000 for solutions in dilute sodium chloride is consistent with less deformation of the helix in 1.0M sodium chloride than in 0.001M sodium chloride. It would thus appear that in a strong electrolyte solution there is some protection against, or inhibition of, deformation of the DNA helix (i.e. denaturation), but not against intramolecular hydrogen bond rupture. The explanation of this behaviour probably lies in the observation by Thomas[53] that DNA solutions may be denatured by dilution to very low sodium chloride concentrations (e.g. 10^{-4}M) for there are then insufficient cations to neutralise the DNA

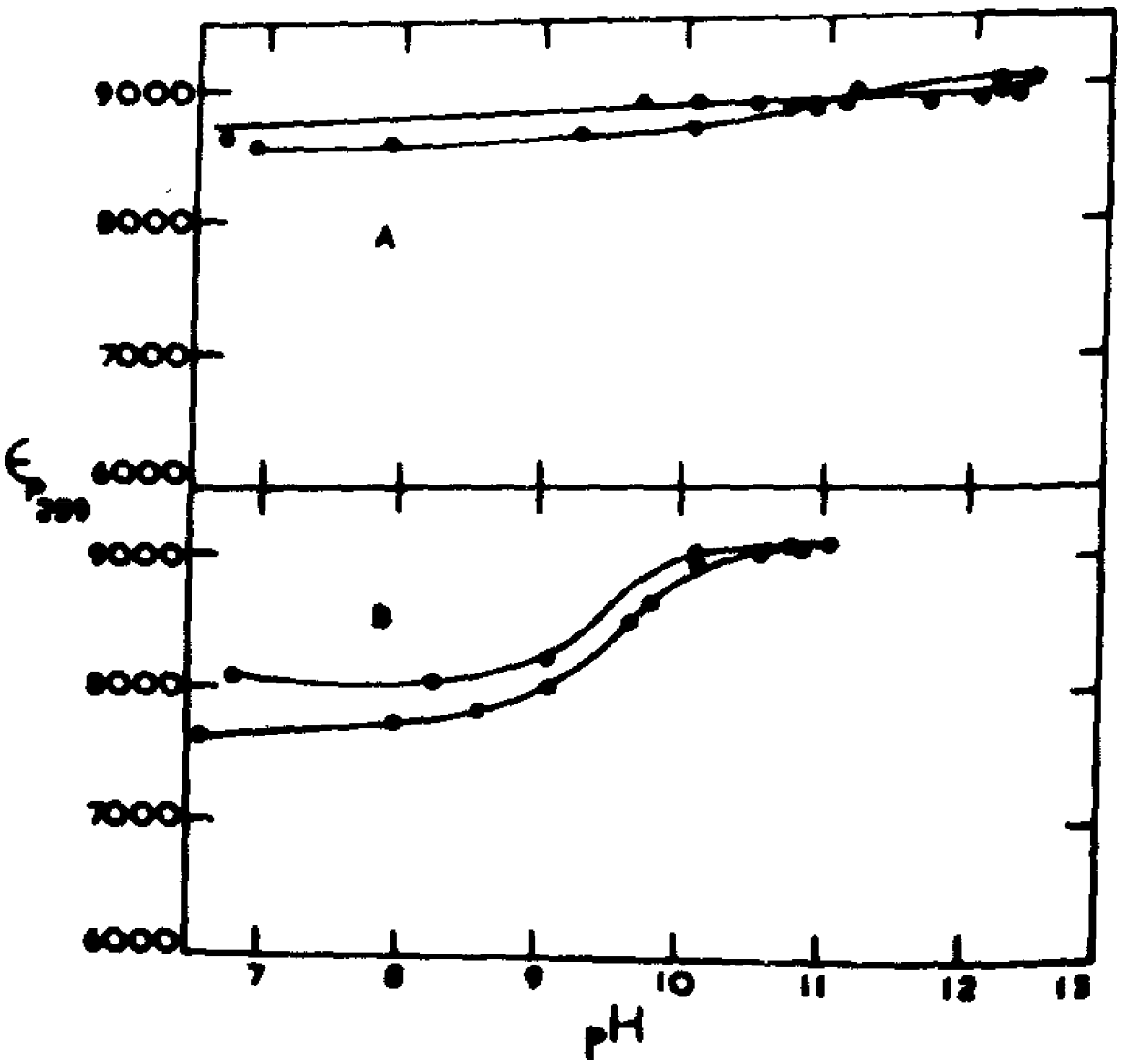


Figure 17. Spectrophotometric titration curves in A, 0.001M sodium chloride, and B, 1.0M sodium chloride, of a heated solution of DNA in 0.001M sodium chloride

phosphate groups which may repel each other sufficiently strongly to cause a deformation of the helix. In a strong sodium chloride solution the rupture of intramolecular hydrogen bonds would permit any deformation of the helix, but this is restricted by the presence of a high concentration of sodium ions around the phosphate groups. The absence of such ions at more dilute sodium chloride levels permits repulsion of DNA phosphate groups to aid deformation of the helical structure. Experimental evidence in support of this theory was readily obtained by heating a solution of DNA in 0.001M sodium chloride and diluting two aliquots for titration, one with 0.001M sodium chloride, and one with 1.0M sodium chloride. Each solution was then titrated to pH 12 and back to neutrality and the curves plotted are illustrated in figure 17. The spectrophotometric titration behaviour was thus shown to be a function of the ionic strength of the solution and the experiment further suggested that the denatured DNA molecule is a slightly deformed helix, rather than a randomly disordered structure, for the lowering of ϵ_P on diluting the heat-denatured solution with 1.0M sodium chloride solution implies deformation to a less extent than in the solutions of low ionic strength.

As an alternative to the attribution of the lower ϵ_P values of denatured materials in 1.0M sodium chloride solution to modifications of neighbouring phosphate group interactions, the difference may be considered a consequence purely of the effect of the

stronger electric field obtaining in the solutions of higher ionic strength on the π orbital interactions of the bases.

The shift of pH at which the steep increase in the forward titration curve occurs on changing from 0.001M to 1.0M sodium chloride solution is probably caused by a change in the pK values of the dissociable groups involved in hydrogen bonding, in the solution of higher ionic strength. The increase in ϵ_P to values of almost 9000 (Table IV) at the extremes of pH may be attributed to the ionisation of the bases for this would alter the electron distribution in the aromatic rings, and hence the absorption.

These spectrophotometric experiments show that the DNA within the DNP complex retains its characteristics and the presence of protein appears to make little modification to its behaviour, for DNP solutions are denatured by heat and alkali in a similar manner to solutions of DNA.

The ultraviolet absorption curve of a solution of histone in 0.001M sodium chloride is shown in figure 10 and has a peak at 275 mμ. At 259 mμ, its absorption is very low ($\epsilon_N = 21$) compared with the absorption of DNA at this wave length ($\epsilon_P = 6650$). The addition of histone and DNA solutions produced a slight increase in absorption at 259 mμ which was greater than would result from the algebraic addition of the two absorptions. Figure 18 shows the increase in absorption which occurred as a solution of histone was gradually added to DNA in 0.001M sodium chloride. As the ratio

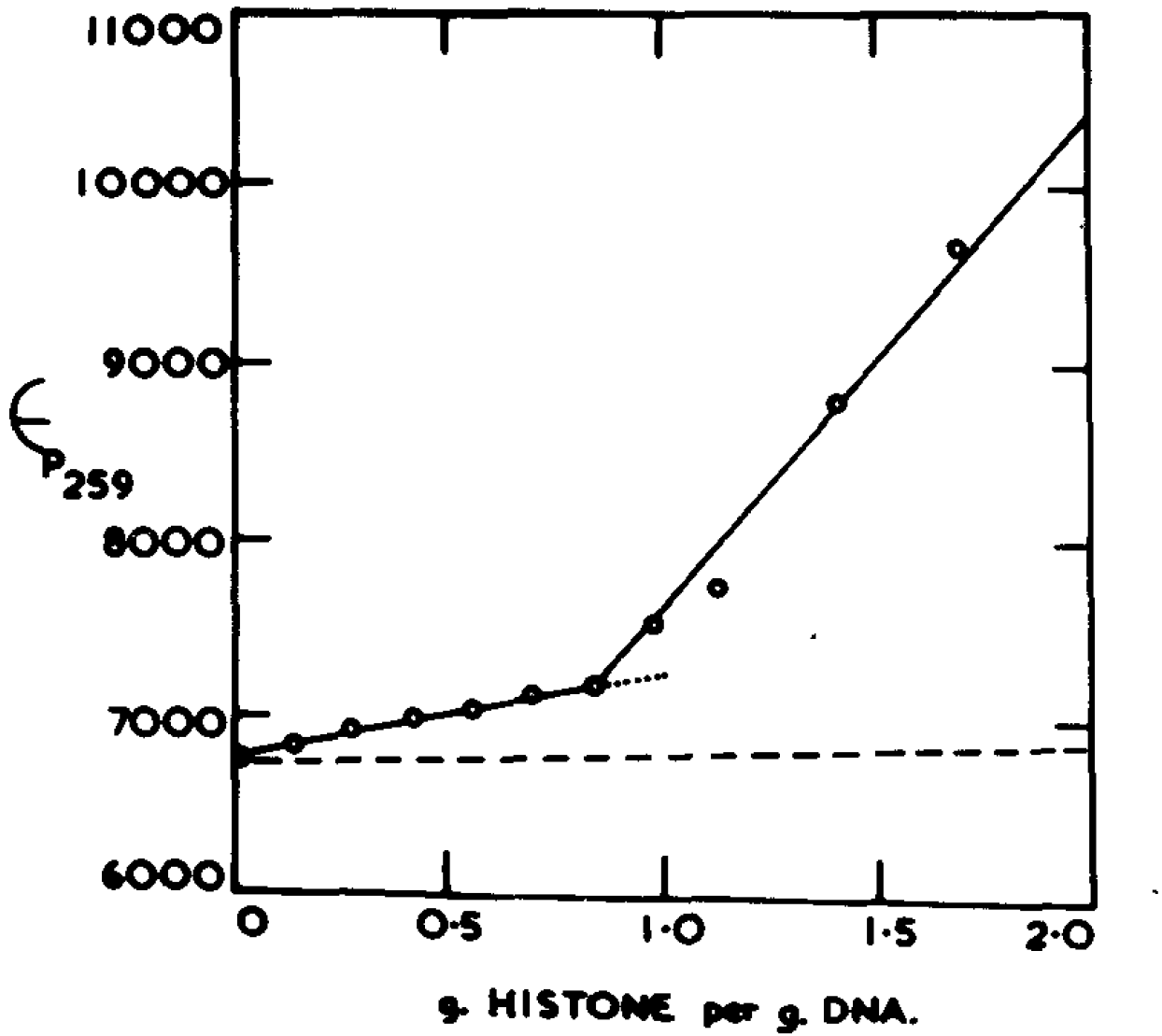


Figure 18. The increase in absorption at 259 mμ of a solution of DNA on addition of histone. The broken line is the algebraic sum of histone and DNA absorptions. The dotted line is the extrapolation to a mixture of equal weights of DNA and histone.

by weight of histone to DNA approached unity the solution became opalescent, and then increasingly turbid resulting in a steeper and less uniform increase in absorption. However, if the initial slope is extrapolated to a weight ratio of 1.0, the ϵ_P value at this point is 7250 which is the value observed in fresh DNP solutions. The pH of the histone solution was 5.6 and that of the DNA solution was 6.8; in a similar experiment using a histone solution at a lower pH (3.1) the solution became turbid almost as soon as additions to the DNA solution were commenced. The broken line of figure 18 represents the algebraic sum of the histone and DNA absorptions.

This experiment indicates that the higher value for the ϵ_P of DNP (7300 compared with 6700 for DNA) is a consequence of interaction between protein and DNA. The modification of electron interactions which the increase suggests probably arises from the proximity of heavily charged protein groups rather than deformation of the helix.

Chapter V. Light Scattering Experiments

The more recent molecular weight determinations on samples of DNA have been made by the use of light scattering methods and the data obtained have also provided information concerning the shape of the molecule.

Scattering envelopes of DNA and DNP solutions at different electrolyte concentrations were obtained both before and after heat treatment of the solutions in a further attempt to assess the influence of the presence of protein on the behaviour of DNA. The light scattering method was also used to measure the molecular weight of two of the histone preparations in order to compare the theoretical weight average molecular weight of dissociated DNP with that observed in strong sodium chloride solution.

Theoretical

The general phenomenon and fundamental theory of light scattering have been known for many years[34], but the conception of using the light scattered by large molecules in solution to determine their molecular weight was first introduced by Putzeys and Brosteaux[109] in 1935 and the rapid developments which have followed arose largely from the work of Debye[35,36]. The principal advantage of the light scattering method is that it permits

determination of a molecular weight without making previous assumptions about the molecule's shape, and simultaneously provides information concerning molecular shape[40].

Rayleigh's original equation for the intensity, i_θ, of light scattered at an angle $\theta°$ to an incident beam of intensity I_o is given by

$$\frac{i_\theta}{I_o} = \frac{8\pi^4 \nu \alpha^2 (1 + \cos^2\theta)}{\lambda_o^4 r^2} \qquad (1)$$

where λ_o is the wave length of the light in vacuo, r is the distance of the scattering molecules from the observer, ν is the number of scattering molecules per unit volume and α is the polarisability which can be replaced by the refractive index, n, using the equality $\alpha = \frac{n - 1}{2}$. Experimental data are usually treated in the form of the reduced intensity (or Rayleigh ratio), R_θ, which is defined by equation 2, or the turbidity, τ, which is a function of the reduction in

$$R_\theta = \frac{i_\theta r}{I_0} \qquad (2)$$

intensity of the incident beam, in passing through a distance l of the system, due to scattering and is defined in equation 3, in which

$$I = I_o e^{-\tau l} \qquad (3)$$

I is the intensity of the transmitted beam. It can be shown that R_θ and τ are related by equation 4[110], and for an ideal solution of a

$$\tau = \frac{16\pi}{3} \cdot R_{90} \qquad (4)$$

macromolecule it can be proved that equation 5 is valid[36]. Here, N is Avogadro's number, M the molecular weight, c the concentration in g./unit volume, n_o the refractive index of the solvent, and n the refractive index of the solution. For simplicity, equation

$$R_{90} = \frac{2\pi n_o^2 (n - n_o)^2 c M}{N\lambda^4} \qquad (5)$$

$$= KcM \qquad (6)$$

5 is generally written and used in the form of equation 6. A similar relationship to equation 5 may be obtained for the turbidity,

$$\tau = HcM \qquad (7)$$

instead of reduced intensity, and this also is usually written in the abbreviated form of equation 7.

The constants, K and H, are characteristic of a given solution, and these equations show a close similarity to the van't Hoff expression for the osmotic pressure, P, of an ideal solution. For real solutions the van't Hoff equation, 8, is modified to the form shown in equation 9 and in a similar way, a virial

$$P = \frac{cRT}{M} \qquad (8)$$

$$\frac{P}{cRT} = \frac{1}{M} + Bc + Dc^2 \qquad (9)$$

coefficient may be introduced into the light scattering expressions giving equation 10[110]. It can now be seen that a plot of $\frac{Kc}{R_{90}}$

$$\frac{Kc}{R_{90}} = \frac{Hc}{\tau} = \frac{1}{M} + 2Bc \qquad (10)$$

against c may be extrapolated to give an intercept at zero concentration which is the reciprocal of the molecular weight.

When the molecular dimensions become comparable with the wave length of the light employed, the simple equations so far used are no longer valid because interference and reinforcement occur amongst light scattered at various parts of the molecule, thus causing i_θ to vary with θ as a complex function of the dimensions of the scattering molecule. A correction factor must therefore be applied to the observed values of i_θ, and this is given by the reciprocal of the particle scattering function, $P(\theta)$, which is a summation over all the scattering elements of the particle and has a complex mathematical definition in terms of the dimensions of various model systems[35,36,111]. Assumptions about the shape of the molecule can, however, be avoided in the method used by Zimm[40] which depends on the fact that the particle scattering factors are unity when θ is zero; the scattering at various angles is therefore measured and an extrapolation made to zero angle. Since $P(\theta)$ is a function of $\sin^2 \frac{\theta}{2}$, Zimm has shown that the extrapolation is best effected from a plot of $\frac{Kc}{R_\theta}$ against $\sin^2 \frac{\theta}{2} + Kc$, where K is an arbitrary constant so chosen that the data from different concentrations become suitably

separated. A grid-like plot is then obtained (figures 21 to 24) which may be simultaneously extrapolated along lines of constant concentration to zero angle, and along lines of constant angle to zero concentration. The two lines produced through these

$$\left(\frac{Kc}{R_\theta}\right)_{\theta=o} = \frac{1}{M} + 2Bc \qquad (11)$$

$$\left(\frac{Kc}{R_\theta}\right)_{c=o} = \frac{1}{MP(\theta)} \qquad (12)$$

extrapolations have equations 11 and 12 respectively and they should meet in a common intercept which is the reciprocal of the molecular weight.

In consequence of the integrating nature of the particle scattering function, the ratio of the initial slope of the line at zero concentration to its intercept affords the calculation of the radius of gyration, ρ_3, of the particle as defined by equation

$$\frac{\text{initial slope } (c=o)}{\text{intercept}} = \frac{16\pi^2 \rho_3^2}{3\lambda^2} \qquad (13)$$

13. This may then be used to evaluate the dimensions of certain model systems[110].

Although a number-average molecular weight can be obtained from light scattering data[112], the value obtained from the treatment described is a weight-average value. The weight average molecular weight, M_w, of any given system is defined by equation 14, where n_i and m_i are the number and weight,

$$M_w = \frac{\sum_i n_i m_i^2}{\sum_i n_i m_i} \qquad (14)$$

respectively of the particles of the ith species[113].

Experimental

Solutions of DNA IV and DNP IV in 1.0M and 0.05M sodium chloride and a solution of DNA IV in 0.002M sodium chloride and of DNP IV in 0.0007M potassium phosphate buffer (pH 6.8) were prepared and a part of each solution was heated at 100°C for 20 minutes. All the solutions were then clarified by centrifuging for about 16 hours in a vacuum centrifuge at 22,000 g. The scattering envelopes of these solutions were measured as described later. Solutions of DNA V and DNP V in 0.001M and 1.0M sodium chloride were prepared and a portion of each solution was heated at 100°C for 20 minutes. Solutions of histone I A and histone III in 0.001M sodium chloride were prepared and all the solutions were then clarified as before and the scattering envelopes of the DNA and DNP solutions were measured, but in the case of the histones the scattering at 90° only was measured.

After clarifying, all the sblutions were transferred by means of pipettes to conical flasks, with externally ground stoppers, for storage. All glass ware with which the clarified solutions would come into contact was thoroughly cleaned with chromic acid,

hot water, and distilled water, and then rinsed in a stream of redistilled acetone and allowed to dry in an oven at about 50°C. The light scattering cells were cleaned by washing with a solution of "Stergene" in distilled water and were then rinsed with much distilled water, followed by redistilled acetone, and were dried in the oven.

Scattering measurements were made at four different dilutions on the DNA and DNP solutions, and at seven or eight dilutions on the histone solutions. The appropriate solvent for dilution of these solutions was clarified by filtering, under pressure through a pad of 'metasil' supported on a No. 5 sintered glass disc, directly into the scattering cell. Aliquots of the solution under examination were then added serially to the solvent and thoroughly mixed by means of a small magnetic stirrer, and scattering measurements were made after each addition of the solution. All filtration and dilution operations were carried out in a dust-free cabinet and the exact dilutions were determined by weighing. The dilution was normally made by successively adding 2, 2, 5, and 5 ml. of the clarified solution to about 25 to 30 ml. of the solvent.

A Brice-Pheonix photometer[39], calibrated against an opal diffusing screen and checked with "Ludox" solutions[114], was employed for measuring scattering intensities; the wave

length of the light used (selected by a blue filter over a mercury lamp) was 436 mμ. The scattering envelope of DNA and DNP solutions was obtained by measuring the intensity of the light scattered from solutions contained in a cylindrical cell at angles from 25° to 135° to the incident beam. The ratio of the intensity of the light scattered at 90° to that of the transmitted beam was measured and the intensity of the light scattered at other angles relative to that of the incident beam was calculated using this ratio. Similar measurements were initially made on the solvent itself and these values were subtracted from those of the solutions to give the scattering due to the solute. In order to compensate for possible changes in the response of the photomultiplier during prolonged use, the sequence of readings was made in a fixed order from 135° to 25° and then back in the reverse order. Each reading was made in duplicate and readings at 90° were observed before and after, and half way through, each sequence, and the average of the two sets of readings at each angle was used. Solutions of DNP in 1.0M sodium chloride gave wide fluctuations in intensity and it was therefore necessary to make readings at 90° at more frequent intervals. In the case of the histone solutions, readings were made only at 90° and a square scattering cell was used.

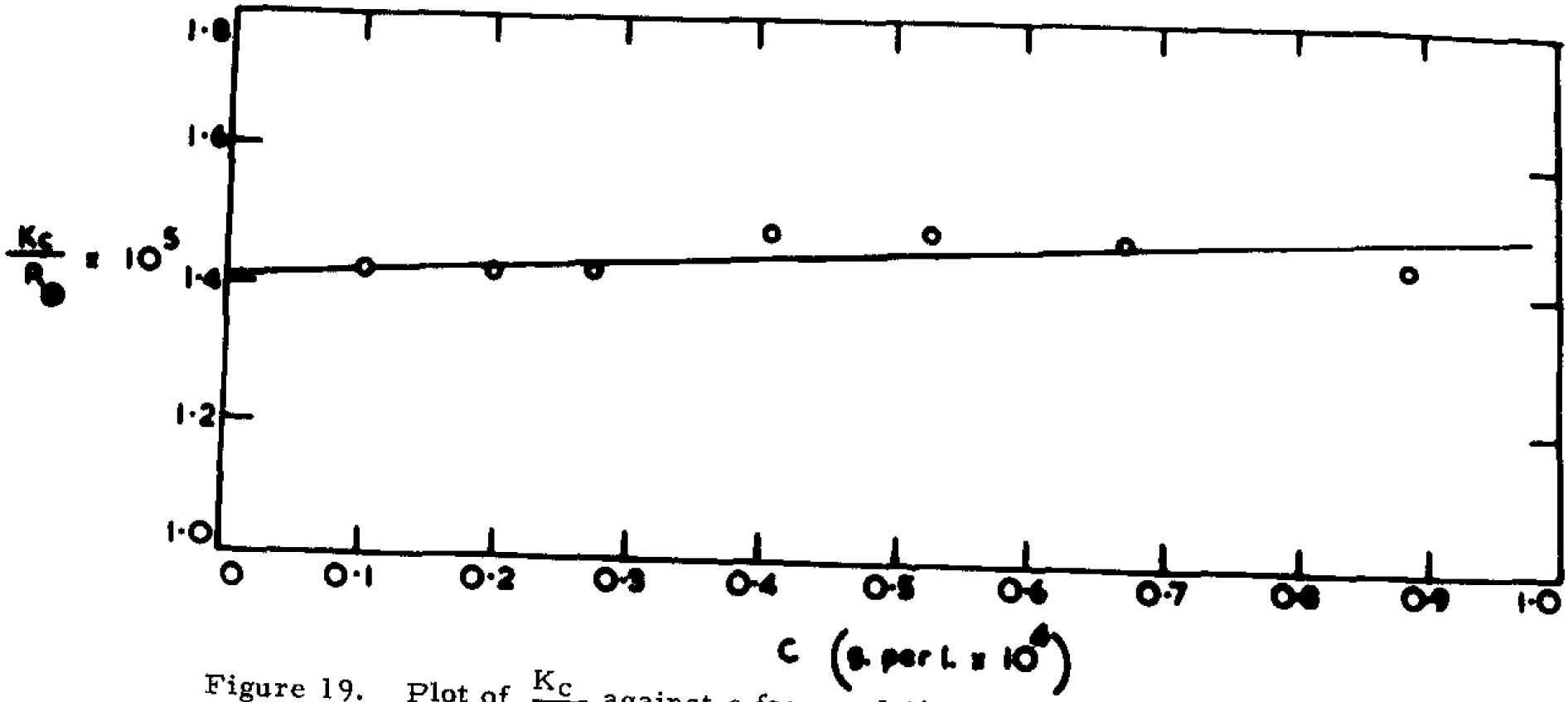


Figure 19. Plot of $\frac{K_c}{R_\theta}$ against c for a solution of histone in 0.001 M sodium chloride.

Results and Discussion

From plots of $\frac{Kc}{R_\theta}$ against c, the molecular weight of two of the histone samples was obtained. The graph from the data of histone III is shown in figure 19 and gives a molecular weight of 72,000, and that obtained for histone I A was 63,000. The constant, K, includes the refractive index increment of the protein, and since facilities for its measurement were not available, the average of the values listed by Doty and Edsall[115] for twelve other proteins was used (0.193). The molecular weights obtained are comparable with those of a number of serum proteins[115], but much lower values, in the region of 10,000, have been calculated from sedimentation experiments with certain fractions of histone[96].

Extensive use has now been made of the Zimm method for plotting data from DNA solutions and values for M of 4 to 7×10^6 and for ρ_3 of 2000 to 2500 have been observed[40-44,110,114]. Similar values were obtained in the present experiments, although in the very dilute electrolytes the readings obtained at angles below 40° were ignored for they would have led to an extrapolation giving a negative intercept of $\frac{Kc}{R_\theta}$, and the data from solutions in 1.0M sodium chloride (figure 21) are probably more reliable, for a shorter extrapolation was possible. The results obtained are included in Table V.

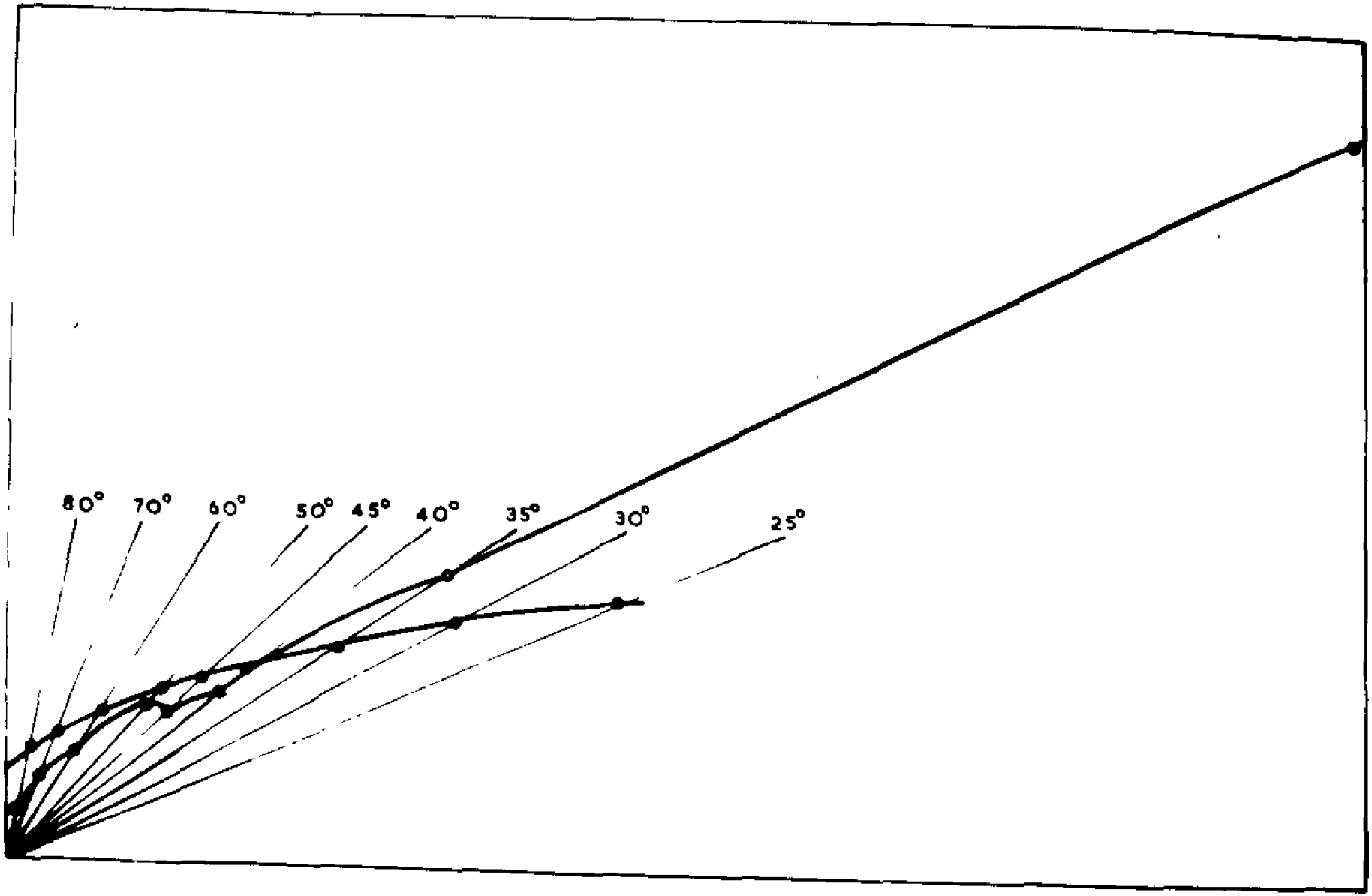


Figure 20. Relative intensity of light scattered at a given angle plotted as a vector along that angle. Open circles, DNP; closed circles, DNA.

None of the data obtained from DNP solutions in dilute electrolytes (i.e. DNP IV in 0.05M sodium chloride and in 0.0007M potassium phosphate buffer, and DNP V in 0.001M sodium chloride) could be represented on a Zimm plot, for the intensities at constant concentration gave a discontinuous curve. Behaviour of this sort is indicative of very large particles[110, 116] and a diagram was constructed showing the relative scattered intensity as a vector along the angle of scattering. A badly kinked envelope, typical of large insoluble particles such as gold or sulphur sols, was obtained which in figure 20 is shown in sharp contrast with the smooth curve produced by the data from DNA in the same solvent. All the data from preparations of DNP in dilute electrolytes thus indicate aggregation of the molecules in these solutions.

Although it proved impossible to measure the molecular weight of DNP in dilute electrolyte solutions, the method was applied to solutions in 1.0M sodium chloride with a little more success. The plots obtained (figure 22) were not, however, as accurate as those for DNA solutions in the same solvent. Less accurate values would perhaps be expected from an extremely polydisperse system such as that which would result from the dissociation of the relatively small protein moiety of the DNP complex. The values obtained for the mean molecular weight

Table V

Preparation	Solvent	DNA		DNP	
		M_w	ρ_3	M_w	ρ_3
IV	0.002M NaCl	6×10^6	2530	v. large	
V	0.001M NaCl	5.9×10^6	2320	v. large	
IV	1.0M NaCl	4.7×10^6	2420	2.9×10^6	1400
IV heated	1.0M NaCl	4.3×10^6	1220	2.8×10^6	2340
V	1.0M NaCl	5.7×10^6	2500		
V heated	1.0M NaCl	4.6×10^6	1770	2.6×10^6	2460

Data obtained from light scattering measurements on solutions of DNA and DNP in dilute and strong NaCl solution

and radius of gyration of the dissociated DNP (IV) were 2.9×10^6 and 1400 respectively. Calculation of the weight average molecular weight of dissociated DNP from equation 14, assuming equal weights of protein and DNA (from the analyses of Toennies and Bakay[54] and of Doty and Zubay[88]) having molecular weights of 6.7×10^4 (mean of molecular weight of histones I A and III) and 5.7×10^6 (Table V) respectively, gives a value of 2.7×10^6 which is in good agreement with the value obtained experimentally. The scattering data from a similar solution of DNP V showed extremely wide fluctuations in intensity and areliable extrapolation could not be made. The value for the refractive index increment used for DNA was 0.194[117] and that for DNP was the value of 0.193 reported by Doty and Zubay[88].

Measurements on solutions of DNA and DNP in 1.0M sodium chloride after heating at 100°C for 20 minutes showed that no significant change in molecular weight had occurred, but a change in the molecular shape was indicated by the different radii of gyration (figures 21, 22). The fall in radius of gyration of the DNA from 2460 to about 1500 (average of values for the two samples) indicates a contraction or folding up the molecule[110,114] as the intramolecular hydrogen bonds are broken. The change in the reverse direction of 1400 to 2400 in the case of DNP is rather surprising and since it is not attended by an increase in molecular

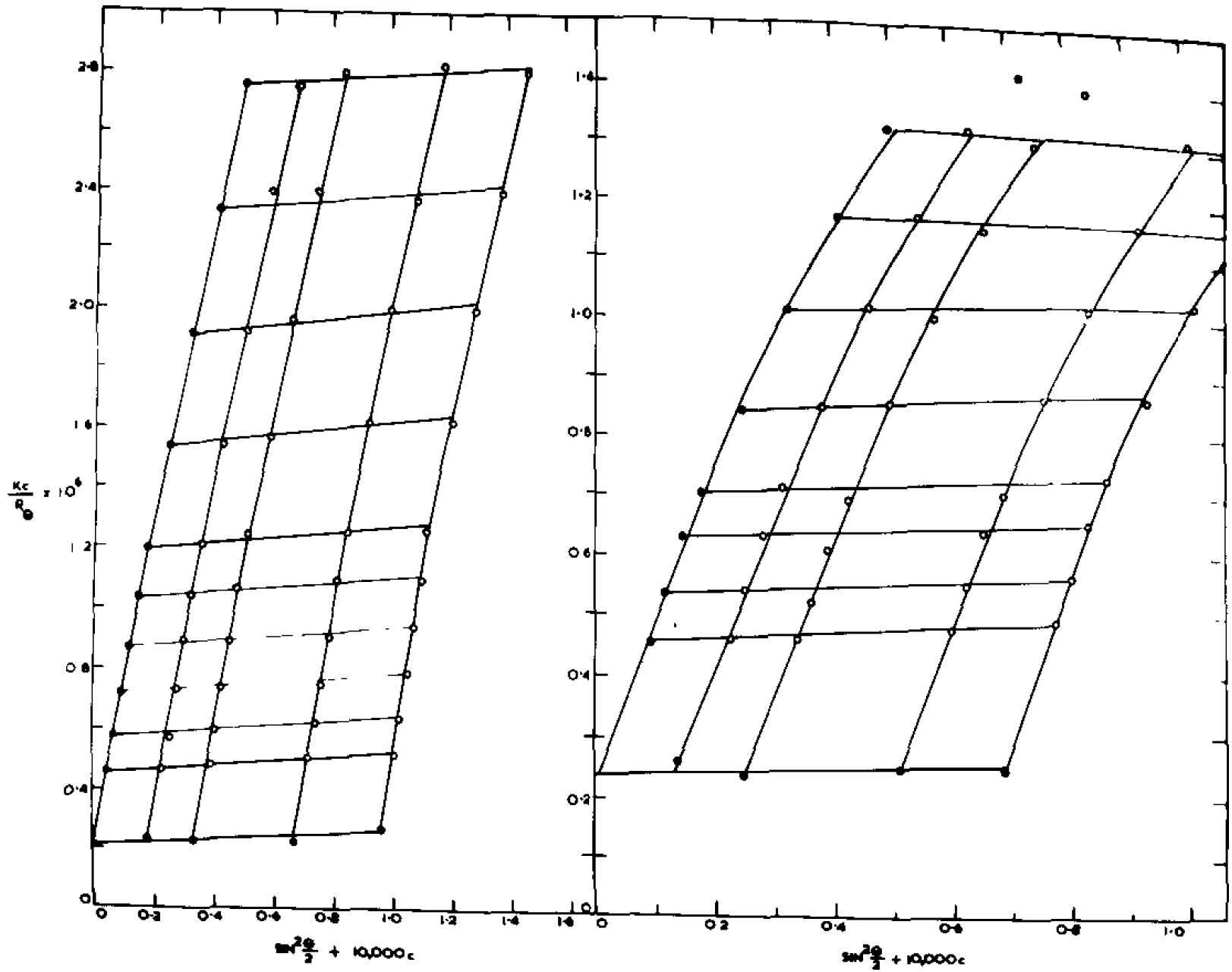


Figure 21. Zimm plots of DNA solutions in 1.0M sodium chloride. Left, original solution and right, solution after heating.

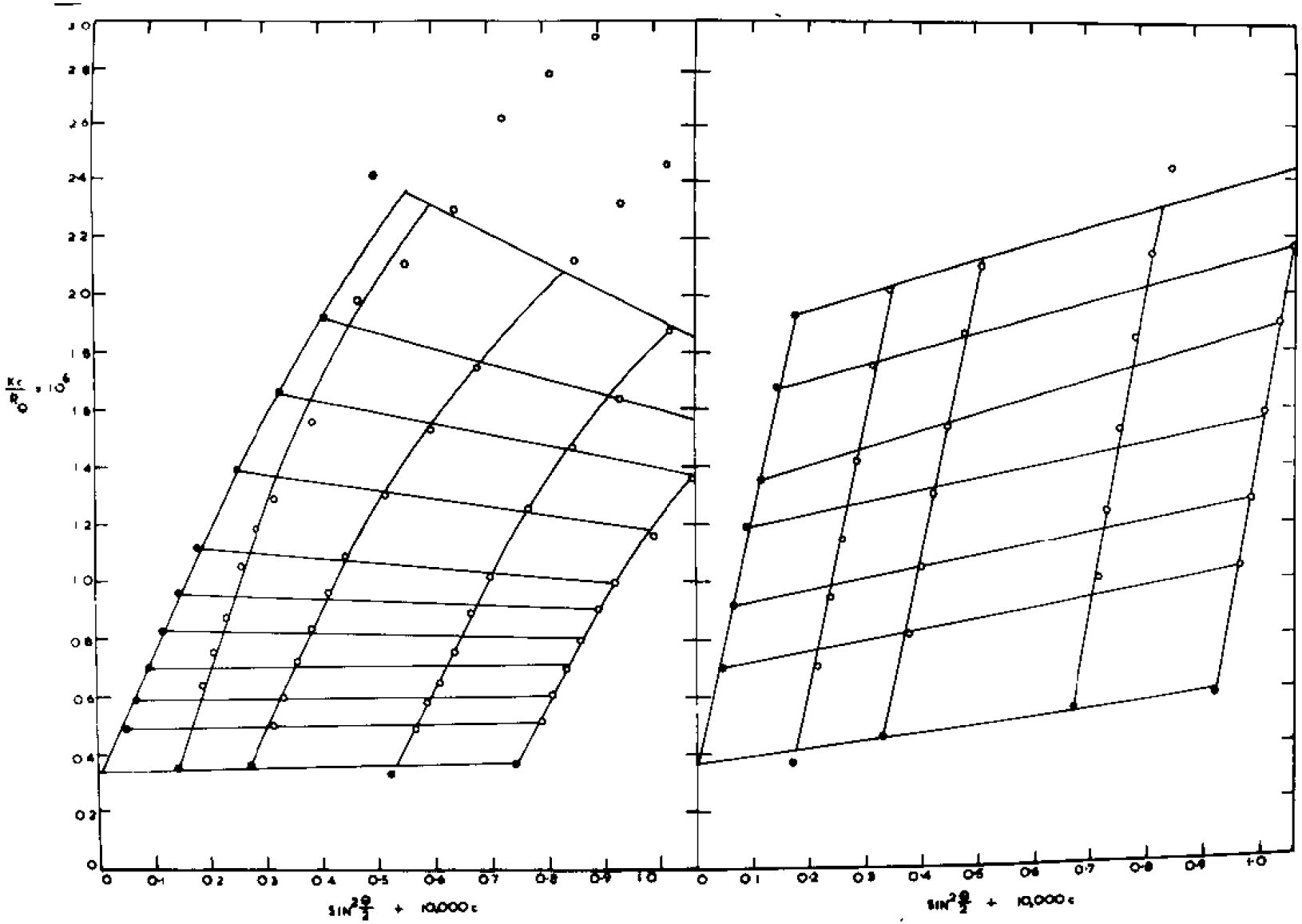


Figure 22. Zimm plot of DNP solutions in 1.0M sodium chloride. Left, original solution and right, solution after heating.

weight, which would occur if denatured protein molecules aggregated or associated with DNA, it can only be inferred that an extension of the DNA molecule occurs on denaturation in the presence of protein.

The recently published results of Doty and Zubay[88] for the molecular weight of undissociated DNP are interesting. A solution of DNP IV in the same solvent as used by Doty and Zubay (0.0007M potassium phosphate buffer) gave a scattering envelope showing aggregation of the solute, but by following precisely the procedure described in a personal communication from Zubay, Preston[117] was able to obtain a true solution of DNP having a molecular weight of about 25×10^6 compared with the value of 19×10^6 reported by Doty and Zubay. The only difference in the procedure used by Doty and Zubay for preparation of the DNP solution from that described in Chapter I (p. 15) is the incorporation of "versene" (disodium ethylene-diamine tetra-acetate) in the tissue washing solution, and the significance of this step in preventing aggregation is discussed in the next chapter.

Chapter VI. Electronmicroscopy

Light scattering envelopes indicate that in solutions of low ionic strength, DNP particles are very large (p.68) and they may therefore be visible under the electron microscope. Comparative samples of DNA and DNP were accordingly prepared for examination.

Experimental

The solutions of DNA (V) and DNP (V) in 0.001M and 1.0M sodium chloride used were those on which light scattering measurements had been made. Small portions of each solution (10^{-4} µl.) were sprayed onto plates and vacuum dried. The specimens were then subjected to low angle carbon shadowing.

The slides were examined in a Philips electron microscope and a photograph was taken of the slide of DNP in 0.001M sodium chloride, under a magnification of 70,000, the maximum of which the instrument is capable.

Preparation and examination of the slides was carried out by Dr. J.R. Majer.

Results and Discussion

No significant particles were visible in either of the DNA preparations, or in that from the solution of DNP in 1.0M sodium chloride. The preparation from a solution of DNP in 0.001M

Figure 23. Electron micrograph of DNP from a solution in 0.001M sodium chloride (x 70,000)

sodium chloride, however, showed a large number of similar sized rods and the photograph reproduced in figure 23 shows a pair of such particles which were selected at random.

The observation of fibrous macromolecules with the electron microscope requires a very clean surface and magnifications of 100,000 or above are desirable[118], and the failure to detect DNA may be due in part to uneven surfaces of the preparations and low resolution of the instrument. The observed particles of DNP are, presumably, aggregates and the dimensions of particles illustrated were: length 2000 mμ, and width 200 mμ, giving an axial ratio of 10.

The aggregation of DNP in solutions of low ionic strength which was suggested by the light scattering data is thus substantiated by the electronmicrograph and it is interesting that the aggregates are in the form of rods rather than spheres. Two mechanisms may be postulated for the aggregation of DNP molecules. Ionic interactions of protein side chains could be responsible for the process, or, more probably, co-ordination of protein nitrogen atoms with bivalent metal ions, such as magnesium, could result in a "broadside on" aggregation represented schematically in figure 24. Aggregation by such a chelation process appears particularly probable when the observations of Doty and Zubay[88] on the molecular weight of DNP are considered. The necessary

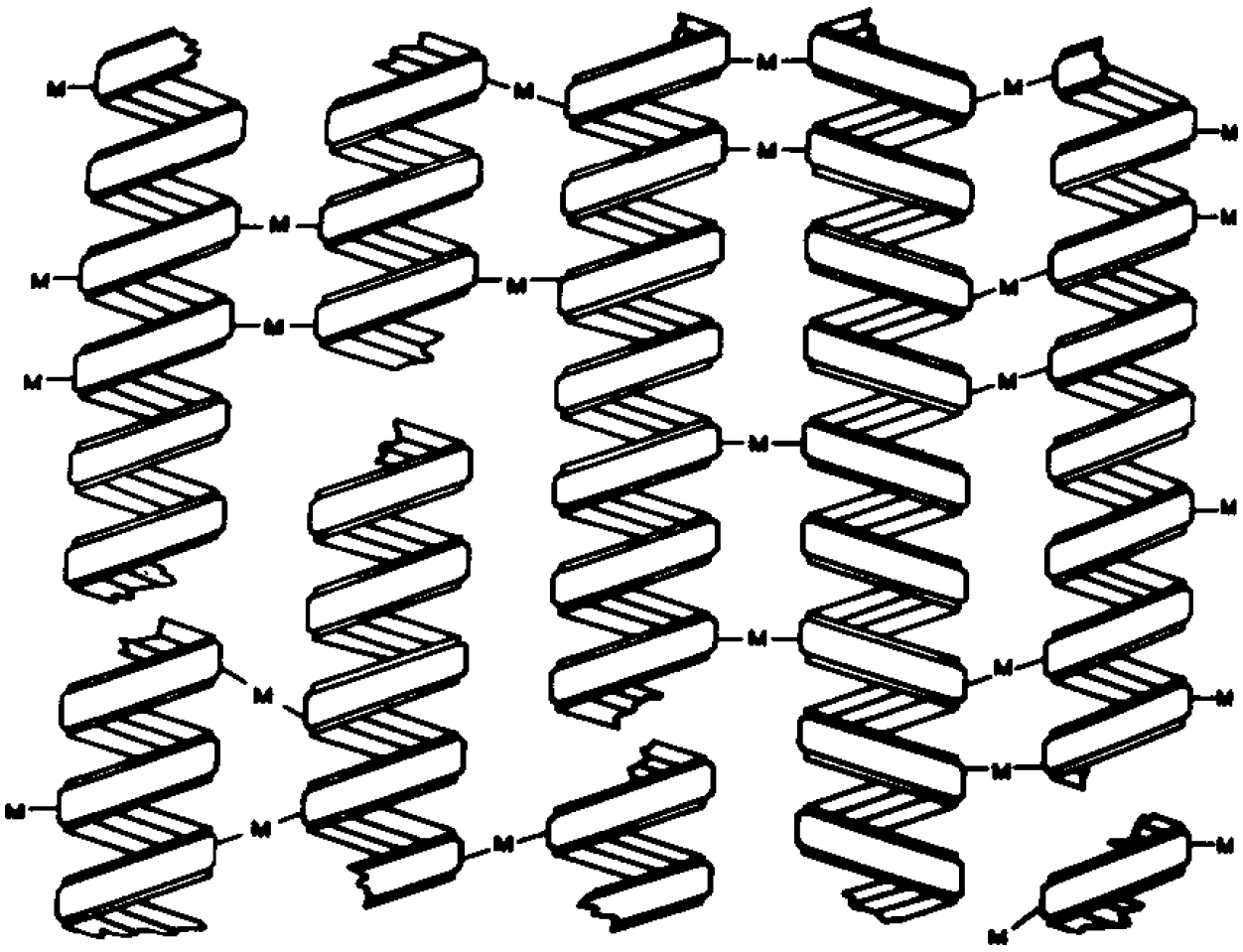


Figure 24. A representation of the aggregation of DNP in the presence of metal ions.

metal ions would not be removed by the washing procedure employed in preparing the DNP samples used here, and would be available for chelation with the protein. Extraction of the tissues with a solution containing a sequestering agent, such as the disodium salt of ethylenediamine tetra-acetic acid, would remove cations (other than monovalent ones) enabling a solution of DNP to be obtained free fromthe ions capable of chelating with the protein. Such a solution would have little tendency towards aggregation, and hence its suitability for molecular weight measurements.

Conclusion

A reasonably reproducible preparation of DNP was obtained from calf thymus glands by a mild extraction procedure which did not dissociate the complex. Solutions of this material at low electrolyte concentrations were shown by light scattering methods to be highly aggregated and this was confirmed by electron microscopy, but the particles observed under the electron microscope may be larger than those in solution due to further aggregation during drying of the slide. The dissociation of the DNP complex in a solution of high ionic strength was demonstrated by ionophoresis in 0.4M sodium chloride solution, and by measurement of the average molecular weight in 1.0M sodium chloride solution, when a value equal to the weight average molecular weight for equal weights of histone and DNA was obtained. Paper ionophoresis was also used to demonstrate the dissociation of the complex in solutions of low ionic strength at pH 13.

The low solubility of DNP in solutions of low ionic strength precluded accurate potentiometric titration, but some hysteresis characteristic of DNA was observed although the presence of the protein of the DNP complex greatly modified the shape of the titration curve. Spectrophotometric titration is a technique more applicable to very dilute DNA solutions and a series of comparative

experiments with DNA and DNP solutions was made. Shaek and Thompsett had previously shown that such titrations of DNA in the alkaline region give hysteresis curves analogous with those from potentiometric titrations[26] and present experiments showed that spectrophotometric titration of DNA in the acid region produced a curve similar to that obtained in the alkaline region. The experiments with DNP solutions showed that the hysteresis of the spectrophotometric titration curves obtained by Shaek and Thompsett using DNP prepared by the Mirsky and Pollister procedure was in fact a property of the native DNP and not a reconstituted artefact.

From the spectrophotometric studies, it was apparent that DNP is denatured, by heating or by treatment with alkali, in a similar manner to DNA. Parallel experiments with solutions of DNP in 0.001M and 1.0M sodium chloride showed that denaturation proceeded in a similar way to that of DNA whether the complex was whole or dissociated. It was concluded further from the spectrophotometric studies that at high ionic strength (1.0M sodium chloride) there was some restriction on the deformation of the DNA helix, (i.e. a partial protection against denaturation) in both DNP and DNA itself, under conditions where the intramolecular hydrogen bonds were broken. The ultraviolet absorption behaviour of DNP was, in fact, almost that of DNA and it appears from an experiment with mixtures of histone and

DNA that the slightly higher ϵ_P value of fresh DNP preparations over that of DNA is a result of DNA-histone interaction rather than an integration of their individual absorptivities.

Two main conclusions arise from the experiments discussed in these pages. The presence of the protein of the DNP complex can result in a high degree of aggregation in solutions of low ionic strength, although it has recently been shown by Doty and Zubay that a non-aggregated solution of DNP can be obtained if a special washing procedure is adopted.[88] As a result of DNA-protein complex formation, denaturation of DNP by heating is slightly slower than that of DNA, otherwise there appears to be little modification of the DNA properties.

The features of DNA solutions consequent upon its unique helical structure are also exhibited by DNP solutions. It is therefore to be concluded that DNA retains its unique helical configuration within the complex, as one would anticipate, and as is suggested by the X-ray diffraction patterns of fish-sperm DNP.[71]

PART TWO

THE REACTION OF MANNOSE WITH GLUCOSAMINE HYDROCHLORIDE

Introduction

Nitrogen-containing disaccharides have been synthesised enzymically by α-galactosyl transfer to 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine) and 2-acetamido-2-deoxy-D-galactose (N-acetyl-D-galactosamine) using an extract of autolysed Trichomonas foetus organisms[119]. A number of galactosides of N-acetyl-glucosamine has also been obtained by the incubation of extracts of Lactobacillus bifidus var. pennsylvanicus[120], Escherischia coli[121], and several animal tissues[122] with lactose and N-acetyl-glucosamine. In the course of an attempt to demonstrate the transglycosylation of D-mannose to 2-amino-2-deoxy-D-glucose hydrochloride (glucosamine hydrochloride), an interesting reaction between these two sugars was observed.

The production of a disaccharide containing glucosamine could be followed ionophoretically, for in acetate buffer at pH 5 glucosamine itself would migrate towards the anode at about twice the speed of its disaccharide, thus effecting a rapid separation. When such experiments were commenced and solutions were spotted onto a strip of paper and dried with a stream of hot air from an electric hair drier in the customary manner, a control of D-mannose and D-glucosamine hydrochloride solutions was found to produce a spot on the ionophoretogram

attributable to a disaccharide. It was soon established that the compound responsible for this spot arose entirely from heating this mixture on the paper, and that both an amino group and a reducing (aldose) group were necessary for the formation of the compound.

Similar compounds may quite possibly arise as artefacts in the chromatographic analysis of the hydrolysates of many natural products, particularly polysaccharides, unless heating on the paper is avoided, and the reaction appears to be similar to the condensation on paper of glucose and ammonia which was observed by Bayly in the chromatographic analysis of urine samples from diabetics[123,124]. Investigation of this reaction of mannose with glucosamine hydrochloride was undertaken and it was soon apparent that an unstable compound reminiscent of the glycosylamines produced from aliphatic amines[125] was formed by a condensation process.

The condensation of reducing sugars with amines has been known for almost a century[126] and although two isomeric products were isolated from the reaction of D-glucose with p-phenetidine in 1925[127], it is only in relatively recent years that the complexity of reactions between reducing sugars and amines has been fully appreciated. Reactions analogous with the condensation of aldehydes with amines have been demonstrated between reducing sugars and primary and secondary aromatic and aliphatic amines,

hydrazines, hydroxylamine, urea, guanidine, and amino acids, peptides, and proteins. Initially, condensation occurs to form a glycosylamine, but this may be followed by a sequence of rearrangements and degradation reactions leading, eventually, to the production of complex brown polymers.

The first synthetic compounds were obtained as glass-like products by Schiff[126] on heating dry glucose with aniline or p-toluidine, but Sorokin isolated crystalline derivatives by carrying out the reaction in alcoholic solution[128]. Numerous modifications of this simple reaction have permitted products to be obtained from many different aldoses and a large number of aromatic amines of varying basicity[129]. Preparation of the corresponding derivatives of ketoses has, however, proved to be very difficult and only a few have been obtained, and these in poor yields. The best preparative methods for ketose derivatives appear to be those described by Barry and Honeyman[130] in which the amine and ketose are heated in anhydrous ethanol in the presence of catalytic quantities of the amine hydrochloride. The presence of an amine hydrochloride catalyst is also employed in the preparation of alkylamine derivatives of glucose when the two reactants are heated together[125]. In contrast with their aromatic analogues, aliphatic amines appear to condense more readily with ketoses than with aldoses[131].

The initial products from the condensation of reducing sugars with amino compounds are best regarded as arising from the cyclic hemi-acetal form of the sugar rather than its aldehydo form. It is then readily apparent that the product will be a glycosyl derivative and not a Schiff base. Although a number of names has been applied to these condensation products, only two may be used on a systematic basis[129]. The condensation compound furnished by glucose and ammonia is called glucosylamine, for it may be regarded as ammonia with one of its hydrogens replaced by the glucosyl radical, and the products derived from simple amines are regarded as derivatives of such glycosylamines. The derivative from glucose and aniline would thus be N-phenyl glucosylamine, but where it is more convenient the compound may be named as the glycosyl derivative of the amine, which in this case would be N-glucosylaniline. The term "glycosylamine" is used as a general name for compounds derived by condensation of reducing sugars with amines (and hence we may have aldosylamines and ketosylamines) and "glucosylamine" refers to the derivative of glucose and ammonia.

Condensation between the imino group of glucosylamine and a second molecule of glucose to form diglucosylamine has been demonstrated[124]. Sjollema obtained this compound in a crystalline form from glucosylamine on boiling in methanol for several hours[132], and it was also encountered as a by product

from the deacetylation of glucose pentaacetate with ammonia in ether[133]. The reaction of glucose with ammonia is, however, particularly complex, for no fewer than fifteen components have been chromatographically separated from an aqueous reaction mixture[134], but a compound analogous with diglucosylamine has been isolated from the reaction of aniline with an excess of β-phenylglyceraldehyde[135]. In comparison with the formation of diglycosylamines, condensation products have been obtained from the reactions of glucose with cyclohexylamine[125] and fructose with octadecylamine[131] which contain two molecules of the amine to one of the sugar, and similar compounds have been reported from the reaction of aldehydo sugars with primary aromatic amines where glycosylamine formation is impossible[136].

Although glycosylamines of aromatic amines are readily crystallised from alcohol solutions, and indeed are widely used in the characterisation of sugars[129,137], they can undergo a re-arrangement (loc. cit.) and are unstable in aqueous systems even at normal temperatures[138]. The glycosylamines of aliphatic amines are rapidly hydrolysed to the original reactants in aqueous solutions[125,139,140] and, since these solutions are basic, alkaline degradation of the sugar usually accompanies the hydrolysis[141].

In his work on the condensation of glucose with p-phenetidine, p-anisidine, and p-toluidine, Amadori obtained two structurally

different isomers which were not anomers. One of these was much more labile than the other, particularly towards acid hydrolysis, and this he regarded as a glycosylamine, but he wrongly attributed a Schiff-base structure to its isomer. Kuhn and Dansi[142] proved that the more labile isomer was in fact the N-substituted glycosylamine and showed that the stable isomer was a molecular rearrangement product, but incorrectly assigned to it a branched-chain structure. From studies of the compounds obtained on hydrogenation, the rearrangement products were shown by Kuhn and Weygand[143] to be 1-amino-1-deoxy-2-ketoses, and they named the rearrangement after Amadori. The rearrangement was first observed when the glycosylamine was heated, but an increased yield was obtained in the presence of acid catalysts[144], and the intramolecular change has now been accomplished in a number of ways[145]. The best of these appears to be the method used by Hodge and Rist[146] of heating the glycosylamine in alcoholic solution in the presence of a relatively large quantity of a compound containing an activated methylene group (malonic acid is a good example) and a catalytic quantity of a secondary amine. At first, the rearrangement was thought to be peculiar to the derivatives of aromatic amines, but, using the procedure devised by Hodge and Rist, the N-substituted 1-amino-1-deoxy-2-ketose was obtained from the glycosylamines of all classes of amines.

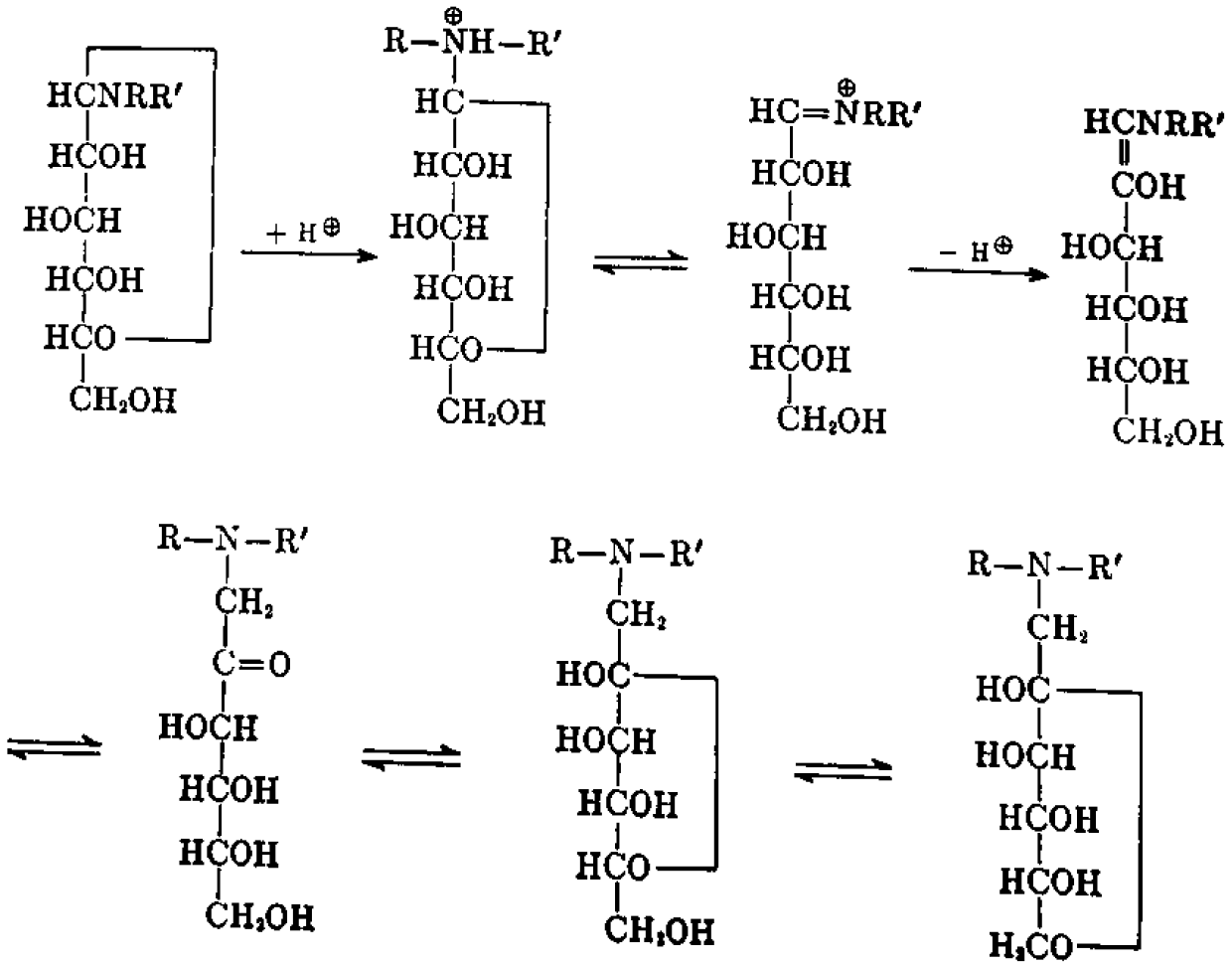


Figure 25. Mechanism for the Amadori rearrangement suggested by Isbell.[147]

Experiment shows that the Amadori rearrangement is catalysed by acids and a number of detailed mechanisms has been proposed[145]. One of these, suggested by Isbell[147], is represented by the sequence of changes illustrated in figure 25. The nitrogen atom of the N-substituted aldosylamine accepts a proton from an acid catalyst to form an ammonium ion which is presumed to be in equilibrium with the cation of the Schiff base. Movement of the double bond is then explained on a basis of electron flow towards the positive nitrogen atom, making C_1 transiently positive. The consequent electron flow from C_2 to C_1 weakens the C-H bond on C_2 permitting the expulsion of the hydrogen atom from C_2 as a proton to leave the enol form of the ketose. Tautomeric shift to the keto form would be favoured by its tendency to form one of the more stable ring systems. The essential features are/presence of the proton as a catalyst and the 1-2 enolisation which occurs as the proton is discharged. That 2-deoxy and 2-substituted aldoses do not undergo the rearrangement[146,148,149] may be taken as evidence for the 1-2 enolisation stage of the process.

Derivatives of amino acids prepared by refluxing a methanolic solution of glucose and the amino acid[150], or by heating a syrup of the reactants and separating the products by ion-exchange chromatography[151], were found to have similar properties to compounds obtained from the same starting materials by method of Hodge and Rist (loc. cit.) which furnishes the Amadori

rearrangement product. An interesting reaction involving a repetition of the rearrangement process has recently been reported in which an excess of glucose is heated as a paste with glycine to give a difructose derivative instead of a diglycosylamine[152].

Proof of the structure of Amadori rearrangement products from glucose and aromatic amines was obtained by studying the products obtained on hydrogenation (loc. cit.), but the rearrangement product may be readily distinguished from its isomeric glycosylamine by the application of a number of colour tests, and by examination of the products of hydrolysis[148]. Hydrolysis of glycosylamines by water or hot, dilute acids yields the corresponding amine and reducing sugar. Similar treatment of the Amadori rearrangement compound does not, however, produce fructose and the amine. Usually, the hydrolysis products of these compounds comprise the amine, a furfural derivative, much insoluble, dark brown material, and a very small quantity of the original aldose. The formation of furfural derivatives from ketoses on heating is well known[153] and occurs by dehydration.

Decomposition of the Amadori rearrangement compounds with the development of brown colours and insoluble, brown, polymeric material, which occurs on hydrolysis, can also occur spontaneously and the importance of the Amadori rearrangement in the browning reaction of carbonyl-amino systems has been

emphasised in a comprehensive review by Hodge[148]. Conversion of the 1-amino-1-deoxy-2-ketoses to the brown polymers is a highly involved process which originates with dehydration and, or fission of the sugar chain whilst in combination with the amine. The dehydration may produce either furfurals or reductones depending on the conditions obtaining. When solutions of the Amadori compounds in aqueous acid are heated, 5-hydroxymethyl-2-furfural (HMF) is obtained from hexose derivatives, and furfural from pentose derivatives, and the dehydration is believed to occur whilst the sugar is in combination with the amine. The Schiff base of the furfural is thereby formed and hydrolysis yields the aldehyde and amine, and hence the ketose as such can never be recovered by hydrolysis of an Amadori rearrangement compound.

The formation of furfural derivatives requires the removal of three molecules of water, but dehydration to reductones can occur by the loss of two molecules of water. A crystalline reductone was obtained when glucose was heated with an excess of piperidine in the presence of malonic acid at 80°C and the same compound was obtained by heating N-galactosyl piperidine or 1-deoxy-1-N-piperidino-fructose in the dry state, or on long standing of the latter compound at room temperature[146]. An analogous compound was obtained from the N-glucosyl derivative of monoethanolamine

by spontaneous dehydration in air.

Both the furfurals and reductones readily become discoloured and the browning of their solutions is greatly accelerated by the presence of amino compounds[140,148]. Since the structure of the reductones formed by sugar dehydration is not known, most studies of model reactions have been made with ascorbic acid, and the dehydro-reductone appears to be an essential precursor to the development of brown colours[148]. That both furfural (or HMF) and reductones should arise from dehydration of the sugar and exhibit similar tendencies towards colour development is less surprising than may initially appear, for the reductones may possess a structure similar to that of furfural without ring closure. Hydrolytic cleavage of HMF could furnish a conjugated diene-diol analogous with $\alpha\alpha'$-dihydroxy muconic acid which has been shown by polarographic methods to possess reductone properties[148,154]. It is quite possible that open chain diene-diols of this sort may be interm ediates in the formation of HMF and furfural.

The products of dehydration are by no means the only precursors to colour formation and the eventual production of insoluble, brown, polymeric materials which are often referred to as melanoidins. Fission of the main sugar chain may accompany the reactions already described, for in addition to the well known fragmentation of sugars in alkaline solution, the degradation of reducing sugars in neutral and in slightly acid solutions with the

formation of acetol, pyruvaldehyde, pyruvic acid, and diacetyl has been reported[155]. In alkaline solutions, these and many more degradation products, including α-β unsaturated aldehydes and reductones, have been isolated[148], and the separation of pyrazines and imidazoles from the reaction of glucose with ammonia[134] furnishes additional evidence of splitting of the carbon chain. Dealdolisation (i.e. reverse of the aldol condensation) is the accepted mechanism for sugar fragmentation and the process should therefore be catalysed by amines and their salts. Such an effect has been demonstrated in the catalysis of the dealdolisation of diacetone alcohol by amines, but not by general base catalysts (ref. 148, p. 935), and since amines are liberated from either the glycosylamine or its rearranged form, their potential in promoting both sugar fission and aldol condensation of the products must be considered as an integral part of the browning process.

Particularly in amino acid systems, the Strecker degradation is another side reaction which may be important in polymer formation. This degradation of an α-amino acid to a deaminated aldehyde containing one carbon atom less than the original amino acid could be the means of a transamination incorporating nitrogen into the melanoidins, and would be initiated by reductones formed by dehydration or as fission products.

No visible changes in solutions result from these

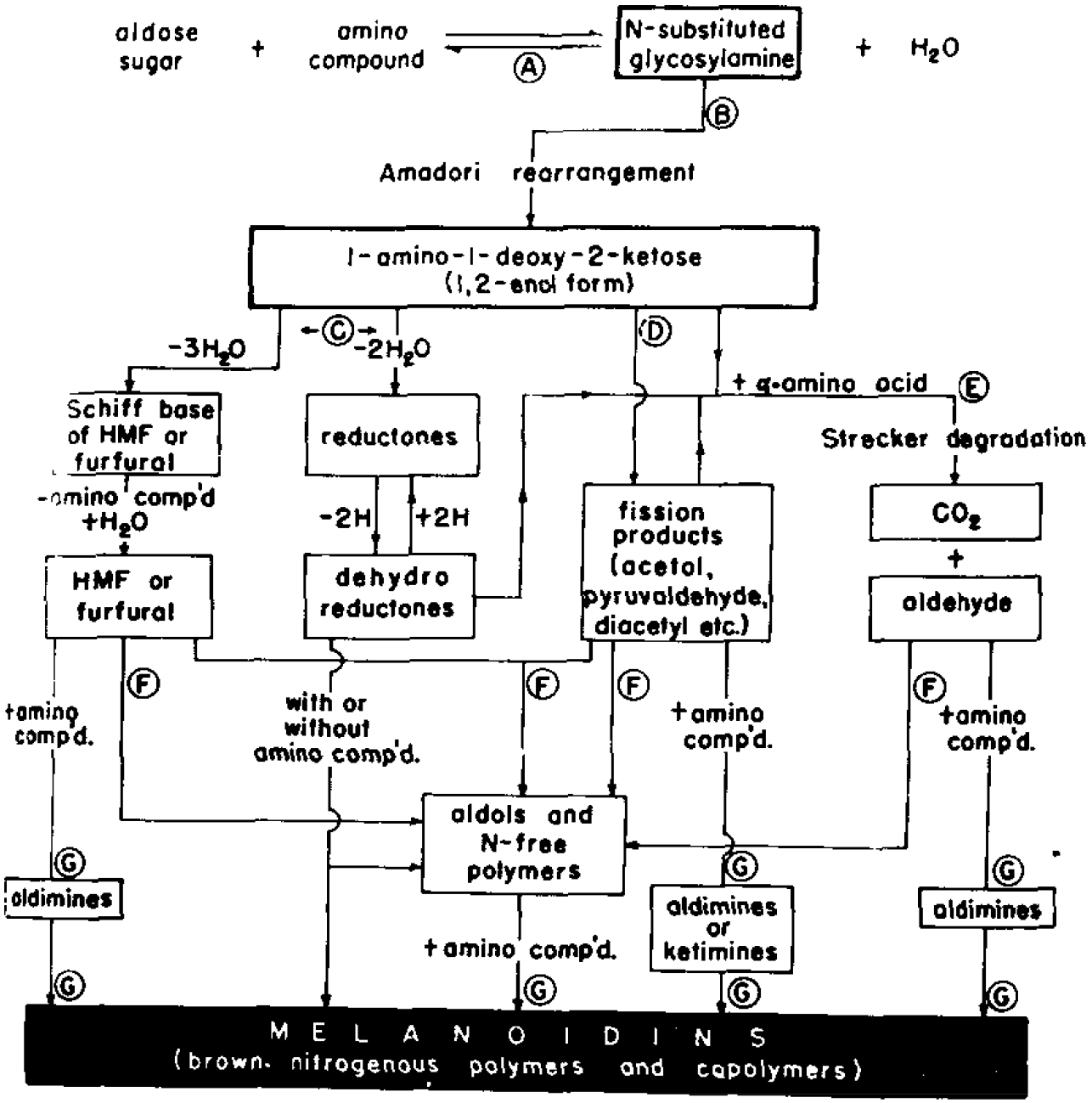


Figure 26. The browning reaction scheme of Hodge.[148]

preliminary reactions, but they are accompanied by the development of strong absorption in the ultraviolet. In the later stages of the browning process the various intermediates polymerise to form unsaturated, fluorescent, coloured compounds which lead eventually to the melanoidins. The final polymerisation processes are believed to be effected mainly through aldol condensations and aldehyde-amine polymerisations, although the formation of reactive heterocyclic nitrogen compounds may also be important.

The various stages outlined for the browning process in reducing sugar-amine systems have all been proved to lead to melanoidin formation in appropriate model systems and Hodge has integrated them into a scheme which shows how the various possibilities may be combined in the production of the final insoluble polymers[148]. This is reproduced in figure 26 and it emphasises the complex corollaries devolving upon the simple condensation of reducing sugars with amines. It may be mentioned that in addition to carbonyl-amino systems, browning by caramelisation and oxidative processes (both enzymic and non-enzymic) are also important in the development of melanoidins in non-amino systems, and that many of the later stages postulated for these processes are analogous with those in the carbonyl-amino group of reactions.

It is difficult to evaluate adequately the importance of carbonyl-amine condensations in natural processes. The browning reaction is only one of many complex sequences initiated by this

simple condensation mechanism, but if affords an excellent example of the far-reaching consequences of a single initial reaction and illustrates how a series of simple addition, rearrangement, and dehydration processes may provide the basis of the formation of an almost limitless range of materials. The preceding pages have indicated the importance of the reaction of carbonyl functions with amines in the production of natural flavouring and colouring matters (see also refs. 145 and 148); the importance of the Amadori rearrangement in the biosynthesis of tryptophan has been pointed out by Hanley-Mason[156]; the condensation of sugars with nitrogenous bases has led to the synthesis of nucleotides[157] and of riboflavin[158], and the acylation of glucosamine-1:3:4:6-tetraacetate with carbobenzoxy glycyl and alanyl chlorides followed by deacetylation to form N-glycyl- and N-alanyl-glucosamine[159] has suggested a means for the incorporation or conversion of carbohydrates into peptides and proteins[160].

The reaction of mannose with glucosamine hydrochloride is a particularly interesting case in this important group of condensations because it is effected in a neutral medium, rather than the basic medium normally prevailing when condensations occur between a sugar and the free amine. Furthermore the amine used here is itself a reducing sugar and the possibility of its glycosidic group being involved in a condensation to form a disaccharide should

not be overlooked, particularly under conditions where molecular movement may be severely restricted. The formation of anhydro sugars could well precede the production of glycosidically linked sugars, for the chemical synthesis of di- and oligosaccharides has been accomplished using 1-2 anhydro sugars[161]. Two types of product might thus be anticipated from the reaction, though the product of a condensation between the reducing group of mannose and the amino group of glucosamine may be but the starting point for another series of reactions and a multiplicity of products.

Investigations of the conditions under which the sugars react, the generality or scope and extent of the reaction, and attempts at the isolation of the reaction products and observations on their properties and structure are described in the following pages.

Discussion

The Production and Properties of Compound A

The new basic component detected on ionophoretograms of D-mannose and D-glucosamine hydrochloride mixtures could be a consequence of heating these two sugars together on paper, or it could result from glucosamine hydrochloride itself. It was readily demonstrated that the component, which was designated "compound A", was only produced when mannose and glucosamine hydrochloride were heated together on the paper. Compound A was not formed when either sugar was heated separately, or when a mixture of mannose and glucosamine hydrochloride was dried on the paper without the application of heat (Expt. 2, p. 138).

The presence of compound A was first observed after spraying with ninhydrin, which showed that it possessed a primary or secondary amino group. Compound A also gave positive reactions with alkaline silver nitrate, the Elson-Morgan reagent, triphenyl tetrazolium chloride, and aniline hydrogen phthalate, but not with naphthoresorcinol (Expt. 3, p.138). From these tests it was deduced that compound A had a reducing group and contained hexosamine, but did not contain a ketose component. Its ionophoretic mobility with respect to glucosamine (M_{GA}) suggested that it was a disaccharide carrying one basic group.

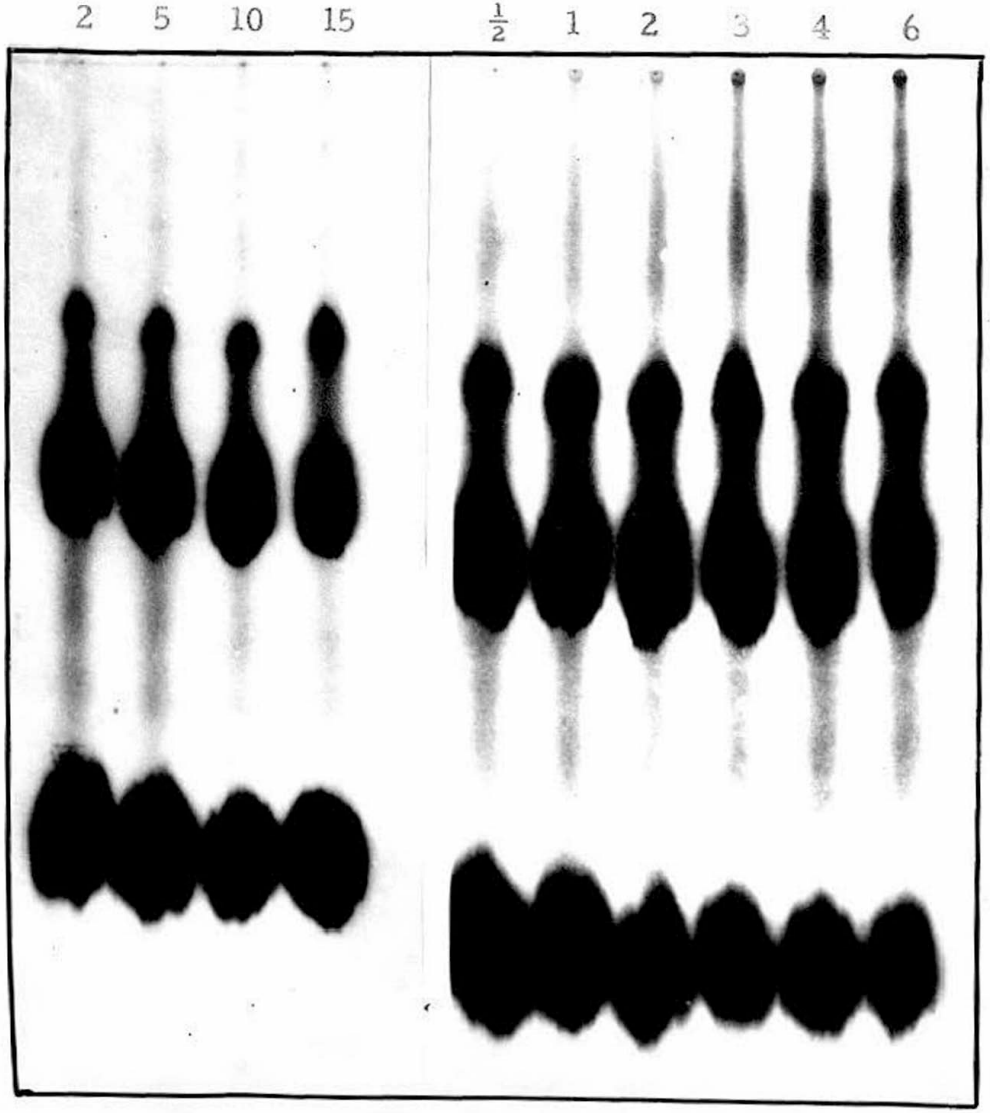


Figure 27. Chromatograms after heating at 100°C for periods indicated (left, minutes and right, hours)

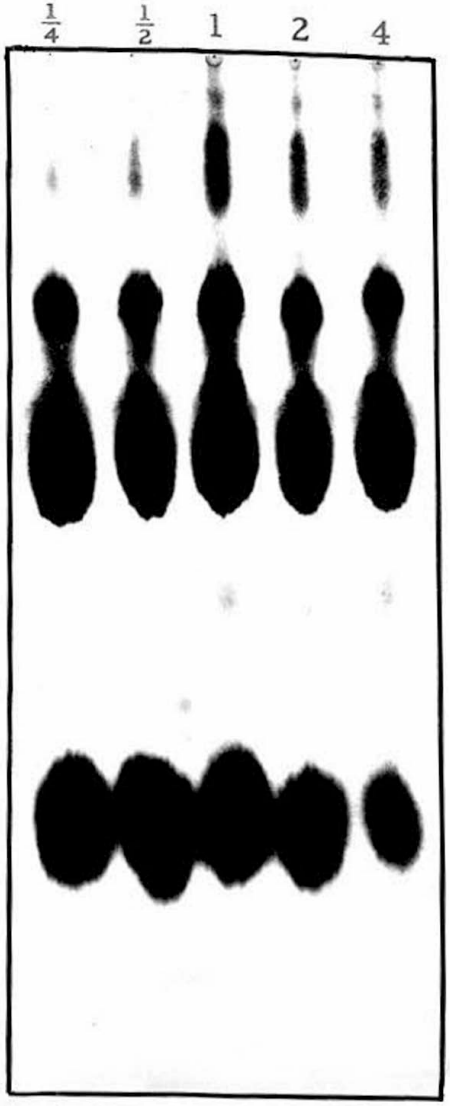


Figure 28. Chromatogram after heating at 120°C for periods (hours) indicated

The optimum conditions of heating on paper for the formation of compound A were investigated (Expt. 5, p.140) and both ionophoretograms and paper chromatograms were prepared of the mixture heated at 100°C and 120°C for varying periods of time. Ionophoretograms of mixtures heated at 100°C for periods of fifteen minutes to four hours, and chromatograms of mixtures heated at 100°C for periods of two minutes to six hours, showed a gradual increase in the intensity of the spot due to compound A during the first hour, but little change thereafter (see figure 27). Compound A had an $M_{G\,A}$ value of 0.56 and its movement on the paper chromatogram relative to glucosamine hydrochloride ($R_{G\,A}$) was 0.58, and that relative to mannose (R_M) was 0.32. Small amounts of other compounds appeared to be formed in addition to compound A when the reaction was carried out at 120°C (figure 28). At 80°C, the reaction was much slower than at 100°C and at 125°C a series of compounds having $M_{G\,A}$ values lower than that of compound A was formed. The preferred conditions of heating were therefore periods of at least fifteen minutes at 100°C.

The Scope and Nature of the Reaction

Information concerning the functional groups involved in the formation of compound A was obtained by heating several other mixtures of amino sugars with reducing sugars and their derivatives (Expt. 4, p.139). The combinations used are listed in Table VI and the spots formed by compounds analogous with compound A

	Glucosamine hydrochloride	N-acetyl glucosamine	3:4:6-tri-O-methyl glucosamine hydrochloride	Galactosamine hydrochloride	Glucosaminol hydrochloride	2-amino-glycerol hydrochloride
Glucosamine hydrochloride	-	-		-		
N-acetyl-glucosamine	-	-				
3:4:6-tri-O-methyl glucosamine hydrochloride			-			
Galactosamine hydrochloride	-			-		
Glucosaminol hydrochloride					-	
2-amino-glycerol hydrochloride						-
Mannose	+	-		+	+	+
3:4-di-O-methyl-mannose	+					
α-methyl-mannoside	-					
Mannitol	-					
Galactose	+			+		
Fructose	-					
Xylose	+					
Ribose	+			+		
2-deoxy-ribose	+					
Sodium glucuronate	-					
Sorbitol	-					
Dulcitol	-					
Glucose	+		+	+		
1-deoxy-glucose	-					
α-Methyl glucoside	-					
3-O-methyl glucose	+					
2:3-di-O-methyl glucose	-					
3:4-di-O-methyl glucose	+					
2:3:6-tri-O-methyl glucose	-					
2:3:4:6-tetra-O-methyl glucose	-		-			
Maltose	-					

Table VI. The Combinations of Sugars Examined

were termed "D spots". Since D spots were not produced from N-acetyl-glucosamine and a reducing sugar, or from glucosamine hydrochloride and non-reducing sugars (mannitol, α-methyl-mannoside, sorbitol, dulcitol, and α-methyl glucoside), the formation of D spots must be a consequence of reaction between the reducing group of a sugar and the amino group of a second compound. The reaction appears to be a general one between aldoses and amino sugars, but will not proceed with ketoses.

A necessary condition for the formation of compounds giving a D spot appears to be that the C_2 atom of the reducing sugar should not be substituted. Steric hindrance is the most probable cause of this and experiments with the various methyl ethers of glucose (an ionophoretogram of some of these is illustrated in figure 29) favour this explanation, for a D spot was not obtained with 2:3-di-, 2:3:6-tri-, and 2:3:4:6-tetra-O-methyl-D-glucose, but was obtained with 3-, and 3:4-di-O-methyl-D-glucose, and 3:4-di-O-methyl-D-mannose. 2-O-methyl-D-glucose was not available for examination, but the failure of N-acetylglucosamine to react with glucosamine hydrochloride is another indication of steric hindrance.

A second explanation which cannot be ruled out is that compound A would be expected to be a glycosylamine (since it results from a carbonyl-amine condensation) which could undergo an Amadori rearrangement to a 1-amino-1-deoxy-2-ketose, for the

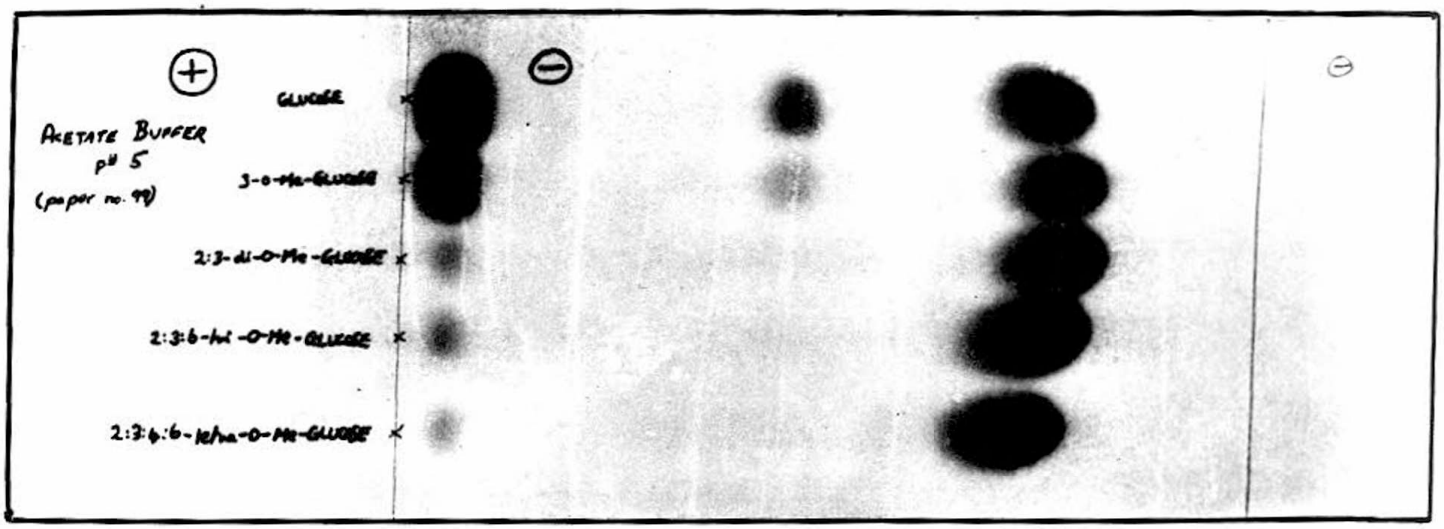
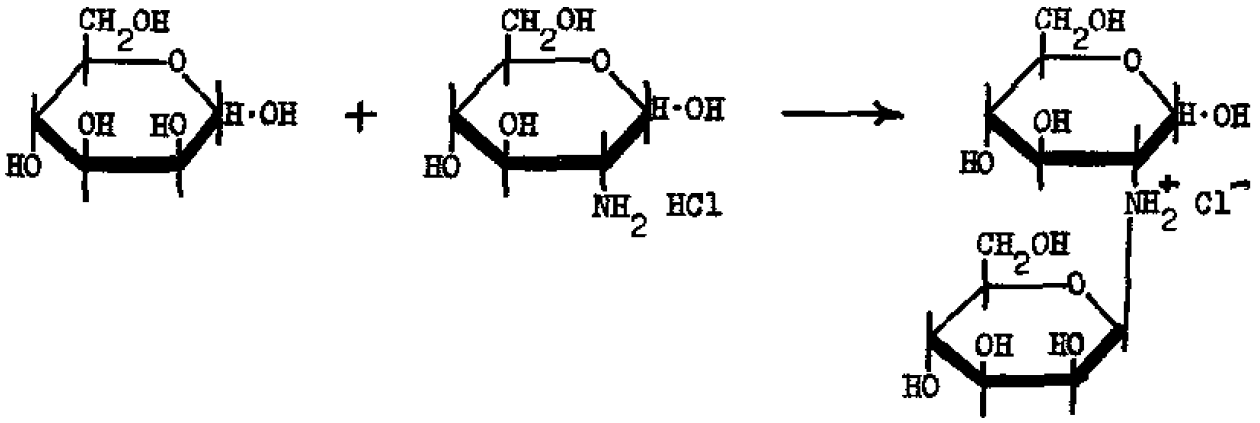


Figure 29. Ionophoretogram of glucose and some methyl ethers after heating on the paper with glucosamine hydrochloride.

reaction is effected by heating in the presence of acid in that the amine hydrochloride is used. This rearrangement, however, cannot occur if the C_2 carbon atom of the sugar is substituted, but since compound A does not appear to be a ketose, an explanation attributing the failure of the reaction to the blocking of an Amadori rearrangement is less tenable than the steric hindrance hypothesis .

From its reactions in the colour tests and the indications of the experiments using the different sugar combinations, compound A would appear to be a glycosylamine and a formal equation may be represented as shown below. The product would



have reducing properties by virtue of the glycosidic group of the glucosamine part of the molecule, and would react with ninhydrin, whereas a Schiff-base, being a tertiary imine, would not.

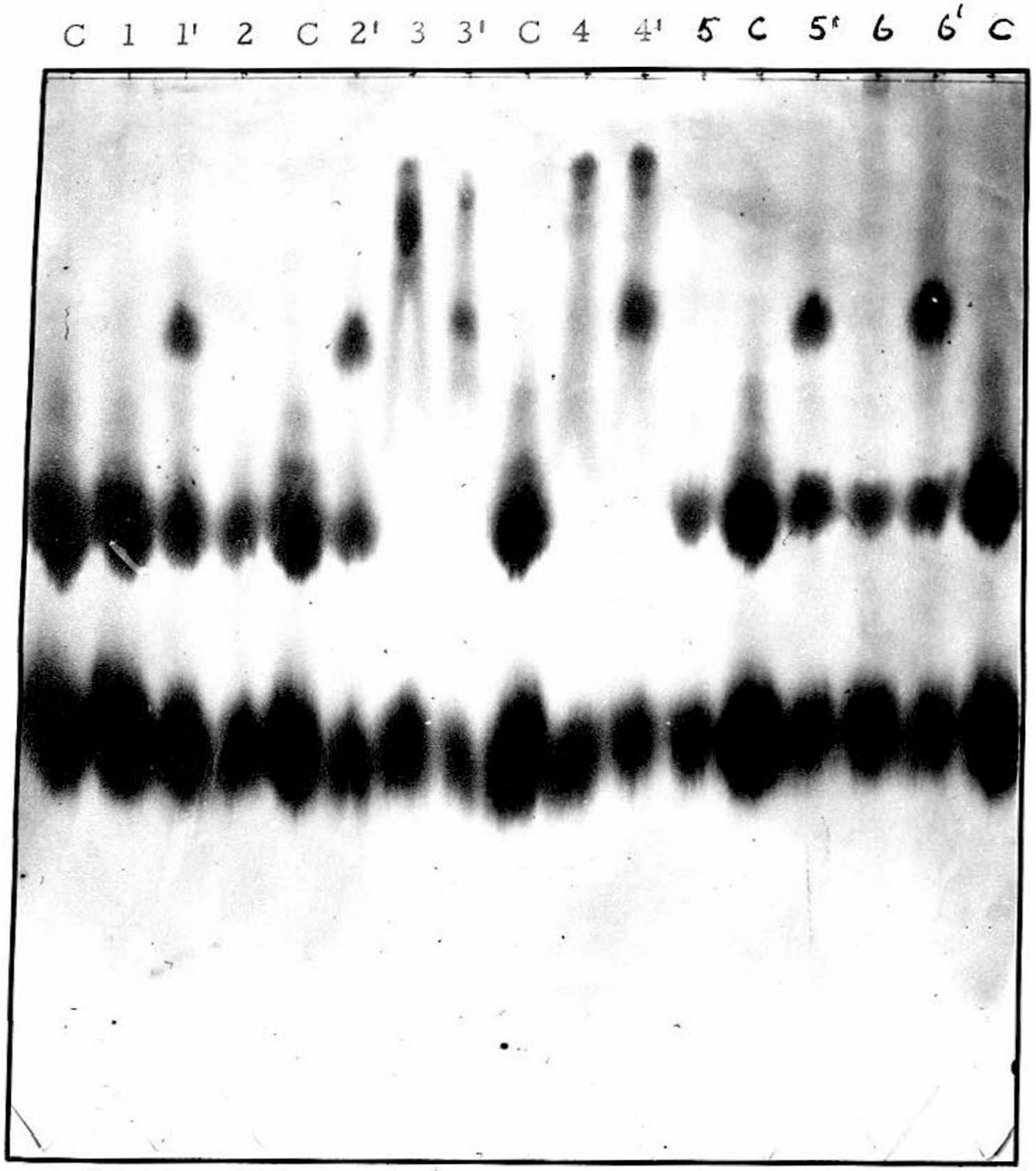


Figure 30. Chromatogram of samples of mannose and glucosamine hydrochloride heated in solution at various pH values.

C Reference samples of mannose and glucosamine hydrochloride.

1 Aqueous solution
2 Formate buffer, pH 2
3 Citrate buffer, pH 4
4 Citrate buffer, pH 6
5 Barbiturate buffer, pH 8
6 Barbiturate buffer, pH 10

The prime indicates additional heating after application to the paper

Preparation of Compound A

Preparation of compound A in aqueous solution was attempted by heating mannose and glucosamine hydrochloride in buffer solutions covering the pH range 2 to 10. The solutions were heated in sealed tubes (Expt. 7, p. 141) and then examined chromatographically, but compound A was not detected on the chromatogram. If, however, these solutions were applied to the paper and heated, the now familiar D spot (R_{GA} 0.54 to 0.6) was produced in each case as is illustrated in figure 30. An interesting feature demonstrated by this chromatogram is the effect of citrate (solutions 3 and 4 in figure 30) on the R_M value of glucosamine: due, in all probability, to the formation of glucosamine citrate, the value is depressed from 0.68 to about 0.18. The same effect was observed when a solution of glucosamine hydrochloride containing citric acid was applied to paper without heating and a chromatogram developed in n-butanol-ethanol-water, and the behaviour is similar to the formation of two spots when a chromatogram of glucosamine hydrochloride is developed with n-butanol-acetic acid-water. The two spots are attributed to glucosamine hydrochloride and glucosamine acetate, and modifying effects of this sort of solvents on the chromatographic behaviour of amino sugars has been discussed by Walker[162].

Attempts to isolate compound A by the conventional preparative chromatography and ionophoresis methods whereby the appropriate band of the paper is simply eluted with water[163] were unsuccessful, for when these eluates were freeze-dried and re-analysed they were found to be no longer homogeneous (Expts. 6 and 8, pp. 141,143). Ionophoretograms of the separated material showed a streak from the D spot (M_{GA} 0.56) to the base-line, together with a small amount of glucosamine, and chromatography indicated the presence of mannose, glucosamine hydrochloride and compound A (R_{GA} = 0.57). If compound A is a glycosylamine, this is perhaps to be expected, for the instability of glycosylamines in aqueous systems has been demonstrated[138] and the glycosylamines of aliphatic amines are particularly labile in such media[125].

Instead of eluting the bands of chromatograms and ionophoretograms which were indicated by the guide strips, a narrow strip was cut from these bands and chromatographed again without elution by sewing the strips into corresponding holes cut in the base line of new sheets of paper. Controls of mannose and glucosamine (heated on the paper) were placed either side of these strips in the same manner and the chromatogram was developed in the usual way and stained with alkaline silver nitrate. The "re-run"

Figure 31. "Re-run" chromatogram of a strip cut from the compound A zone of a preparative chromatogram.

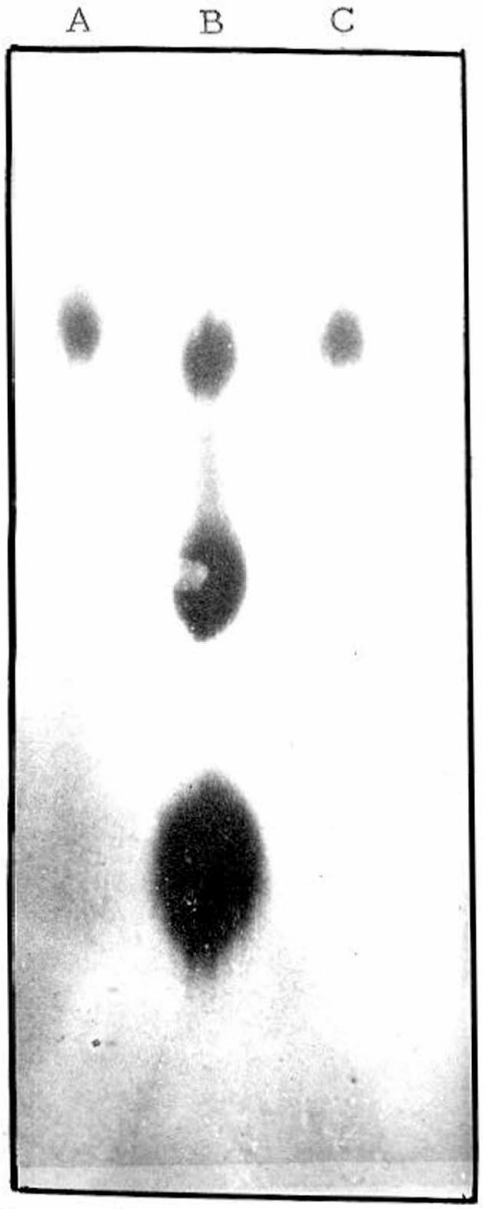


Figure 32. Chromatogram of compound A isolated after "re-chromatography" of A, a sample separated ionophoretically, and C, a sample separated chromatographically. B is a control. (see text).

chromatogram of a strip taken from a preparative chromatogram is illustrated in figure 31 and a distinct band due to compound A ($R_{G\,A}$ about 0.63) can be seen. This experiment was repeated using larger strips from a preparative chromatogram and a complete strip width from an ionophoretogram, and this time the position of the compound A bands was located by staining guide strips; the appropriate bands were then cut out and eluted with dry methanol. After evaporation to low volume (under reduced pressure at room temperature) the methanol solutions were examined by paper chromatography which showed clearly that they were solutions of compound A, almost chromatographically pure. Figure 32 shows the chromatogram of these solutions together with a control of mannose and glucosamine hydrochloride heated on the paper.

Although the separation of compound A was effected in this fashion, the method was far too tedious for the preparation of the substance in quantity and other possibilities were examined (Expt. 9, p.145).

Glycosylamines of aromatic amines were first obtained by heating the amine and sugar together[126], and a similar experiment was performed in which glucosamine hydrochloride was added to molten mannose. The solution of the resulting dark brown mass was shown by ionophoresis and chromatography to contain a sub-

stance having a M_{GA} value of 0.57 and a R_{GA} value of 0.58, which was doubtless compound A, but the presence of a large number of other substances was also indicated. Less drastic conditions of heating were employed but there was no indication of the production of compound A when a finely ground mixture of mannose and glucosamine hydrochloride was maintained at 110°C for several hours, or when a stiff paste of these two sugars with a little water (as used in the preparation of ureido sugars[164]) was heated at 100°C for six hours.

An attempt to produce compound A by refluxing a methanolic solution of mannose with glucosamine hydrochloride was unsuccessful and so attention was again focussed on the reaction of the sugars supported on cellulose.

A slurry of cellulose powder in an aqueous solution of mannose and glucosamine hydrochloride was freeze-dried and the solid suspension so obtained was heated at 100°C for ninety minutes. This procedure was employed because it would provide a greater surface area than the reaction on sheets of paper and if cellulose column chromatography could be employed, the reaction products would be obtained in a form convenient for application to the column. Chromatographic analysis of an aqueous eluate from a small portion of the heated cellulose suspension showed that compound A was present, and a separation of the mixture on a column of cellulose was attempted (Expt. 10, p. 146) because

isolation of compound A had been achieved by the repeated chromatography procedure. This, however, was unsuccessful, for the various eluate fractions contained mannose, glucosamine hydrochloride, and compound A and the material was therefore recovered by evaporation of the butanolic solution. A partial separation of this mixture by means of an ion-exchange resin (Amberlite IR 120) was tried, but again without success (Expt. 11, p. 147).

Attempts at Separation of the Reaction Mixture by Chromatography, Solvent Extraction and Crystallisation

Resolution of the mixture on cellulose columns or separation of compound A from mannose and glucosamine hydrochloride on a column of charcoal and celite[165] might be facilitated by acetylation of the amino groups. Compound A was prepared by heating mannose and glucosamine hydrochloride on cellulose powder followed by elution of the sugars with water and this mixture was then selectively N-acetylated using the procedure of Roseman and Ludowieg[166] (Expt. 12, p. 148). Chromatographic analysis of the final product indicated, however, that only a very small amount of compound A or its N-acetyl derivative remained, together with mannose and N-acetylglucosamine, and a considerable quantity of a new, fast moving component (R_{GA} 2.5) which was probably an anhydro-glucosamine. The method certainly held little promise for the separation of the reaction mixture.

Solvent extraction and fractional crystallisation procedures were then considered. The reaction was carried out on cellulose powder and the aqueous eluate was freeze-dried. Portions of the solid so obtained were separately extracted with a number of solvents and the extracts were analysed ionophoretically (Expt. 13, p. 148). The solvents used were methanol, ethanol, n-butanol, acetone, ether, dioxan, and chloroform. Of these, dioxan appeared to extract only mannose, and chloroform removed mannose and a little glucosamine hydrochloride. All the alcohols extracted mannose, glucosamine hydrochloride and compound A, although in varying proportions, but acetone removed only a little mannose, and none of sugars, apparently, was soluble in ether. It appeared from these preliminary tests that one component at least might be removed from the reaction mixture with either dioxan or chloroform, and Soxhlet extraction of a reaction mixture on cellulose was carried out with these two solvents. After twenty-one hours extraction, ionophoresis experiments showed that dioxan had removed an appreciable amount of mannose, and chloroform a small amount of mannose and possibly a trace of compound A, from the cellulose. A final attempt to employ solvent extraction was made by allowing dioxan to percolate slowly through a column of the cellulose reaction mixture. Although a considerable quantity of mannose was removed in this way, the cellulose retained too

much mannose, along with the glucosamine hydrochloride and compound A, for the process to be economically useful.

Glucosamine hydrochloride may be crystallised from aqueous acetone[167] and its removal from the mixture with mannose and compound A was attempted by this means. The aqueous eluate from a reaction mixture on cellulose powder was freeze-dried and dissolved in a little water. Part of the glucosamine hydrochloride was removed by slow crystallisation with acetone, and also with dioxan (Expt. 14, p. 150). A number of crops of material was precipitated from the dioxan mother liquors by increasing the dioxan concentration and this also resulted in the precipitation of some mannose. Combination of the mother liquors from dioxan and acetone crystallisations resulted in the removal of more glucosamine hydrochloride from solution, and although the final mother liquors still contained mannose, glucosamine hydrochloride and compound A, the proportion of each was roughly equal so that a considerable enrichment of compound A had been effected. This procedure could therefore provide the basis of a useful preliminary treatment to some other separation process, particularly if a chromatographic method was to be used.

Ion-Exchange Chromatography

The spectacular separations of complex mixtures of amino acids by Moore and Stein[168] using ion-exchange chromatography

prompted investigations into the application of these techniques to the fractionation of mixtures of amino sugars, and Crampton[169] has successfully resolved mixtures of a large number of amino sugars on a sulphonated polystyrene resin. Since they have very different migration rates on ionophoresis, compound A and glucosamine have different basicities and they should therefore be separable by ion-exchange chromatography.

An investigation of the possibility of resolving the reaction mixture by this means was made using a small analytical column, and the separation of two distinct fractions giving a positive reaction in the Elson-Morgan test[170,171] indicated that experiments on a larger, preparative column could be profitably pursued (Expt. 15, p. 152). Preparations of material for separation in these experiments were made by heating the reactant sugars on cellulose powder and dissolving the freeze-dried eluate from the cellulose in 0.3N hydrochloric acid. Eluate fractions from the large column were scanned by means of optical rotation measurements, the Elson-Morgan reaction[171], the development of a blue colour with ninhydrin reagent[172], and the intensity of absorption at 284 mμ. The histograms constructed from these observations are shown in figure 33. Optical rotation measurements show that mannose is the first substance to be eluted from the column and is found in the early fractions, which is the position expected of neutral and acidic

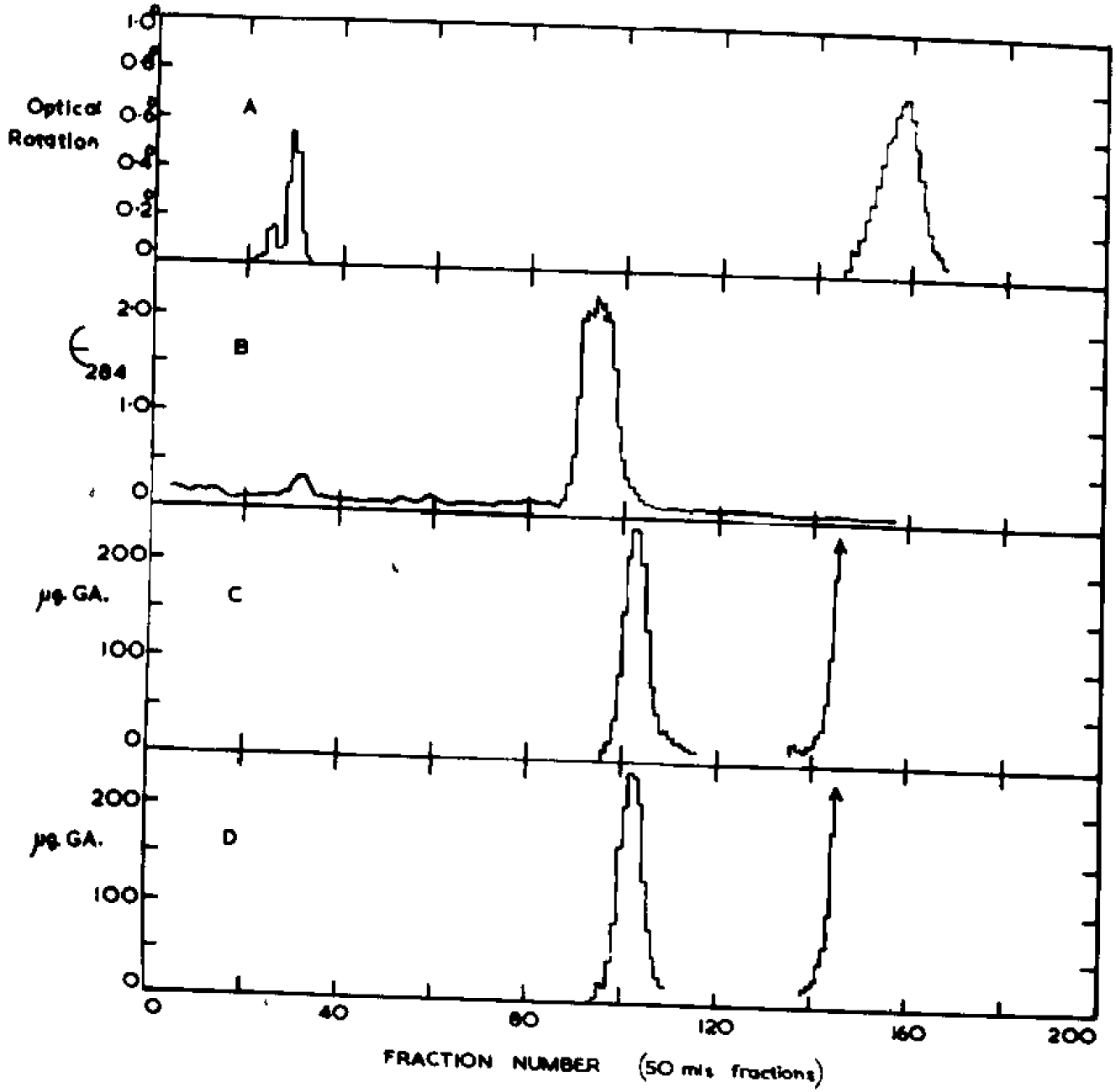


Figure 33. Histograms for ion-exchange chromatographic separations of the reaction mixture. Fractions scanned by, A, optical rotation; B, absorption at 284 mμ; C, Elson-Morgan reaction; D, ninhydrin reaction.

substances. A small peak in the neighbourhood of fraction 100 was indicated by the Elson-Morgan and ninhydrin tests and this was attributed to compound A. From the intensity of the colours developed in these reactions, the concentration of compound A in the fractions was calculated as the concentration of glucosamine hydrochloride in μg./ml., and is recorded in this way on the histograms. The very much larger peak in the region of fractions 145 to 170, which was found by the optical rotation, Elson-Morgan, and ninhydrin measurements, was undoubtedly glucosamine hydrochloride by reason of its magnitude and position, for an authentic sample of glucosamine hydrochloride was eluted from the column in this same fraction range.

The ultraviolet absorption found in fractions 88 to 95 was particularly interesting and was attributed to the presence of a compound B, which will be discussed shortly (p. 106).

Eluate fractions believed to contain compound A were bulked, neutralised to pH 5, and freeze-dried. Initially, the neutralisation was effected with sodium bicarbonate and the freeze-dried material was then extracted with ethanol. Ionophoretic analysis of the yellow-brown solid obtained by evaporation of the ethanol solution showed that it consisted mainly of compound A, with smaller quantities of mannose and glucosamine hydrochloride. In order to avoid the alcohol extraction of the freeze-dried solid and

evaporation of the solution, which was rather time-consuming, the bulked fractions obtained from later separations were neutralised by extraction with a chloroform solution of di-n-octylmethylamine[173]. Care was required in the use of this reagent for the commercial product contained a number of compounds which reacted with ninhydrin. These were removed by washing with aqueous hydrochloric acid, followed by regeneration of the base with aqueous sodium hydroxide solution, until ionophoretic analysis showed that the impurities were removed (Expt. 1, p.137). About 99% of the di-n-octylmethylamine hydrochloride remained in the chloroform phase[173], but the small amount partitioned in the aqueous phase must be removed, for when volumes of the order of 500 ml. are freeze-dried the contamination of a required product with this amine hydrochloride could be considerable. For this reason, the neutralised aqueous phase was washed twice with chloroform and then filtered before freeze-drying. The freeze-dried material so obtained was a yellow-green hygroscopic solid and ionophoresis showed it to be principally compound A, although small amounts of mannose and glucosamine hydrochloride were also present. Several batches of compound A were prepared in this way and the material was dissolved, as required, in a small volume of water and the solutions were usually washed with an

equal volume of chloroform and filtered. This treatment served the threefold purpose of removing any remaining traces of di-n-octylmethylamine hydrochloride, safeguarding the solution against bacterial infection, and ensuring the absence of compound B (loc. cit.). The largest quantity of material which could be conveniently separated in one batch by this process was about 2.5 g. of amino sugar, i.e. about 5 g. of the reaction mixture from which 50 to 100 mg. of compound A were usually obtained.

The stability of compound A at room temperature in aqueous and dilute hydrochloric acid solutions was examined by chromatographic and ionophoretic analysis (Expt. 16, p. 155). Although the acid solution remained almost unchanged during a period of two weeks, the aqueous solution gradually decomposed with the formation of mannose and glucosamine hydrochloride. When the solutions were heated, degradation of compound A occurred and the process was almost complete after six hours. In each case, the decomposition was paralleled by an increasing proportion of glucosamine hydrochloride, but only a small amount of mannose was produced. (See figure 41, p. 124).

Before proceeding with the discussion of the nature of compound A, it is convenient at this stage to consider briefly compound B.

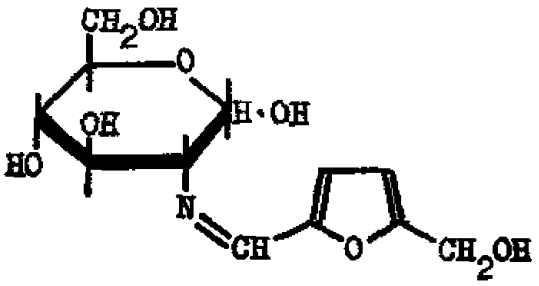
Compound B

The conditions under which compound A is formed are unusual, not only in their physical state, but also in that the reaction is contrived between the reducing sugar and the amine hydrochloride rather than the free base which is normally employed in glycosylamine preparation (loc. cit.). Rearrangement of glycosylamines to substituted 1-amino-1-deoxy-2-ketoses often occurs on heating and in many cases this tendency is accentuated by the presence of acid[145]. Furthermore, the rearrangement is usually accompanied by decomposition of the substituted ketose and in hot aqueous acid solutions the degradation products include, inter alia, 5-hydroxymethyl-2-furfuraldehyde (HMF)[148,150,152]. If compound A is a glycosylamine, the conditions of the reaction would favour this sequence of events and HMF would be expected amongst the reaction products. The presence of HMF could readily be detected by its ultraviolet absorption and if it was found, this would furnish evidence for the occurrence of the Amadori rearrangement.

When the reaction mixture was separated by ion-exchange chromatography, HMF was expected amongst the earlier eluate fractions with neutral compounds such as mannose. The absorption at 284 mμ of the earlier eluate fractions was accordingly measured, but none was found to absorb ultraviolet light. Scanning of the

fractions in this way was continued, however, and strong absorption was exhibited by fractions in the region of 87 to 95. In this way compound B was found. Its ultraviolet absorption spectrum was measured and was almost identical with that published by Wolfrom et al. for HMF[174] (figure 36).

From its behaviour on the ion-exchange column, compound B appeared to be basic and since it was obtained in the presence of a large quantity of glucosamine hydrochloride it was possible that the compound was a Schiff base of HMF and glucosamine as formulated below. The appropriate eluate fractions were com-



bined, neutralised with di-n-octylmethylamine, and freeze-dried. After two or three batches had been treated in this manner, a considerable loss of material was indicated from the relative absorptions and volumes of solutions before and after the isolation of compound B from the eluates. Removal of compound B into the chloroform phase during neutralisation and washing was suspected, for HMF would be soluble in organic solvents, and the distribution of the compound between chloroform and the dilute hydrochloric acid of the eluate was measured (Expt. 20,

p.160). A value of 0.31 at 16°C was obtained for the partition coefficient and a very similar value (0.25 at 25°C.; at lower temperatures a slightly higher value would be obtained) has been reported by Bethge[175] for the partition coefficient of HMF between 3.8N hydrochloric acid and chloroform.

The reason for the loss of compound B could clearly be used to advantage in its isolation, for extraction of the appropriate eluate fractions with chloroform in a continuous extractor would be more convenient than neutralisation of the fractions followed by freeze-drying. For this purpose, however, ether is a better solvent than chloroform because it is more easily removed at low temperature after extraction. The partition coefficient of compound B between 0.3N hydrochloric acid and ether was determined and the average value was 0.26 at 16°C. Ether extraction was therefore employed routinely for the isolation of compound B.

Hydrolysis of Compounds A and B

Studies of the behaviour on hydrolysis of compounds A and B played a most important part in the elucidation of the nature of these compounds. Chromatographic and ionophoretic evidence for the degradation of compound A by water, either hot or cold, and by hot acid has already been mentioned, and from ultraviolet absorption measurements compound B was found to be stable to hot water, but was decomposed by hot, dilute (2N) hydrochloric acid (Expts. 18 and

19, pp.157, 159)

Hydrolysis of compound A by hot and cold water was followed by Elson-Morgan determinations, optical rotation measurements, observation of the intensity of absorption at 284 mμ, and chromatographic and ionophoretic analysis (Expt. 18, p. 158) and the hydrolysis products were separated by ion-exchange chromatography (Expt. 25, p. 164). The solution of compound A had initially no optical rotation and no change was observed during hydrolysis in either hot or cold solutions. It appeared from the chromatography and ionophoresis experiments that the decomposition of compound A was accompanied by the formation of glucosamine hydrochloride and mannose, although the spot on a chromatogram due to the latter was streaked. In hot water, glucosamine hydrochloride was formed together with a small quantity of mannose and a trace of a compound which had R_M and R_{GA} values of 0.77 and 1.44 respectively, which are values obtained for glucose. There was no significant change in the hexosamine concentration, as indicated by the Elson-Morgan reaction, in either case. In the cold solution, no change in absorption at 284 mμ (which was very low, 0.13) was observed, but the absorption at this wavelength gradually increased during the hydrolysis of compound A by hot water (figure 38, p. 118). Also during the hydrolysis by hot water, a gradual darkening in colour occurred and eventually a fine, dark brown precipitate

was produced.

The hydrolysis products were separated by ion-exchange chromatography and the fractions scanned by means of the orcinol-sulphuric acid reaction[176,177], the Elson-Morgan reaction, and (in the case of the solution hydrolysed by hot water) the absorption at 284 mμ. The orcinol reaction showed that a group of neutral sugars fractions was produced from both the solutions (A1 from the solution hydrolysed by boiling and A4 from that hydrolysed in the cold) and fractions giving a positive reaction in the Elson-Morgan test were eluted from the column, in the same position as glucosamine hydrochloride, from both hydrolysates. These were designated A3 from the solution hydrolysed by boiling and A5 from the solution hydrolysed in the cold. Ultraviolet absorbing (284 mμ) fractions (A2) were obtained from solution of compound A which had been boiled and were eluted from the column in the same region as compound B.

The fractions containing neutral sugars (A1 and A4) were neutralised and freeze-dried and fractions A3 and A5, which were probably solutions of glucosamine hydrochloride, were concentrated to low volume in readiness for crystallisation. One of the fractions of A2 was retained for measurement of its absorption curve and distribution coefficients and A2 was obtained as a yellow oil by ether extraction of the remainder. The absorption curve of A2

was almost identical with that of HMF[174] and of compound B (figure 36, p.114) and its partition coefficients for the systems 0.3N hydrochloric acid and chloroform, and 0.3N hydrochloric acid and ether were 0.28 and 0.23 respectively at 17°C. From these pieces of evidence and their positions of elution from the ion-exchange column it seemed that compounds B and A2 were the same and their behaviour on hydrolysis supported this view.

Compounds B and A2 were both unchanged on boiling in water for ten hours, but were both hydrolysed by heating in 2N hydrochloric acid (Expt. 19 and 26, pp.159,167). In neither case was glucosamine hydrochloride found in the hydrolysate and during hydrolysis there was a rapid fall in the intensity of absorption at 284 mμ. The shape of the absorption curves was greatly changed from the original after heating in the acid solution for three hours and the final curves of the solutions of the two compounds were similar. Other than a slight increase in intensity due to unavoidable concentration of the solutions, there was no change in the absorption curves of the aqueous solutions after several hours heating, and when these solutions were applied to the preparative ion-exchange column, the ultraviolet-absorbing substances were eluted in the same positions as previously. The absorption curves of the aqueous solution of compound B before and after heating and of the solution of A2 in hydrochloric acid are shown in figure 36 (A and B respectively).

Identification of Compound B and the Hydrolysis Products of Compound A

In spite of their apparent basic behaviour on the resin column, it now seemed very probable that compounds B and A2 were in fact HMF, for these three compounds had similar ultraviolet absorption curves and partition coefficients in the system, chloroform-aqueous hydrochloric acid. The ultraviolet absorption curves of compound B and A2 were greatly changed by heating with acid (figure 36 B) and it has been reported that HMF is converted to laevulinic acid by heating with acids[153,178,179]. Furthermore, if the compounds had been the previously postulated Schiff base (p. 107), acid hydrolysis would have furnished the aldehyde (although that may have been destroyed by the acid in this case) and the amine hydrochloride, but the latter was not detected.

When compounds B and A2 (both obtained from the acid eluate fractions by ether extraction) were examined by paper chromatography, each showed two spots absorbing ultraviolet light. In each case the faster spot was the more intense and the R_f values of the two spots from each compound were 0.83 and 0.78. It has been reported that 5-hydroxymethyl-2-furfuraldehyde may be converted to 5-chloromethyl-2-furfuraldehyde by treatment with a solution of hydrogen chloride in ether[153,180], and it is therefore quite probable that the two components of the solutions of compounds B and A2 were the 5-hydroxymethyl and 5-chloromethyl derivatives of furfuraldehyde.

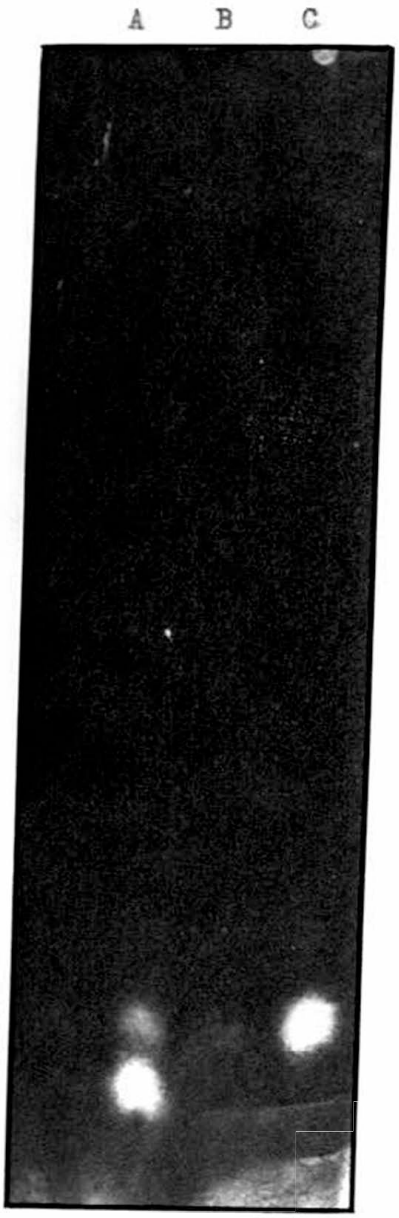


Figure 34

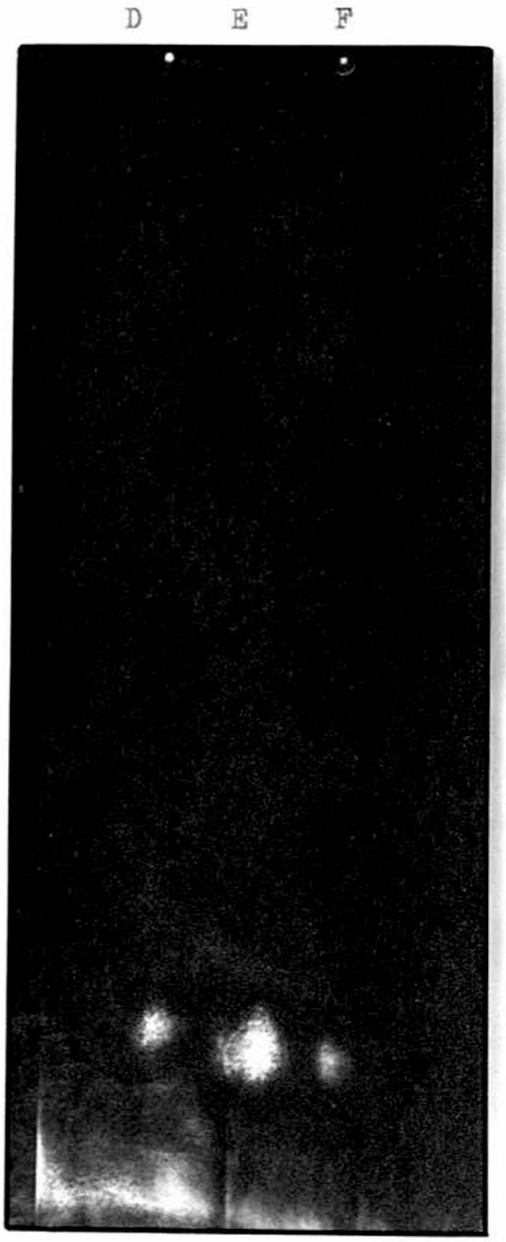


Figure 35

Figure 34. Ultraviolet photograph of chromatogram of compound B samples: A, extracted from acid eluate; B, extracted from neutral solution (A2); C, extracted from eluate after neutralisation.

Figure 35. Ultraviolet photograph of chromatogram of samples of compounds B, A2, and HMF. D is compound B, E is HMF, and F is compound A2.

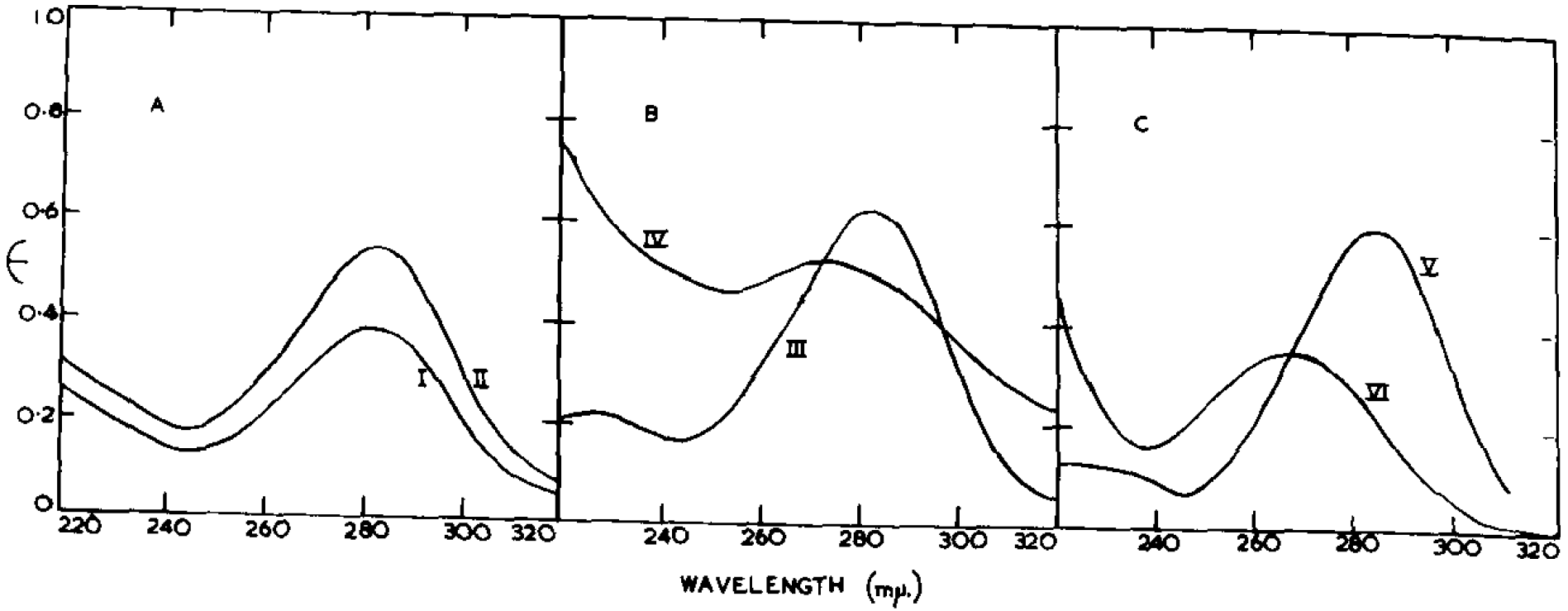


Figure 36. Absorption curves of; A, Compound B before (I) and after (II) heating in aqueous solution. B, compound A2 before (III) and after (IV) heating in 2N hydrochloric acid; C, solutions of HMF (V) and laevulinic acid (VI).

gave three spots each having an R_f value of 0.78. This photograph is illustrated in figure 35, and identically situated spots were obtained on staining with aniline hydrogen phthalate.

The ultraviolet absorption curve of a solution of HMF was obtained (figure 36 C) and was virtually identical with those of compounds B and A2. The absorption curve of a solution of laevulinic acid was measured (Expt. 30, p. 171) and is included with that of the HMF solution. These two curves may be compared with those of compound A2 before and after heating with 2N hydrochloric acid (figure 36 B) and although the curve of A2 after hydrolysis is not identical with that of laevulinic acid, it does have a similarly situated peak and is consistent with the degradation of HMF to laevulinic acid.

A considerable amount of evidence thus indicated that compounds B and A2 were HMF and this was confirmed by the preparation of the 2:4-dinitrophenylhydrazones of the three materials (Expt. 28, p.169). All three derivatives had similar melting points and that of the HMF and compound B derivatives was undepressed on mixing the two. It was therefore concluded that compounds B and A2 were both HMF.

The identity of compound A3, obtained on hydrolysis of compound A with hot water, was soon established as glucosamine hydrochloride (Expt. 29, p. 170) and it was chromatographically

and ionophoretically identical with A5 which was produced by hydrolysis with cold water (loc. cit.). A crystalline product was obtained from the acidic, concentrated solution of A3 by the gradual addition of acetone following the description of Gardell[167]. This material had an infra-red spectrum identical with that of an authentic specimen of α-D-glucosamine hydrochloride[182] and its specific rotation was +69°. The carbobenzoxy derivative of this substance had the same melting point as that of the corresponding derivative of glucosamine hydrochloride and was not depressed on admixture with the latter.

Chromatographic and ionophoretic analyses of the neutral sugars fractions from the hydrolysis of compound A in both hot and cold aqueous solutions (A1 and A4, respectively), showed that neither was homogeneous. They indicated that the major component in each case was mannose and the absorption curve of the coloured solution produced in the orcinol-sulphuric acid reaction had the form typical of mannose as is shown in figure 37 (Expt. 25, p. 164).

The two batches of material were separated by preparative paper chromatography (Expt. 27, p. 168) and two fractions were obtained in each case. One of these (from each material, i.e. A1 and A4) was chromatographically identical with mannose and the other, present in much smaller quantity, had an R_M value of 0.77 which is that of glucose. Crystalline derivatives were not

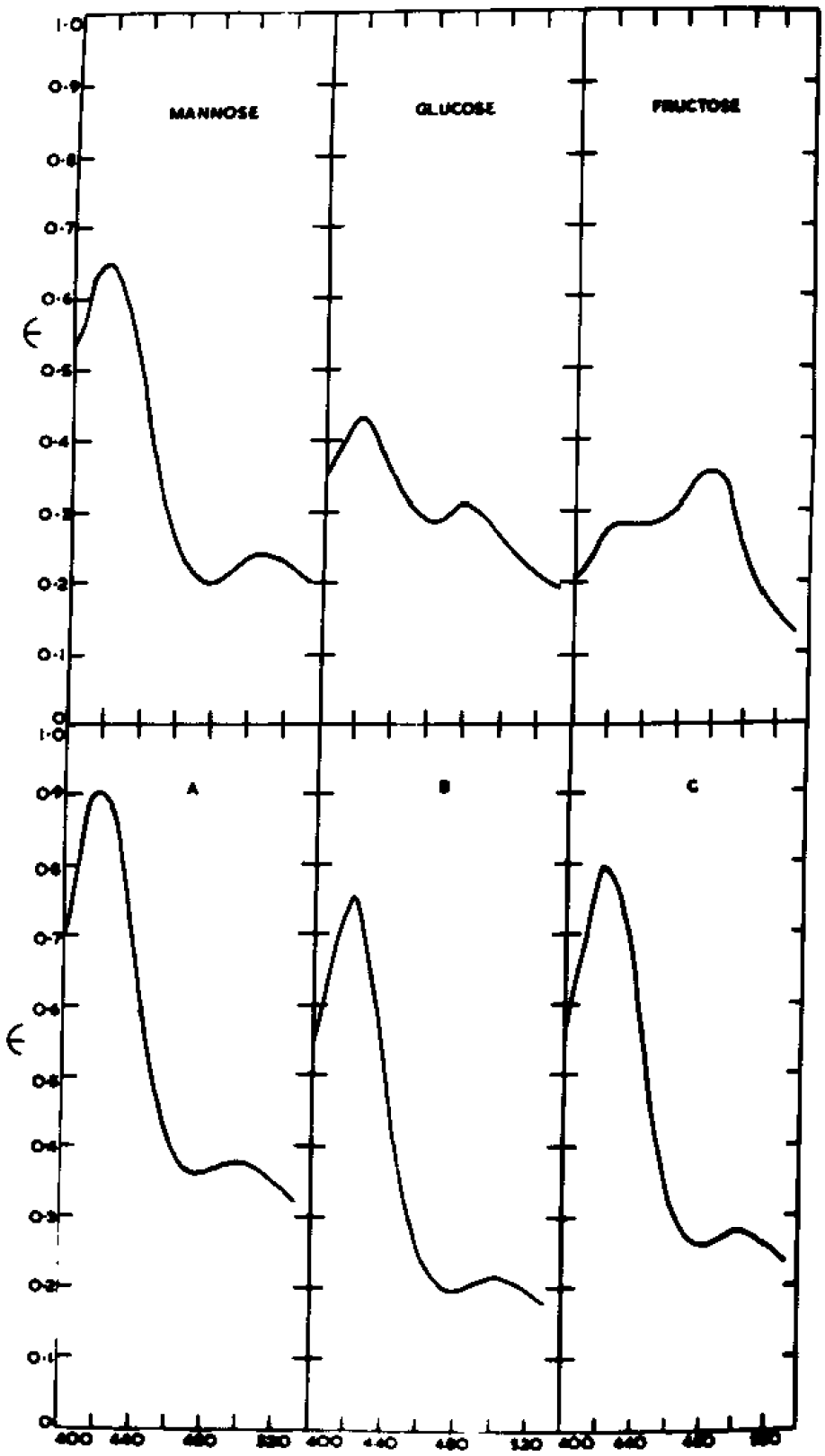


Figure 37. Absorption curves in the orcinol-sulphuric acid reaction. A, compound A; B, neutral sugars obtained from compound A after hydrolysis with water; C, compound C acid hydrolysate.

obtained from these slower moving components, but in addition to the chromatographic evidence, they were ionophoretically the same as glucose. Crystalline p-nitranilides were obtained from the other component of both A1 and A4, though in very low yield (3 mg. from A1, and only a few crystals from A4). Both had the same melting point as the p-nitranilide of mannose[137] and that of the derivative obtained from A1 was not depressed on mixing with an authentic specimen.

Analysis of Compound A

The proportion by weight of mannose to glucosamine hydrochloride in compound A was obtained by carrying out Elson-Morgan and orcinol-sulphuric acid determinations on a solution of compound A directly as eluted from the resin column (*Expt. 17, p. 155*) and the result obtained was 95:98 . The theoretical ratio of an equimolecular mixture of mannose and glucosamine hydrochloride is 95:113, and the experimental result is sufficiently close to this to infer that compound A comprises equimolecular proportions of these two sugars. The absorption curve of the colour produced in the Elson-Morgan reaction was measured and a peak was observed at 530 mμ which is characteristic of glucosamine[171], and the absorption curve of the colour developed in the orcinol-sulphuric acid reaction suggested that mannose was present as such (see figure 37), rather than as the rearranged ketose form.

Further evidence in favour of a glycosylamine structure rather than an Amadori rearrangement product was adduced from a number of colour tests (Expt. 31, p.172). The 1-amino-1-deoxy-2-ketose produced by rearrangement of the glycosylamine would decolourise methylene blue and 2:6-dichlorophenolindophenol[148,183], give a pink colour in the Seliwanoff test on gentle warming, and also give a red stain with naphthoresorcinol. Compound A gave negative reactions in all of these tests.

The infra-red spectrum of compound A was also consistent with the glycosylamine form, for it did not contain a peak due to a carbonyl group which has been observed in the infra-red spectra of certain Amadori compounds[184].

The Origin of HMF, or Compound B

Although HMF can be obtained from aldohexoses by heating in acid solutions, the reaction is very slow, but it is normally catalysed by the addition of amino acids[174]. It is therefore possible that the production of HMF from compound A on boiling in aqueous solution could arise by interaction of the mannose and glucosamine hydrochloride released by hydrolysis, as well as from compound A itself.

In order to distinguish between these two possibilities, aqueous solutions of compound A, mannose, glucosamine hydrochloride, and a mixture of mannose and glucosamine hydrochloride were prepared at similar concentrations and were refluxed for

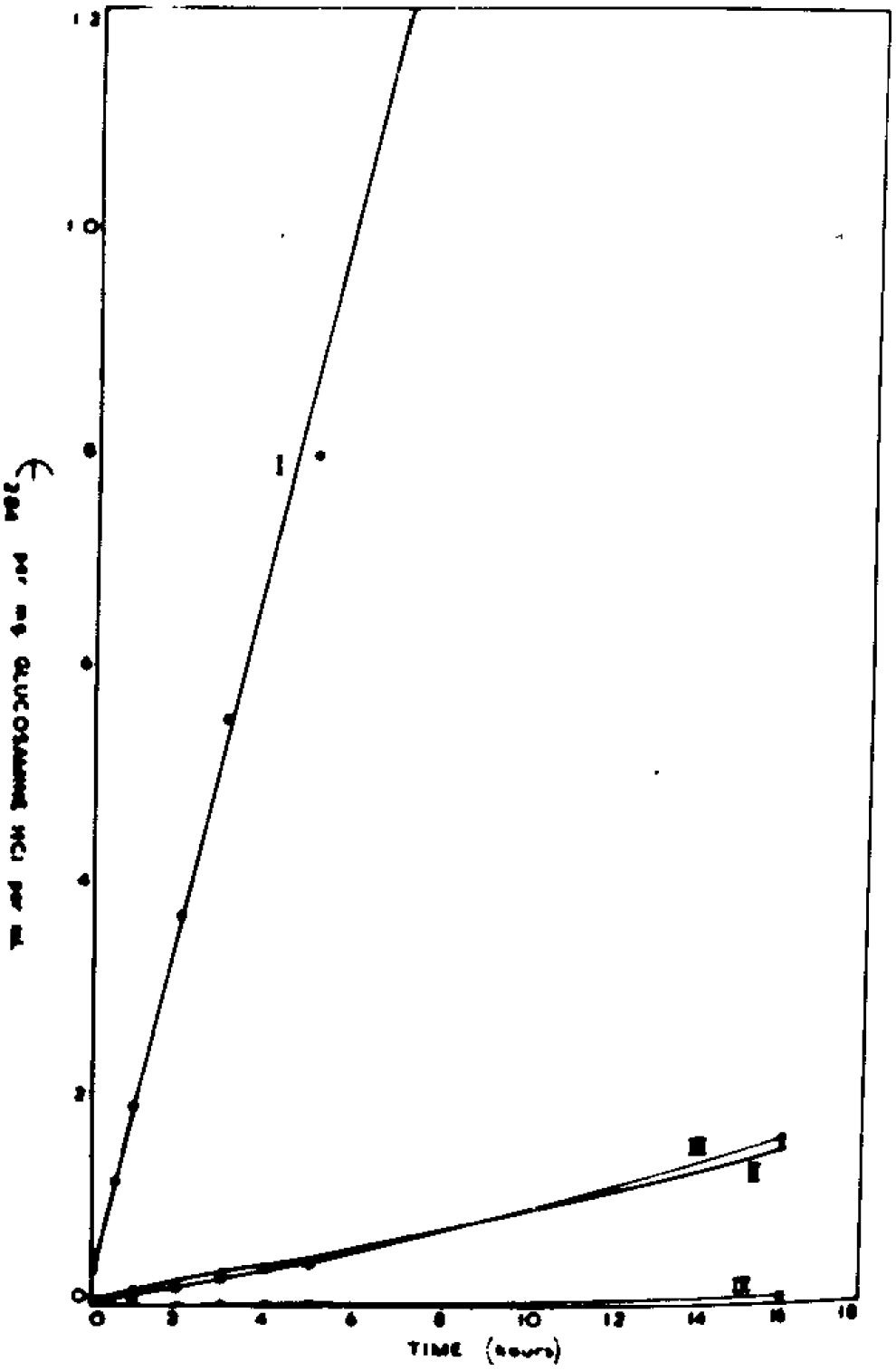


Figure 38. Rate of increase in absorption at 284 mμ on heating solutions of: I, compound A; II, mannose and glucosamine hydrochloride, III, glucosamine hydrochloride; IV, mannose.

several hours. Samples were periodically withdrawn for measurement of the absorption at 284 mμ and the absorption curve of each solution was measured at the end of the heating period (Expt. 34, p. 174). The extinction coefficient per mg. of glucosamine hydrochloride (the concentration being determined by weighing in the case of solutions containing the compound itself, and by the Elson-Morgan assay in the case of compound A; the extinction coefficient per mg. mannose was used in the case of the mannose solution) was plotted against time for each solution and the rate of increase in the case of compound A was much greater than that of the solution of glucosamine hydrochloride or the mixture of mannose and glucosamine hydrochloride. The rate of increase for the two latter solutions was, rather surprisingly, virtually the same, and the solution containing only mannose exhibited hardly any increase in absorption. The rate curves of the four solutions are shown in figure 38. In addition to the higher rate of increase in absorption at 284 mμ, the absorption curves of the heated solutions of compound A and those containing glucosamine hydrochloride have a different form, as can be seen from figure 39 A, that of the compound A solution being the same as that of HMF (figure 36 C, p. 114).

These data are good evidence for the production of HMF by a degradation of compound A itself, rather than by interaction of its primary hydrolysis products. HMF was, however, obtained

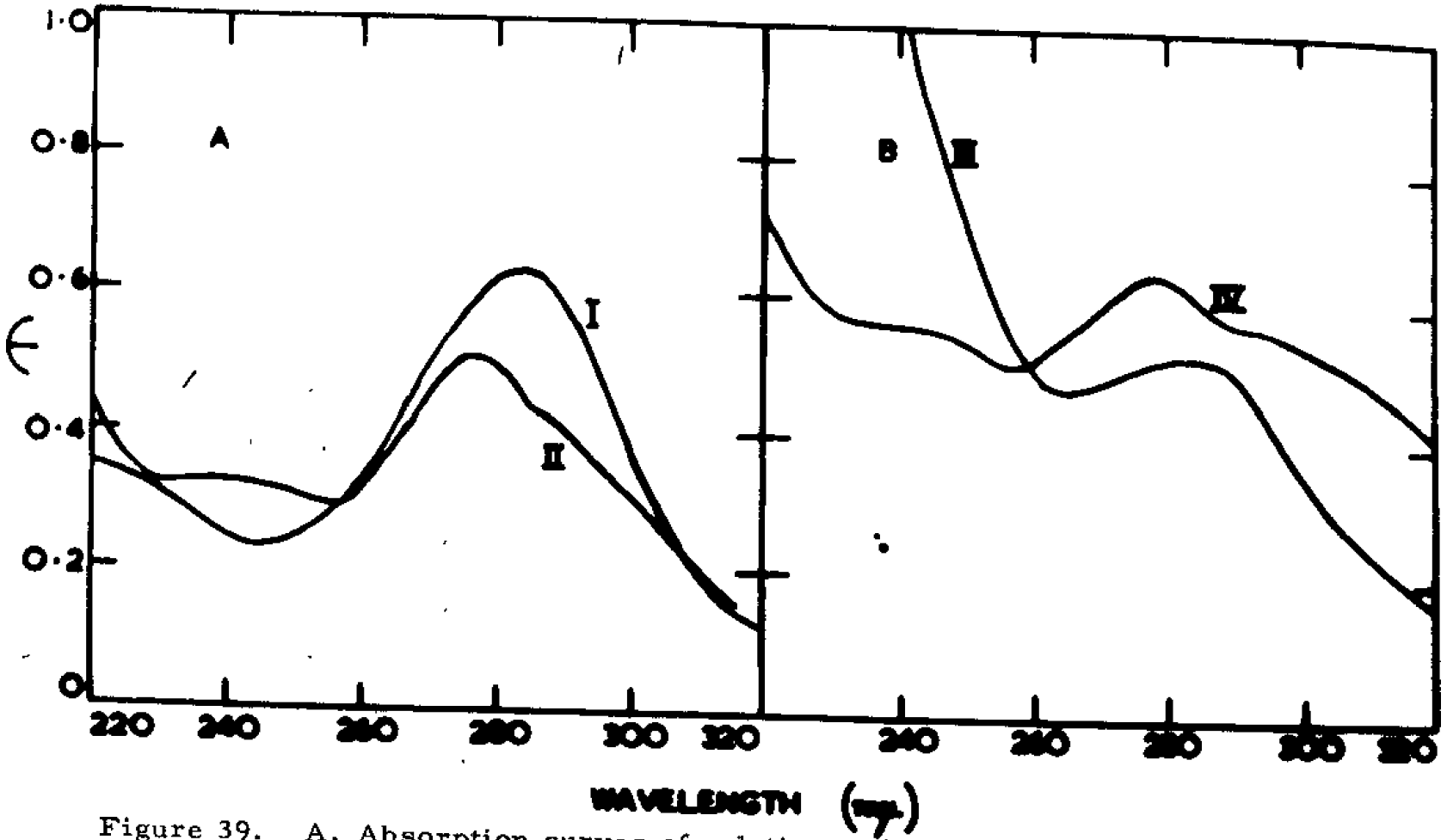


Figure 39. A. Absorption curves of solutions of: I, compound A; and II, glucosamine hydrochloride after heating for several hours.
B. Absorption curves of solutions of: III, mannose, and IV, glucosamine hydrochloride, obtained after heating the sugars on cellulose powder.

directly from the reaction mixture on cellulose powder, as well as from the degradation of compound A, and in the former instance it may have been formed by the action of heat on either mannose or glucosamine hydrochloride in the dry state.

Control experiments were therefore carried out in which cellulose powder was separately slurried in solutions of mannose and glucosamine hydrochloride, and the slurry then freeze-dried and heated in the usual way (Expts. 32 and 33, p.173). The absorption curves of the aqueous eluates from the cellulose are shown in figure 39 B. Although the absorption of the solution of the mannose after heating could be attributed to HMF, the quantity was negligible when compared with that obtained from the reaction mixture of mannose and glucosamine hydrochloride, as is shown in Table VII. The absorption curve of the corresponding solution

Table VII

Sugar Heated on Cellulose	E
Mannose + glucosamine hydrochloride	2.5
Mannose	0.025
Glucosamine hydrochloride	0.06

Development of ultraviolet absorption on heating mannose and glucosamine hydrochloride on cellulose at 100°C for $1\frac{1}{2}$ hours. E is the extinction coefficient of the eluate containing 1 mg. of glucosamine hydrochloride (or mannose) per ml.

of glucosamine hydrochloride, which is also shown in figure 39 B, differs from that of HMF and has the same form as the curve obtained from heated solutions of glucosamine hydrochloride (figure 39 A). Again, there is a large quantitative difference from the absorption of solutions obtained by heating the mixture of mannose and glucosamine hydrochloride on cellulose. Glucosamine is known to undergo self condensation to form substituted pyrazines[148] and it is possible that these, or similar substances, are responsible for the absorption observed in the solutions of glucosamine hydrochloride.

The HMF isolated from the reaction mixture was thus shown to result from the degradation of compound A rather than the dehydration of mannose itself during the heat treatment. In order to ascertain whether HMF had its origin in the mannose or glucosamine moieties of compound A, recourse was made to an experiment using isotopic carbon (Expt. 35, p. 175).

The reaction was carried out in the usual way using C^{14}-generally labelled mannose and compounds A and B were isolated from the mixture; B was removed by ether extraction and A was separated by ion-exchange chromatography. A solution of compound A was refluxed for several hours during which the characteristic darkening and formation of insoluble brown material occurred, and the generation of HMF was indicated by the onset of absorption at 284 mμ. Paper chromatograms of compound B and the heated

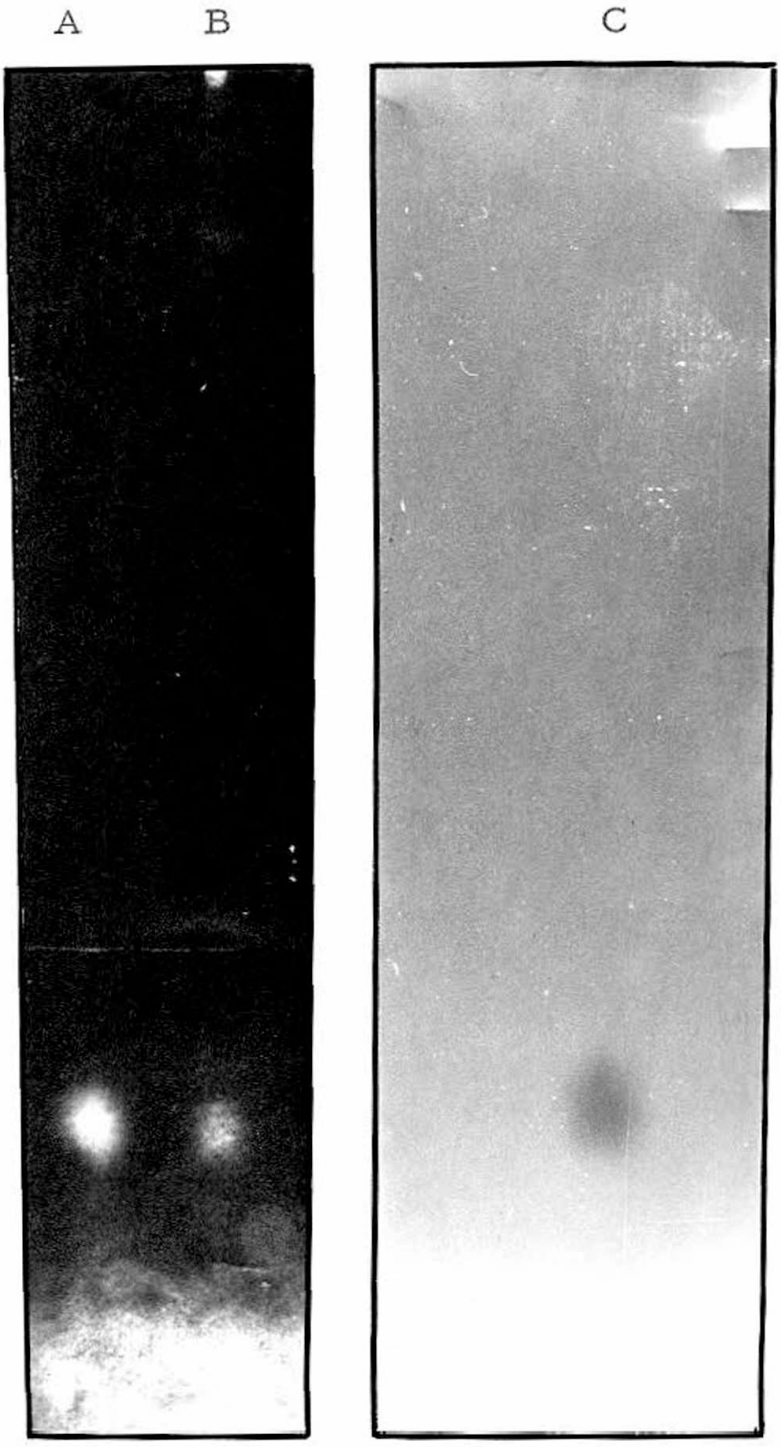


Figure 40. Ultraviolet photograph (1) and autoradigraph (2) of compound B (spots B and C) from a reaction mixture using C^{14}-mannose. Spot A is a reference sample of HMF.

solution of compound A, each carrying a reference sample of HMF, were developed and two spots having R_f values of 0.78 were located on each chromatogram, and were photographed using ultraviolet light. Autoradiographs of the two chromatograms were set up and when these were developed spots were observed on the film corresponding exactly with the spots located by ultraviolet photography. The autoradiograph and ultraviolet photograph of the chromatogram of compound B are reproduced in figure 40.

The HMF obtained from both the reaction mixture and the degradation of compound A was therefore radioactive and must have been formed from the mannose, and not from the glucosamine part of compound A.

The Structure of Compound A

The data concerning compound A may now be summarised. It is formed by condensation of the aldehydic group of mannose with the amino group of glucosamine hydrochloride, for unless these two functional groups are available the compound is not obtained. It comprises equimolecular quantities of mannose and glucosamine hydrochloride, and its ease of hydrolysis, and the compounds furnished on hydrolysis with cold water (principally mannose and glucosamine hydrochloride), are indicative of a glycosylamine structure. Colour reactions favour a glycosylamine rather than an Amadori rearrangement compound or a Schiff base (the latter would not react with ninhydrin or the Elson-Morgan

reagent). In boiling water, the hydrolysis was accompanied by the production of HMF and by the development of a brown colour and eventual precipitation of melanoidins. The infra-red spectrum showed that compound A did not contain a carbonyl group, whereas a peak at about 1720 $cm.^{-1}$ in the spectra of some Amadori compounds has been attributed to this functional group[184].

The only satisfactory interpretation of these data is that compound A is a glycosylamine. Production of HMF and melanoidins from this molecule requires an Amadori rearrangement to form 1-deoxy-1-glucosamino fructose. The colour tests for this class of compound were applied during and after the heating of compound A, as well as initially, but no indications of the formation of an Amadori compound, or of the presence of fructose (by chromatography and staining with naphthoresorcinol) were obtained. It would therefore appear that the Amadori compound is dehydrated as fast as it is formed to give the Schiff base of glucosamine and HMF, which in turn is hydrolysed to glucosamine and HMF itself. The mutual presence of HMF and glucosamine (either the base or its hydrochloride) would be expected to promote browning and melanoidin formation which could proceed through the various pathways already outlined (pages 84 to 88, and figure 26).

Unequivocal proof of the structure of a substance as labile as compound A is difficult if not virtually impossible to obtain, for the usual approaches employing oxidation, reduction, and

methylation cannot be used since they would be carried out on a mixture of compound A and its hydrolysis products. Similarly, optical rotation studies would need careful interpretation, as has been pointed out by Barclay et al.[138], for the species under examination is constantly changing. Nevertheless, the information derived from hydrolysis and degradation is sufficient, when considered in conjunction with supplementary information from colour reactions, to enable a structure to be assigned with reasonable confidence.

The structure suggested for compound A is N-mannopyranosyl-glucosamine hydrochloride. Since the compound is not a Schiff base, the hemi-acetal form of mannose must be involved and the pyranose form is more probable than the furanose ring. In conjunction with the relatively high positive specific rotation of glucosamine hydrochloride (+69°) and the low positive value for mannose (+14°), the negligible rotation of the glycosylamine is indicative of the β-configuration of the glycosidic linkage.

The sequence of reactions involved in the formation and degradation of compound A is included in figure 43.

Compound C, Its Formation and Properties

Heating of the mannose-glucosamine hydrochloride reaction mixture at temperatures in excess of 120°C has been shown to produce a multiplicity of products (p. 92), and from one preparation

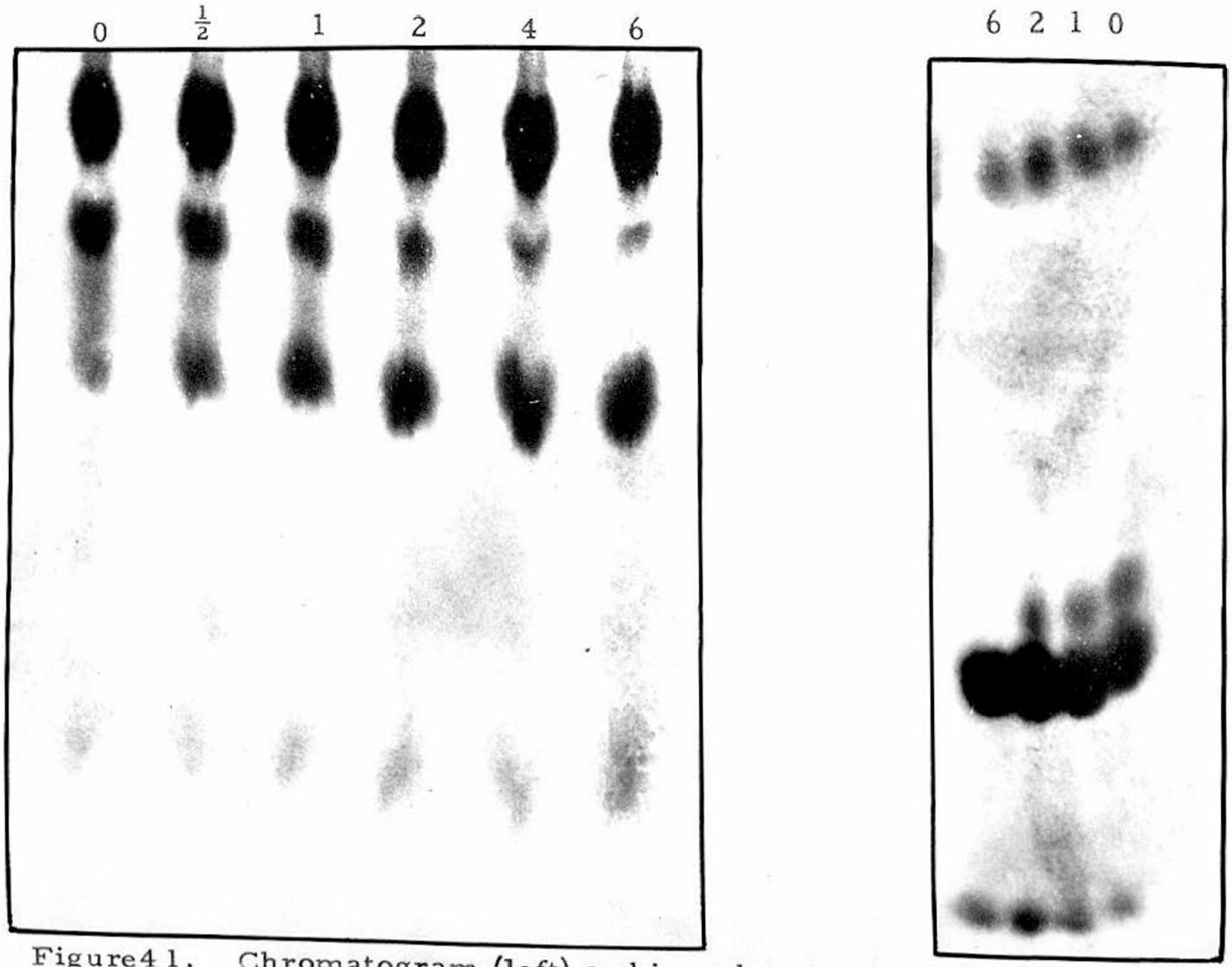


Figure 41. Chromatogram (left) and ionophoretogram of a solution containing compounds A and C after boiling for the periods (hours) indicated.

which had inadvertantly been heated at 120°C for three hours, the fractions eluted from the ion-exchange column in the position normally occupied by compound A were found to comprise two components (Expt. 36, p. 177). One of these was the now well-known compound A, or N-mannosyl glucosamine, but the other moved much more slowly on paper chromatography, having R_M and R_{GA} values of 0.14 and 0.28, respectively. The new compound, which was named compound C, was not resolved from compound A by ionophoresis unless the experiment was prolonged for almost twice the normal length of time. Compound C was then observed to have moved slightly faster than compound A, their respective M_{GA} values being 0.69 and 0.59.

When an aqueous solution containing compounds A and C was heated, chromatographic analysis showed the expected gradual decomposition of compound A, but compound C appeared to be quite stable. This at once suggested a method for the separation of the two compounds, or rather the recovery of compound C, for if the solution was boiled until the glycosylamine (compound A) was completely degraded, the new mixture which resulted could be resolved by ion-exchange chromatography. The procedure outlined was followed (Expt. 37, p. 178) and the hydrolysis of compound A was followed ionophoretically and chromatographically. A chromatogram of samples removed during the first six hours is shown in figure 41, and after twelve hours the removal of compound A was virtually

complete. Separation of the components of this solution on the ion-exchange chromatography column afforded a sample of compound C which was chromatographically and ionophoretically pure.

The stability of compound C in aqueous solution, together with its chromatographic and ionophoretic behaviour suggested an O-linked disaccharide and a preliminary examination of the properties of the compound was made by treating it on paper chromatograms with a number of spray reagents. Positive reactions were obtained with aniline hydrogen phthalate, alkaline silver nitrate, ninhydrin, the Elson-Morgan reagent, and alkaline triphenyltetrazolium chloride, but not with naphthoresorcinol. The reactions with ninhydrin and the Elson-Morgan reagent preclude a Schiff base structure, and reaction with the Elson-Morgan reagent also requires that the C_1 atom of glucosamine is unsubstituted[185]. Since it reacts with aniline hydrogen phthalate, compound C is a reducing sugar, and reaction with the triphenyltetrazolium chloride reagent signifies that the hydroxyl group adjacent to the reducing group is unsubstituted[186]. Failure to react with naphthoresorcinol rules out a keto structure and this is supported by the absence of a peak in the region of 1720 $cm.^{-1}$ in the infra-red spectrum[184].

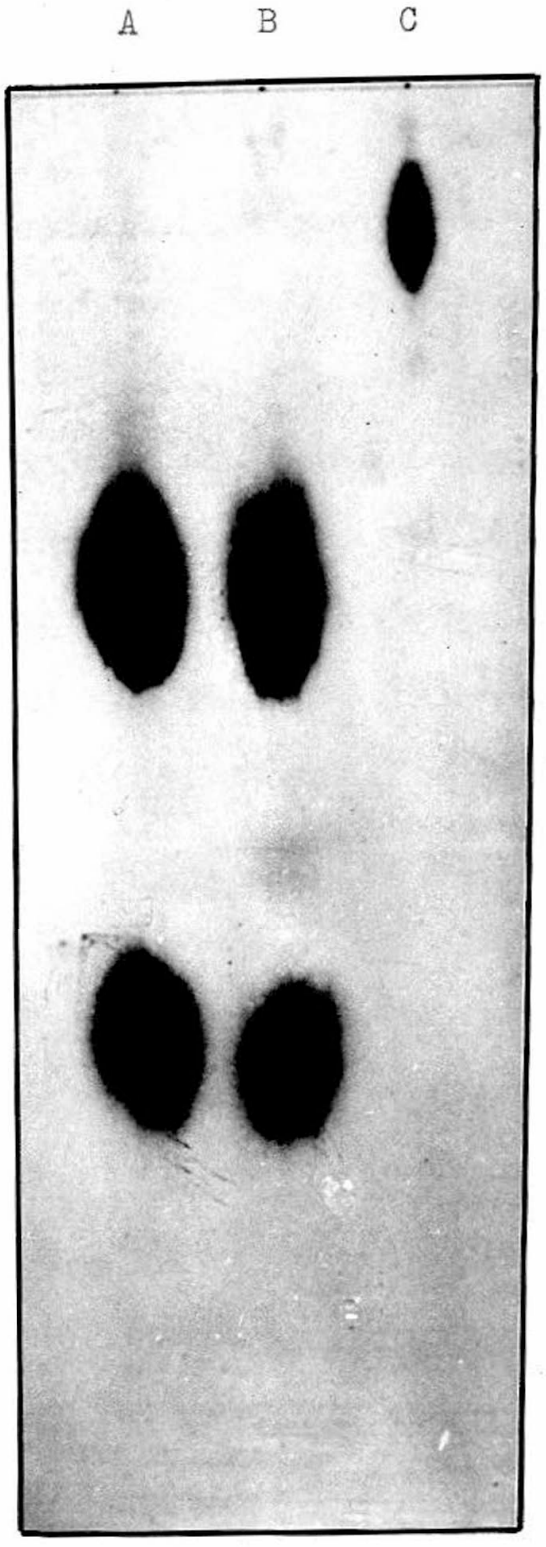


Figure 42. Chromatogram of compound C hydrolysate.
A, reference samples of mannose and glucosamine hydrochloride;
B, hydrolysate of compound C; C, compound C

Although it was quite stable in boiling water, compound C was found to be completely hydrolysed on refluxing in 2N hydrochloric acid for three hours(Expt. 38, p. 180) and the hydrolysate was colourless and had negligible absorption at 284 mμ. Chromatographic analysis showed that the hydrolysate contained only mannose and glucosamine hydrochloride (figure 42) and the proportion by weight of the former to the latter, as determined by the orcinol-sulphuric acid and Elson-Morgan reactions was 64:72. The absorption curve of the colour developed in the Elson-Morgan reaction had a peak at 530 mμ, characteristic of glucosamine, and that of the solution in the orcinol-sulphuric acid reaction was characteristic of mannose (figure 37, p. 116).

An attempt was made to measure the molecular weight of compound C by oxidation with an alkaline solution of sodium hypoiodite[187] (Expt. 40, p. 182) but erroneously low values were obtained when oxidations were carried out at room temperature (two hours) and at 5°C (twenty-four hours). These values (232 and 208, respectively) most probably result from over-oxidation of the amino group, for Jeanloz and Forchielli[188] have shown that solutions of glucosamine are very prone to over-oxidation in this reaction, even at 5°C. Conditions of oxidant concentration and time of reaction have a great influence on the extent of the oxidation and need careful calibration before significant results can be

obtained, and in this case insufficient material was available for the preparation of these data. The experiment therefore only indicated that compound C was a reducing sugar, and was not a monosaccharide.

A granular precipitate of compound C was obtained by the addition of acetone to an aqueous solution (Expt. 39, p. 181), but the material was very hygroscopic. Elemental analysis of the amorphous material showed: C, 36.46; H, 6.53; N, 4.05; and that required for $C_{12}H_{24}O_{10}NCl$ is C, 37.19; H, 6.21; N, 3.62.

The Structure of Compound C

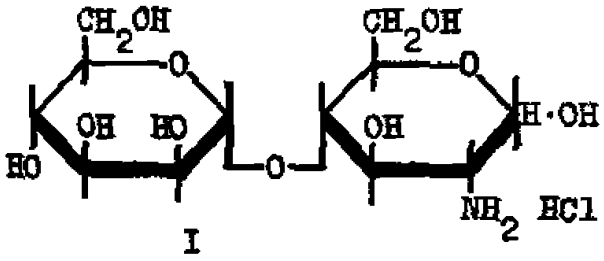
The observed ratio by weight of mannose to glucosamine hydrochloride in the hydrolysate of compound C (64:72) compares favourably with the theoretical ratio of 64:79 for a mixture containing equimolecular proportions of the two sugars. Its stability in water and ease of hydrolysis with acid, which is not accompanied by a browning reaction, indicate that compound C is an O-glycoside and not an N-glycoside. Reaction with aniline hydrogen phthalate and oxidation by sodium hypoiodite show that it is a reducing sugar and it comprises equimolecular proportions of mannose and glucosamine hydrochloride. Its $M_{G\,A}$ value of 0.69 and $R_{G\,A}$ value of 0.28 support the formulation of compound C as a disaccharide of mannose and glucosamine hydrochloride. A higher saccharide, which would need to be at least a tetrasaccharide,

is very improbable for this would have a molecular weight of about 700 and over-oxidation with sodium hypoiodite does not normally proceed to the extent of consuming more than two moles of oxidant when carried out at low temperatures[188].

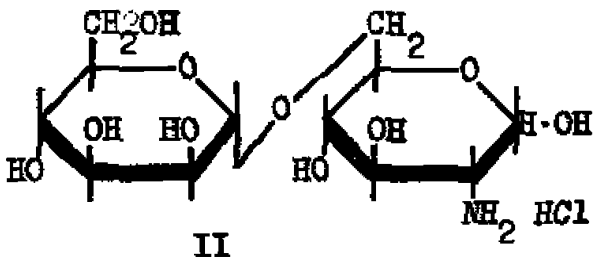
Seven O-glycoside structures may be written for a reducing disaccharide containing mannose and glucosamine hydrochloride. Following the nomenclature recommendations of The Chemical Society[189], these are the hydrochlorides of 3-, 4-, and 6-O-D-mannosyl-2-amino-2-deoxy-D-glucose, and 2-, 3-, 4-, and 6-O-(2-amino-2-deoxy-D-glucosyl)-D-mannose. Several of these possibilities were eliminated by consideration of the reaction with the Elson-Morgan reagent. Derivatives of glucosamine in which the glycosidic group is blocked do not give this reaction, and substitution of the C_3 hydroxyl group of glucosamine results in a shift of the absorption peak from 530 mμ to 510 mμ[185]. Thus, none of the four 2-amino-2-deoxy-D-glucosyl-D-mannose structures is permitted and this interpretation of the Elson-Morgan reaction is supported by the ease of acid hydrolysis, for the proximity of an amino group on C_2 would greatly hinder acidic hydrolysis of a glycoside[190,191]. The absorption curve of the coloured solution furnished by compound C in the Elson-Morgan reaction had a peak at 530 mμ, and not at 510 mμ as would be the

case with 3-O-mannosyl-2-amino-2-deoxy-glucose.

Only two of the seven structures remain and these are the hydrochlorides of 4-, and 6-O-mannosyl-2-amino-2-deoxy-glucose which are formulated below as I and II, respectively.



I



II

These two structures could be differentiated by their behaviour on oxidation with sodium metaperiodate, for I would be expected to absorb four moles of periodate and release two moles of formic acid per mole of the sugar, whereas II would absorb five moles of periodate with the production of three moles of formic acid, per mole of the sugar. Oxidation of compound C with a solution of sodium metaperiodate was carried out at room temperature (Expt. 41, p.183) and after four hours, 4.2 moles of periodate had been absorbed and 2.1 moles of formic acid released. This result is closer to that expected from structure I than to that from structure II, but the significant point is that it is slightly greater than the theoretical value for structure I, for Jeanloz and Forchielli[188] have shown that the consumption of periodate by glucosamine and its derivatives is a complex function of reaction conditions, and the lowering of pH attending the release of formic acid in unbuffered solutions retards the oxidation so that the experimental value would

be expected to be somewhat lower than the theoretical. A control experiment with glucosamine hydrochloride gave lower values than the theoretical for both periodate uptake (4.5 moles compared with 5.0) and formic acid release (3.4 moles compared with 4.0), after four hours. It therefore appeared very probable that the experimental values for compound C were also lower than the theoretical, in which case they favour structure II rather than structure I, for the latter would have shown a periodate uptake and formic acid release appreciably less than 4.0 and 2.0 moles, respectively, per mole of sugar.

Structure II was therefore tentatively assigned to the disaccharide and its specific rotation of +55° suggests the presence of an α-glycosidic linkage, for the specific equilibrium rotations of mannose and glucosamine hydrochloride are, respectively, +14° and +71°. It may be noted that 6-O-D-mannopyranosyl-2-amino-2-deoxy-D-glucopyranose is a more probable product than the 4-O-D-mannopyranosyl isomer if the disaccharide formation involved an anhydro sugar intermediate in a manner analogous with that of the chemical synthesis of oligosaccharides reported by Haq and Whelan.[161]

Conclusion

Although there appears to be no reaction between mannose and glucosamine hydrochloride in aqueous solution, even when refluxed for several hours, a complex mixture of products is obtained when the two sugars are heated together in the molten state. Heat treatment in the dry state may be controlled if the

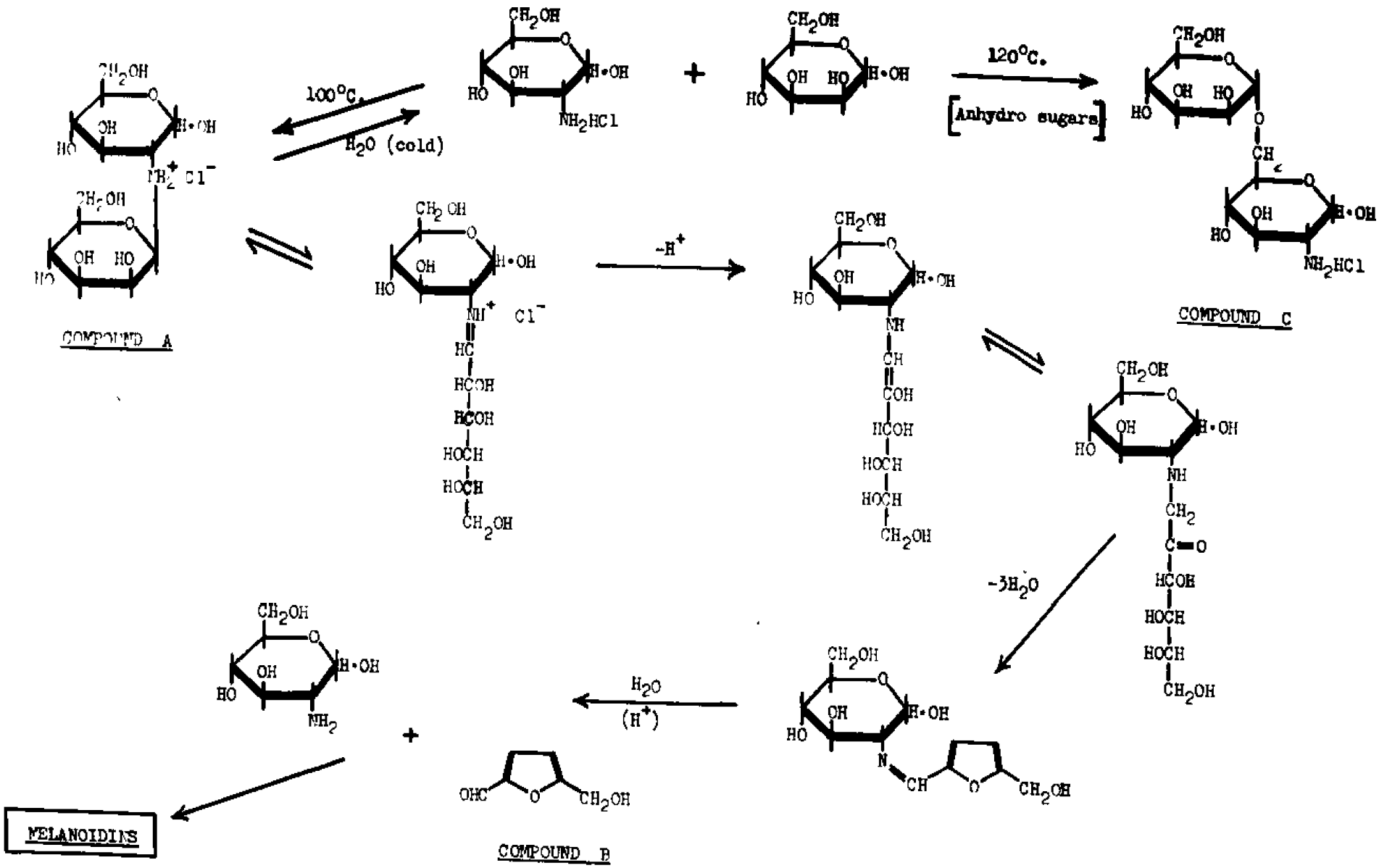


FIGURE 43. A SCHEME FOR THE REACTION OF MANNOSE WITH GLUCOSAMINE HYDROCHLORIDE.

reactant sugars are supported on cellulose, which is conveniently done by freeze-drying a slurry of cellulose powder in a solution of the sugars. When the reaction is carried out at temperatures not exceeding 100°C, N -mannosyl glucosamine and one of its degradation products, HMF, are obtained, but at slightly higher temperatures (120°C) an O-mannosyl glucosamine is obtained in addition to the products formed at the lower temperature. A sequence of reactions believed to be involved in these processes is summarised in the scheme shown in figure 43. The yields of the products were very low, that of the glycosylamine being about 2 to 3% of the theoretical (larger quantities may be formed but decomposed during extraction) and that of the O-mannosyl glucosamine being about 1% of the maximum theoretical yield.

Formation of the glycosylamine is accompanied by its degradation in a browning reaction sequence, for HMF is obtained from the reaction products and the solution of products (at the same concentration) from the reaction at 100°C is golden brown in colour whilst that from the reaction at 120°C is a dark reddish-brown. The significance of the glycosylamine formation may be of little more than academic interest in the chemistry of browning processes in natural products, but it must be constantly borne in mind when analysing mixtures or hydrolysates containing amino sugars (or other amino compounds) and reducing sugars by paper chromatography, or ionophoresis, for unless heat treatment is avoided these products may arise as artefacts.

The Behaviour of Some Aldehydes on a Sulphonated Polystyrene Resin

Compound B was first obtained in the eluate from ion-exchange chromatography and was eluted from the column in the fractions immediately preceding compound A which was known to be basic (figure 33, p. 103). The assumption that compound B was also basic therefore seemed reasonable and a Schiff base of HMF and glucosamine was suspected, but the compound was later shown to be HMF.

The rather surprising behaviour of HMF on the resin column can be explained by either of two processes. The compound could in fact be exhibiting true basic properties in the strong acid solution used (0.3N), possibly by the formation of an oxonium ion, or the retention of the HMF on the column could be due to some other mechanism of interaction with the resin. No evidence of basic behaviour was obtained from the ionophoresis of HMF in 0.75M formate buffer at pH 2.

In order to differentiate between these two possibilities, experiments were carried out using two other aldehydes (Expt. 24, p.164). A solution of furfuraldehyde in 0.3N hydrochloric acid was applied to the ion-exchange chromatography column and was retained by the column slightly longer than was HMF. Furfuraldehyde and HMF are structurally similar and both

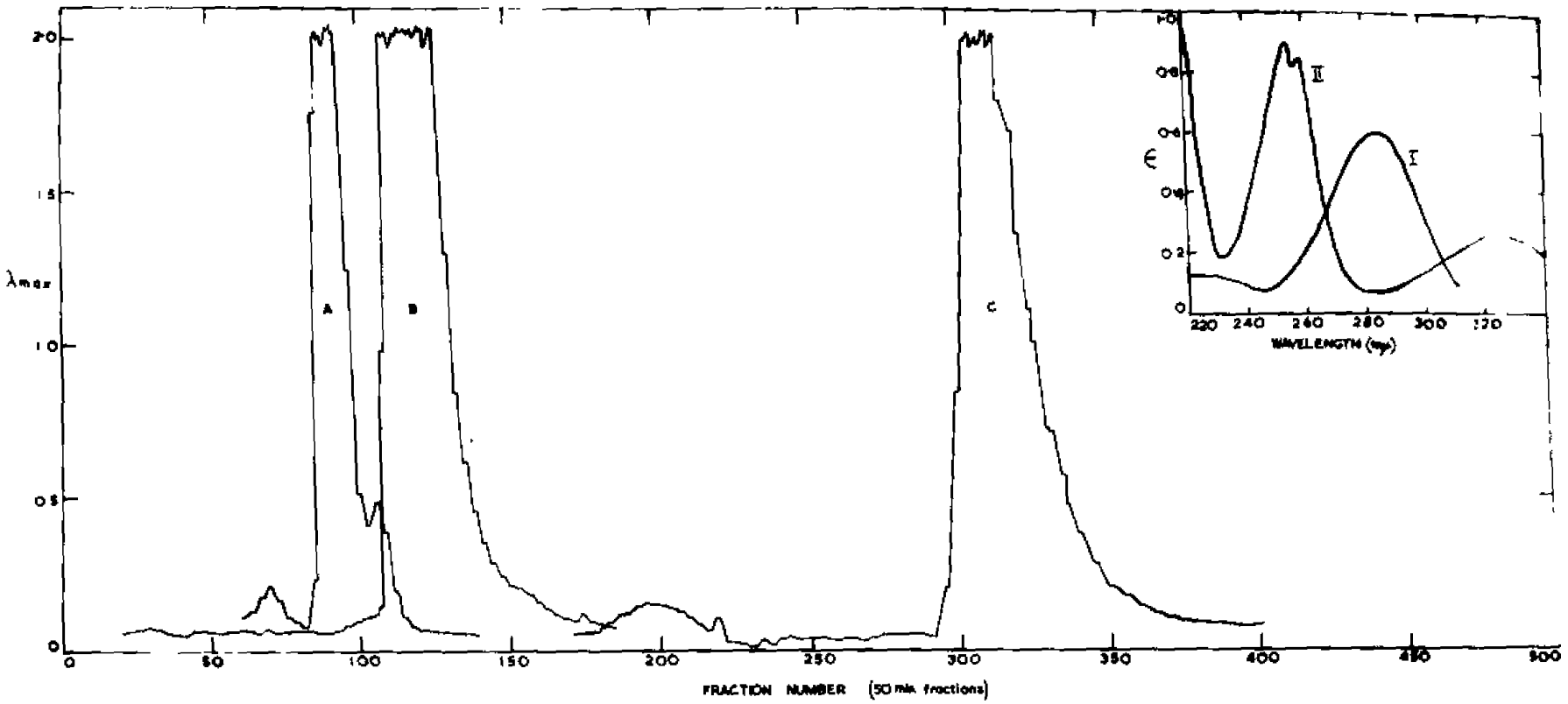


Figure 44. Histogram showing the position of elution of HMF (A), furfuraldehyde (B), and salicylaldehyde (C), from a column of sulphonated polystyrene resin (Zeokarb 225). Inset are the absorption curves of HMF (I), and salicylaldehyde (II).

could possibly behave as a weak base, but a non-heterocyclic aromatic aldehyde could not. An aromatic aldehyde was desirable for its ease of detection in the eluate fractions, but the choice of a compound was governed largely by solubility in an aqueous system. A saturated solution of salicylaldehyde in 0.3N hydrochloric acid was found to be suitable and the aldehyde was very firmly held by the resin, being eluted in fractions 300 to 330. This suggests very strongly that the retention of the aldehydes on the column is due to some interaction of the aldehyde group with the resin, rather than the ionic linkage of a base with the anionic resin. Further support of an aldehydo-group - resin interaction was obtained when ionophoresis of the three aldehydes in 0.3N hydrochloric acid failed to provide any evidence for their behaviour as bases.

The formation of bisulphite addition compounds by aldehydes is well known, and these derivatives are furnished by furfuraldehyde[192], although they are unstable in aqueous solution and an equilibrium mixture is established below pH 5[193]. By analogy with this reaction, the interaction with the resin

$$RCHO + NaHSO_3 \rightleftharpoons RCHOHSO_2ONa$$

$$RCHO + R'SO_2OH \rightleftharpoons RCHOHSO_3R'$$

could be attributed to the formation of a weak addition compound with the sulphonic acid function of the resin. The analogy is readily seen from the above equations.

If this mechanism is correct, the reaction should occur with all aldehydes, and provided that an adduct has a suitable instability constant in the acid solution, it could be eluted from the ion-exchange resin in a useful fraction range. Calibration of a resin column with a number of known aldehydes would then be possible and a very useful new method for the resolution of mixtures of aldehydes, or of aldehydes with neutral sugars such as are frequently obtained in carbohydrate studies, would be available.

Experimental

1. General Methods

A. Paper Chromatography

Unless stated to the contrary, paper chromatographic separations were effected on Whatman No. 1 filter paper, the chromatograms being developed, usually for about 60 hours, with the organic phase resulting from a mixture of n-butanol, ethanol, and water in the proportion 4:1:5. The solutions applied to the paper were allowed to dry without the application of heat.

The following reagents were used for staining the dried chromatograms.

i) Ninhydrin[194], for compounds containing a primary or secondary amino group.

ii) Elson-Morgan reagent[195], for glucosamine and certain derivatives.

iii) Aniline hydrogen phthalate[196], for reducing sugars.

iv) Alkaline silver nitrate[197], for reducing sugars and sugar alcohols.

v) Naphthoresorcinol[195], for ketoses.

vi) Alkaline triphenyltetrazolium chloride reagent[198,197] for various sugars.

B. Paper Ionophoresis

Ionophoretic analysis was carried out on strips of Whatman

No. 3 filter paper (usually 57 x 11 cm.) using 0.75M formate[199] (pH 2), 0.2M acetate[87] (pH 5), or 0.12 M borate[200] (pH 10) buffer solutions in an apparatus described by Foster[85]. A potential difference of about 500 to 600 volts (i.e. about 8 or 9 volts/cm.) was usually applied for 4 hours. After drying, the ionophoretograms were treated with one of the detection reagents previously listed. Acetate buffer was used in all experiments where no statement to the contrary is made, and solutions applied to the paper were usually allowed to dry without the application of heat.

C. Ion-exchange Chromatography

Zeokarb 225 (200 to 400 mesh) $4\frac{1}{2}\%$ cross-linked resin (sulphonated polystyrene) was used for chromatographic fractions as described by Crumpton[169]. A small column (43 cm. x 1 cm.^{2}) was used for analytical purposes, but a large column of resin (53 cm. x 50 cm.^{2}, 1.8 Kg., or 4 lbs., of resin) was employed for preparative separations. The resin was carefully washed before use by twice taking through the sodium form with 2N sodium hydroxide solution and regenerating with 2N hydrochloric acid. Between each of these processes, the resin was washed twice with distilled water and was finally washed thoroughly with 0.3N hydrochloric acid which was the solvent invariably used for elution of the columns. Eluate fractions (normally 1 ml. from the analytical column and 50 ml. from the preparative column) were collected using an automatic fraction cutting machine which worked on the

rocking siphon-balance principle (J. W. Towers, Ltd.).

Recovery of materials from the acid eluate fractions was effected either by ethanol extraction of the freeze-dried mixture obtained after neutralising to about pH 5 with sodium bicarbonate, or by neutralisation through extraction with a 10% (v/v) solution of di-n-octylmethylamine in chloroform[173] and freeze drying. It was found necessary to remove various amine impurities from the di-n-octylmethylamine by washing with 2N hydrochloric acid and regenerating the free base by shaking the chloroform solution with 2N sodium hydroxide solution. Equal volumes of the two phases were used and the chloroform solution was washed with distilled water between each operation. Four such treatments were required before ionophoretic analysis showed that compounds reacting with ninhydrin had been completely removed. The neutralised solutions were washed twice with chloroform and filtered before freeze-drying.

2. Production of Compound A on Ionophoretograms

Solutions of glucosamine hydrochloride (5% w/v) and of a mixture of mannose and glucosamine hydrochloride (5% w/v of each) were applied side by side to a strip of No. 3 filter paper and heated for about 2 minutes in a stream of hot air from an electric hair-drier. Two similar spots were applied alongside the previous ones, but were not heated. After iono-

phoresis the paper was sprayed with ninhydrin reagent and mauve stains, due to glucosamine, were produced about 15cm. (towards the anode) from the base line in the case of each solution applied to the paper. From the spot of the mixture which had been heated, but not from the other three spots, a second, weaker stain slowly appeared about half-way between the glucosamine stain and the base line and had a M_{GA} value of 0.56. Stains in this position relative to glucosamine were generally termed "D spots" and that obtained from mannose and glucosamine hydrochloride was attributed to the presence of compound A.

3. Detection of Compound A with Various Reagents

Five spots of a solution of mannose and glucosamine hydrochloride were spaced along the base line of an ionophoretogram and heated by means of a hair drier for 2 to 3 minutes. After ionophoresis the paper was cut lengthways into five strips, each carrying one of the original spots. The strips were severally stained with ninhydrin, Elson-Morgan reagent, aniline hydrogen phthalate, alkaline silver nitrate, and naphthoresorcinol. All the reagents but naphthoresorcinol produced two stains, one attributable to glucosamine, and one to compound A. Another ionophoretogram carrying two spots of the mixture was prepared in a similar way and cut into strips, one of which was stained with ninhydrin to provide a control, and the other

with alkaline triphenyltetrazolium chloride reagent and stains attributable to both glucosamine and compound A (M_{GA} 0.57) were obtained on each strip.

4. Combinations of Sugars Producing D Spots on Ionophoretograms

Ionophoretic separations of several mixtures of sugars with amino sugars were carried out after heating the mixtures on the paper. The effect of heating on the various amino sugars alone was determined in control experiments. Heat treatment was effected either with a hair drier as previously described, or in an oven at 100°C during about 20 minutes. The ionophoretograms were stained with either ninhydrin or alkaline silver nitrate and Table VI indicates the mixtures which furnished D spots.

The amino sugars used were glucosamine hydrochloride, 3:4:6-tri-O-methyl-glucosamine hydrochloride, N-acetyl glucosamine, glucosaminol hydrochloride, galactosamine hydrochloride, and 2-amino-glycerol hydrochloride. The sugars with which they were mixed were the following, though by no means all of the possible combinations were made. Mannose, α-methyl mannoside, 3:4-di-O-methyl mannose, mannitol, galactose, fructose, xylose, ribose, 2-deoxy-ribose, sorbitol, dulcitol, glucose, 1-deoxy-glucose (poly galitol), 3-O-methyl glucose, 2:3-di-O-methyl glucose, 3:4-di-O-methyl glucose, 2:3:6-tri-O-methyl glucose,

2:3:4:6-tetra-O-methyl glucose, α-methyl glucoside, sodium glucuronate, and maltose. Two spots of 5% w/v solutions were applied to the paper.

An ionophoretogram, stained with alkaline silver nitrate, of glucose and some of its methyl ethers after heating with glucosamine hydrochloride is shown in Figure 29 (p.94).

5. Optimum Heating Conditions for Compound A Production

Spots of a solution of mannose and glucosamine hydrochloride were successfully applied along the base-lines of a strip of ionophoresis paper and a sheet of chromatography paper and the papers were heated in an oven at 100°C. for varying periods of time after each application. In this way, heating periods of 2 minutes to 6 hours were covered and after ionophoresis and chromatographic development of the respective papers, they were stained with alkaline silver nitrate. Compound A was observed on the chromatograms as the slowest moving of three spots, having R_{GA} and R_M values of 0.58 and 0.32 respectively. Both ionophoretigram and chromatogram showed a gradual increase in the intensity of compound A during the first hour of heating, but little change was discernible thereafter.

The experiment was repeated using a temperature of 120°C. and in this case small amounts of other compounds appeared to be formed in addition to compound A. The two chromatograms are illustrated in Figures 27 and 28 (p. 92).

Comparative ionophoretograms of mixtures heated for half-an-hour at 80°C., 100°C., and 125°C., showed that compound A formation was much less rapid at 80°C. than at 100°C., and that at 125°C. a series of spots was produced between the spot due to compound A, and the base-line.

6. Preparative Ionophoresis

A solution of mannose and glucosamine hydrochloride (0.1 ml., 5% $^{w}/_{v}$ of each sugar) was applied as a streak across the width of a strip of No. 3 filter paper and the paper was heated in an oven at 100°C. for half-an-hour. After ionophoresis, guide strips were cut from the edges and centre of the paper and were stained with alkaline silver nitrate to locate the position of compound A. The appropriate band was then cut from the paper and eluted with distilled water, and the extract was filtered and freeze-dried. The material so obtained was dissolved in a little water and examined ionophoretically. Only a faint D spot was observed, together with a small amount of glucosamine and most of the material, apparently decomposition products, remained at the base line or formed a streak extending between the D spot and base-line.

7. Reactions in Aqueous Solution

Mannose and glucosamine hydrochloride were dissolved in a number of buffer solutions in the pH range 2 to 10 and these solutions (about 1 ml., containing 5% $^{w}/_{v}$ of each sugar) were heated in sealed tubes for $1\frac{1}{4}$ hours at a temperature of 100° to 120°C.. The buffer

solutions used were: O.1 M formate buffer, pH 2;[199] O.1M citrate buffer, pH 4 and 6;[201] and O.2M barbiturate buffers, pH 8 and 10.[202] The same procedure was followed with an aqueous unbuffered solution of the two sugars. After heating, the solution in formate buffer remained virtually colourless, the two solutions in citrate buffer were only very slightly yellow, the unbuffered solution was yellow-brown in colour, the solution in barbiturate buffer at pH 8 was a pronounced golden-brown, and that at pH 10 was dark brown in colour.

All the solutions were examined ionophoretically and chromatographically and none was found to contain compound A. Several chromatograms of the solutions were prepared and in addition to the spots of the six solutions which were applied to the paper and allowed to dry without heating, spots of each solution were heated on the paper. Six such papers were prepared and two were developed normally (p. 135), two were developed with the organic phase of a mixture of n-butanol-water-acetic acid (4:5:1), and two with the organic phase of a mixture of n-butanol, ethanol, water, and ammonia (s.g. 0.880) in the proportion 40:10:49:1. After thorough drying, one chromatogram from each development was stained with ninhydrin and the other with aniline hydrogen phthalate. All six chromatograms showed that compound A was absent from each of the six solutions, but was formed in each case by heating on the paper.

The chromatograms developed with n-butanol-ethanol-water-ammonia showed very bad streaking of the glucosamine spots, and

those developed in butanol-water-acetic acid produced two spots from glucosamine, both being quite close together. In addition, the glucosamine spot was streaked and very much retarded on the paper (R_M reduced from the normal value of 0.68 to about 0.18) in each of the citrate buffer samples (figure 30, p.95). A chromatogram of solutions of glucosamine hydrochloride in citrate buffer (pH 6) and in aqueous citric acid exhibited the same behaviour when developed in n-butanol-ethanol-water and stained with ninhydrin.

8. Preparative Chromatography.

A solution of mannose and glucosamine hydrochloride (5% $^{w}/_{v}$ of each; 1 ml.) was applied across the width of a sheet of No. 3 filter paper and the paper was heated in an oven at 100°C. for half-an-hour. After development, guide strips were cut from the edges and from within the chromatogram and stained with alkaline silver nitrate in order to locate compound A. The appropriate band was then cut from the paper, eluted with distilled water, and the aqueous solution was filtered and freeze-dried. The product was extracted in a small volume of water (about 0.1 ml.) and examined by ionophoresis and paper chromatography. Three spots were obtained in each case and could be attributed to mannose, glucosamine hydrochloride, and compound A.

A second chromatogram was prepared as before, but instead

of eluting the band containing compoundA, a narrow strip, 8 mm. x 5 cm. was cut from this band and sewn into a hole of precisely the same dimensions cut in the base-line of a new sheet of No. 3 filter paper. Controls of mannose and glucosamine hydrochloride heated on the paper were placed at either end of the strip and were cut out and sewn back into the paper in order to be truly comparable with the experiment. The new sheet of paper was then developed and stained with alkaline silver nitrate and a distinct band due to compound A (R_{GA} about 0.63) was obtained. A photograph of the chromatogram is reproduced in figure 31 (p. 97). A repetition of the experiment was made using strips from an ionophoretogram and again, a distinct band due to compound A was observed. Strips from preparative ionophoretograms and chromatograms were examined ionophoretically by sewing into corresponding holes cut into the base line of a new strip of No. 3 filter paper. Although separation of a distinct band due to compound A was observed on staining with alkaline silver nitrate, the separation was less complete than by chromatography and larger amounts of mannose and glucosamine hydrochloride were found.

The "double chromatography" experiment was repeated using longer strips from a preparative chromatogram (1 cm. x 12 cm.) and a complete strip width from an ionophoretogram, and this time the position of compound A on the second chromatogram was determined by staining guide strips with alkaline silver nitrate. The appropriate

bands were eluted with dry methanol and the two solutions were evaporated to low volume under reduced pressure at room temperature and examined by paper chromatography. A reference spot of mannose and glucosamine hydrochloride heated on the paper showed that the two samples examined were solutions of compound A, almost pure, chromatographically, (figure 32, p. 97).

9. Other Experiments on the Preparation of Compound A

A small amount of mannose (0.1 g.) was melted in an ignition tube and an equal amount of glucosamine hydrochloride was added. The resulting dark brown mass was allowed to cool and then dissolved in water (2 ml.) and the solution analysed by ionophoresis and paper chromatography. A large number of spots, including those due to compound A and glucosamine hydrochloride, was produced when the papers were stained with ninhydrin.

A finely ground mixture of mannose and glucosamine hydrochloride was maintained at 110°C. for several hours, and a stiff paste of these two sugars with a little water was heated at 100°C. for 6 hours. In both cases, ionophoretic analysis showed that only the two original sugars were present after the heat treatment.

Glucosamine hydrochloride (0.1 g.) was added to a methanolic solution of mannose (0.1 g. in 2 ml.) and the mixture was refluxed for several hours. No spot due to compound A was observed on staining ionophoretograms of the mixture sampled

after periods of up to 12 hours, although after this time the solution was pale yellow. Very little glucosamine hydrochloride appeared to dissolve.

A slurry of cellulose powder (16 g.) in a solution of mannose and glucosamine hydrochloride (0.8 g. of each in 100 ml. distilled water) was freeze-dried and heated in an oven at 100°C. for $1\frac{1}{2}$ hours. A small portion of the cellulose was eluted with water and chromatographic analysis showed the eluate to contain mannose, glucosamine hydrochloride, and compound A.

10. Separation of Compound A on a Cellulose Column

Cellulose powder (about 500 g., Whatman standard chromatographic grade) was slurried in the organic phase of a mixture of n-butanol, ethanol and water (4:1:5), and the slurry was packed into a column (57 cm. x 12.5 $cm.^{-2}$). A disc of filter paper was placed on top of the column which was then drained almost free of the accumulated head of solvent. A preparation of compound A on cellulose powder (see above) was made into a thick slurry with the solvent and carefully poured onto the top of the column. When this had settled down, the column was eluted with the solvent and fractions of 50 ml. were collected automatically.

Samples from alternate fractions were spotted onto filter paper and stained with alkaline silver nitrate. Most of the 40 fractions examined (of 80 collected) gave weak stains, but more intense stains were obtained from fractions 22 to 29 and 45 onwards. Fractions 22

to 29 were bulked and evaporated almost to dryness under reduced pressure at 30°C or less, and the fractions beyond 45 were divided into batches of 5 and similarly concentrated. The samples so obtained were analysed ionophoretically and all furnished spots corresponding with mannose, glucosamine hydrochloride, and compound A. The samples were therefore all bulked and evaporated to dryness to yield 150 mg. of material.

11. Partial Separation with Ion-exchange Resin

Part of the material (60 mg.) eluted from the cellulose column, as described above, was dissolved in water (2 ml.) and mixed with moist ion-exchange resin (IR 120, *hydrogen form*) (25 ml.). The resin was transferred to a column with a little water and was then washed with distilled water. Fractions of 5 ml. were collected (automatically) and after the 12th fraction, 0.25N hydrochloric acid replaced water as the eluant. The first 15 fractions were neutral, but succeeding ones were acidic and a total of 33 fractions was collected. The acid fractions were neutralised with silver carbonate and all the fractions were then separately freeze-dried. No residue was obtained from fractions 4 to 19. Residues from the other fractions were examined by paper chromatography and ionophoresis and fractions 1, 2 and 3 contained mannose and compound A, but no glucosamine hydro-

chloride. Fractions 20 to 23 contained small quantities of glucosamine hydrochloride and compound A (the spot due to compound A was badly streaked), but no mannose. Later fractions were not analysed.

12. N-Acetylation of Glucosamine Hydrochloride and Compound A

Mannose and glucosamine hydrochloride were heated together on cellulose powder as described on p.146 except that 1.5 g. of each sugar and 10 g. of cellulose were used. The cellulose was eluted and washed with water (80 ml.) and the solution was acetylated using the method of Roseman and Ludoweig[166]. The solution was then freed of un-acetylated amino compounds by passing down a column of cation exchange resin (IR 120, hydrogen form, 10 ml.) and was finally freeze-dried. A solution of some of the freeze-dried product was analysed by paper chromatography and on staining the chromatogram with alkaline silver nitrate, spots attributable to mannose and N-acetyl glucosamine were found, together with traces of compound A and a large amount of a fast moving substance (R_{GA} 2.5) which was found in the region usually occupied by anhydrosugars.

13. Solvent Extraction Methods for Separation of the Reaction Mixture

A suspension of mannose and glucosamine hydrochloride in cellulose (p. 146) was heated at 100°C for 1 hour and eluted

with water. The solution was freeze-dried and portions (30 mg. each) of the product were severally slurried in one of the following seven solvents (0.3 ml.); methanol, ethanob, n-butanol, acetone, chloroform, dioxan, and diethyl ether. The mixtures were thoroughly shaken, and after standing for 6 hours the supernatant liquids were examined ionophoretically. The methanol, ethanol, and butanol extracts all contained the three components of the mixture, though in different proportions. The dioxan extract contained only mannose, the acetone extract a small amount of mannose, the chloroform extract small quantities of mannose and glucosamine hydrochloride, and the ether extract did not contain any of the three components, viz. mannose, glucosamine hydrochloride, and compound A. The dioxan extracted material was treated a second time with a similar volume of dioxan and ionophoretic analysis showed that the extract again contained only mannose, and that the remaining solid contained all three components of the mixture.

A second suspension of mannose and glucosamine hydrochloride in cellulose was prepared using 3 g. of each sugar and 15 g. of cellulose. The mixture was heated at 100°C for 1 hour and two portions of 5 g. were extracted, one with dioxan and the other with chloroform, in Soxhlet thimbles for 21 hours. Ionophoresis indicated that the cellulose samples after extraction retained all three components of the mixture, and that the dioxan

contained only mannose (though in reasonable quantity) and the chloroform extract contained a small amount of mannose and possibly a trace of compound A.

A third batch of the sugars on cellulose was prepared (5 g. of each sugar and 30 g. of cellulose) and after heating as before it was slurried in dioxan and packed into a column (diameter, 2.5 cm.). The column was washed with dioxan (about 1 litre) by slow, downward percolation (during about 24 hours) and was then allowed to drain. After extrusion from its container, the column of cellulose was divided into two equal parts and each was sucked dry on a Buchner funnel. A small sample from each part was extracted with water and these solutions, and the dioxan eluate, were analysed by ionophoresis. Only mannose was found in the dioxan eluate, but both extracts from the cellulose still contained all three components although a reduction in the intensity of the mannose spot was discernible.

14. Separation by Fractional Crystallisation

Part of a freeze-dried mixture (about 3 g.) obtained from heating mannose and glucosamine hydrochloride on cellulose was dissolved in water (10 ml.) and the concentrated solution was divided into two equal parts.

a) Glucosamine hydrochloride was crystallised from the solution by the gradual addition of acetone to the cold aqueous solution containing methanol, exactly as described by Gardell[167]. The crystals were filtered and washed with acetone, and both crystals and mother liquor were examined by ionophoresis. The glucosamine hydrochloride crystals contained only a little mannose and the mother liquor contained all three components of the mixture.

b) The aqueous solution was cooled to about 10°C and dioxan at room temperature (its m.pt. is 12°C) was added until precipitation began to occur. After standing in the refrigerator for 2 hours, the precipitate was filtered and washed twice with dioxan. Ionophoresis showed that it contained nothing other than glucosamine hydrochloride. The addition of more dioxan to the mother liquor resulted in further precipitation and the solid was again filtered, washed, and analysed ionophoretically. This procedure was repeated twice again, but the operation was done at room temperature to avoid the crystallisation of dioxan. The second crop of precipitate contained glucosamine hydrochloride and a small amount of mannose; the third crop contained glucosamine hydrochloride, an increased quantity of mannose, and a trace of compound A; and the fourth comprised similar quantities of all three components.

On bulking the mother liquor from the fourth dioxan precipitation with that from the acetone crystallisation, a further precipitate was obtained which was mainly glucosamine hydrochloride. The final mother liquor contained all three components of the original mixture, but probably in roughly similar amounts.

15. Resolution of the Mixture by Ion-exchange Chromatography

A large batch of compound A was prepared as previously described (p. 146) using 25 g. of mannose, 25 g. of glucosamine hydrochloride, and 200 g. of cellulose powder. An aliquot from the aqueous eluate estimated to contain about 2 mg. of amino sugar was applied to an analytical column of ion-exchange resin (Zeokarb 225, p. 136) and the remainder was freeze-dried. The column was eluted with 0.3N hydrochloric acid using a flow rate of 1 to 2 ml. per hour, and 1 ml. fractions were collected. Every fifth fraction was examined for hexosamines (or certain derivatives) using the Elson-Morgan reaction[170] exactly as described by Svennerholm[171]. Intermediate fractions were also analysed where the presence of amino sugars was indicated by the development of a pink or red colour in the test solution. Fractions 85 to 91 contained a small amount of material giving a positive reaction (the total being equivalent to about

50 μg. of glucosamine hydrochloride) and a much more intense coloration was obtained from fractions 121 to 144, and was attributed to the presence of glucosamine hydrochloride.

The freeze-dried reaction mixture was dissolved in 0.3N hydrochloric acid (400 ml.) and 50 ml. aliquots of the solution were applied to the preparative ion-exchange column (p. 136) for separation. The column was eluted with 0.3N hydrochloric acid using a flow rate of about 130 ml. per hour and fractions of 50 ml. were collected. The fractions were examined as before and a positive reaction was obtained from fractions 96 to about 106 and from fractions 140 to 175 when fraction collection was ceased: the latter group of fractions gave a much more intense coloration than the former. The first group of fractions (i.e. 96 to 106) was assumed to contain compound A and the second (140to 175), glucosamine hydrochloride. Fractions 96 to 106 were bulked, neutralised with either sodium bicarbonate or di-n-octylmethylamine (p. 137), and freeze-dried. Analysis of the material by ionophoresis showed that it was mainly compound A, but small quantities of mannose and glucosamine hydrochloride were present.

Several batches of material were separated in this way and the amount of material applied to the column in one batch normally contained 2 to 3 g. of amino sugar, i.e. 4 to 6 g. of

the mixture. The yield of compound A was usually 50 to 100 mg.

The ninhydrin reaction, measurement of optical rotation (using a 2 dm. tube), and measurement of ultraviolet absorption were used in addition to the Elson-Morgan reaction for scanning the fractions of certain batches. Unless stated otherwise, the wavelength used in ultraviolet absorption measurements was 284 mμ. Ninhydrin determinations were carried out by the method of Yemm and Cocking[172] after almost neutralising the acid solution (1 ml.) by adding an equal volume of 0.25N sodium hydroxide solution and diluting with 1 ml. of the citrate buffer solution used in the reaction.

The optical rotation measurements enabled mannose to be detected in the early fractions (20 to 33) and also indicated the position of glucosamine hydrochloride but was much less sensitive than colorimetric methods. The ninhydrin reaction gave exactly the same indications (quantitatively) as the Elson-Morgan reaction. Ultraviolet absorption measurements showed the presence of a compound in fractions 87 to 95 which did not react with ninhydrin or the Elson-Morgan reagent and had no optical activity. This absorption was attributed to a compound B and the absorption curve (220 to 320 mμ) of a suitable fraction was measured. The full range of analyses of fractions from a typical separation is shown

in the form of a histogram in figure 33 (p. 103).

16. Stability of Compound A

Samples of compound A prepared by ion-exchange chromatography were dissolved in water and in 2N hydrochloric acid. Chromatographic analysis of both solutions was carried out at various time intervals from 10 minutes to 10 days. The solution in hydrochloric acid (aliquots were neutralised with silver carbonate for chromatography) appeared to be virtually unchanged after two weeks, but decomposition in the aqueous solution was indicated by a gradual reduction in the intensity of the spot from compound A and a corresponding increase in intensity of spots due to mannose and glucosamine hydrochloride. Parts of the two solutions were refluxed at 100°C for 6 hours and the resulting dark brown solutions were examined chromatographically. In each case, compound A had almost completely disappeared and a considerable amount of glucosamine hydrochloride, but only very little mannose, was formed.

17. Analysis of Compound A

Hexosamine and hexose determinations were made on aliquots diluted from a fresh solution of compound A using the Elson-Morgan[171] and orcinol-sulphuric acid[177] reactions respectively. The absorption curves of the coloured solutions were obtained in each case and those of the respective

solutions were characteristic of glucosamine[171] and mannose. From the intensity of absorption at 530 mμ in the case of the Elson-Morgan reaction and 505 mμ in the case of the orcinol reaction, the glucosamine hydrochloride and mannose concentrations of the aliquots were obtained by interpolation from calibration curves constructed from standard solutions of the respective sugars.

The absorption curves furnished by mannose and compound A in the orcinol-sulphuric acid reaction are depicted in figure 37 (p. 116) and the calibration curve was linear over the range 0 to 100 μg. of mannose. These data for mannose are not reported in the literature cited which is concerned principally with glucose and galactose. The presence of glucosamine hydrochloride had no effect on the reaction of mannose with the orcinol-sulphuric acid reagent, and the Elson-Morgan reaction was unaffected by the presence of mannose.

The glucosamine hydrochloride and mannose concentrations of the diluted solution of compound A were 98 μg./ml. and 95 μg./ml. respectively. Thus the molar proportion of glucosamine hydrochloride to mannose in compound A is approximately unity. The infra-red absorption spectrum of compound A (potassium chloride disc) was obtained on a sample

of the appropriate fraction of eluate from ion-exchange chromatography .. The solution was neutralised with anhydrous potassium carbonate and freeze-dried. The spectrum contained no peak corresponding with the presence of a carbonyl group.

18. Hydrolysis of Compound A

Compound A was hydrolysed in both hot and cold water. Four batches of the material (about 300 mg.) from ion-exchange chromatography were bulked and dissolved in distilled water (30 ml.). After extracting with an equal volume of chloroform, the solution was filtered and refluxed for 12 hours and samples were periodically withdrawn for analysis by chromatography and ionophoresis. Optical rotation measurements were made at frequent intervals (using a 0.5 dm. tube) and samples were diluted for hexosamine determinations (Elson-Morgan reaction[171]) and for measurement of the absorption at 284 m μ. The ultraviolet absorption curve was obtained on samples before and after heating.

The ionophoresis and chromatography experiments showed that after 12 hours compound A was almost completely decomposed, and glucosamine hydrochloride and a small quantity of a spot attributable to mannose and traces of a somewhat slower moving compound (R_{GA} 1.44 and R_M 0.77, probably glucose) were formed. There was no measurable optical activity before or after heating,

and there was no significant change in hexosamine concentration. A gradual increase in the intensity of absorption at 284 mμ. occurred and the rate of this increase is shown in figure 38 (p. 118).

Initially the solution was clear and pale, but during heating it gradually darkened and became turbid with the eventual formation of a fine, dark brown precipitate. The supernatant solution was dark golden-brown in colour.

The same series of tests was periodically applied to a solution of compound A in cold distilled water. The solution was prepared by dissolving the product from two separations (about 150 mg.) in 20 ml. of water and extracting with an equal volume of chloroform, and the aqueous phase was filtered. There was no change in the ultraviolet absorption of the hexosamine concentration during four weeks. Chromatography and ionophoresis at intervals of 1, 3, 5, 10, 20, and 30 days showed a gradual change from the initial solution, which contained compound A with only small amounts of mannose and glucosamine hydrochloride, to the final solution which contained only a very small amount of compound A and quite large quantities of glucosamine hydrochloride and mannose. The spot on the chromatograms due to the latter was streaked, however, and extended over the regions normally occupied by mannose and glucose (R_M 1.0 to 0.75).

Duplicate chromatograms of the final solutions from the hot and cold water hydrolysis experiments were prepared and one was

stained with alkaline silver nitrate and one with haphthoresorcinol. The indications of those stained with the former reagent have been described and no spots were observed on either of the chromatograms with naphthoresorcinol. The products of the hydrolyses were separated by ion-exchange chromatography as described later (p. 164).

19. Hydrolysis of Compound B

A batch of compound B, separated by neutralising and freeze-drying the appropriate fractions from ion-exchange chromatography, was dissolved in water, and a second batch was dissolved in 2N hydrochloric acid and the volume of solution was about 20 ml. in each case. Aliquots of each solution were diluted for measurement of the ultraviolet absorption curve, and hexosamine determinations by the Elson-Morgan reaction[171] were carried out on the original solutions. The two solutions were refluxed for several hours and a slight increase in intensity of the initial pale yellow colour was observed. Samples were removed after 30 minutes and hourly after the first hour, and were diluted for ultraviolet absorption measurements (284 mμ.). Hexosamine determinations were made on the undiluted samples. In neither case was the formation of hexosamine observed and the absorption of the aqueous solution was practically unchanged after 10 hours heating. The absorption of the solution in 2N hydrochloric acid was greatly

decreased after an hour's heating and very soon was virtually removed. The absorption curves and the aqueous solution after 10 hours' heating were obtained and are illustrated, with those of the solutions before heating, in figure 36 (p. 114). The solution in hydrochloric acid required less dilution for absorption measurements after heating than before heating (dilution of 40, compared with 80).

The aqueous solution after heating was applied to the ion-exchange chromatography column and ultraviolet absorption was found in the same region of the fractions as previously.

20. Distribution Coefficients of Compound B

Compound B was eluted in fractions 85 to 91 when a batch of the reaction mixture was separated by ion-exchange chromatography (p. 152). The extinction coefficient (or optical density) at 284 mμ. (284) of fraction 86 was 1.80 and this fraction was used for distribution experiments with ether and chloroform.

An aliquot of fraction 86 (11 ml.) was shaken with an equal volume of chloroform and the two phases allowed to separate. The absorption of each phase at 284 mμ. was measured against the appropriate solvent blank (solvent blanks were prepared by shaking equal volumes of 0.3N hydrochloric acid and chloroform and separating the two phases). The aqueous phase was again extracted with an equal volume of chloroform and the absorption (284) of each phase was

again measured. This prooedure was repeated twice more and the distribution or partition coefficient of compound B was calculated as the ratio of the absorption of the two equilibrated phases, i.e. $K_c = \frac{\text{absorption of chloroform phase}}{\text{absorption of aqueous phase}}$. The average value obtained from four measurements (which agreed closely) was 0.31 at 16°C.

Ether was used instead of chloroform in a repeat of the experiment described above and an average value for K_e of 0.26 at 16°C. was obtained.

21. Separation of Compound B by Ether Extraction

A large batch of the mixture obtained by heating mannose and glucosamine hydrochloride on cellulose (p. 152) was eluted with water and freeze-dried. The freeze-dried material was dissolved in 0.3N hydrochloric acid (400 ml.) and the solution extracted with ether using a continuous extraction apparatus. After drying over anhydrous magnesium sulphate, the ether (about 400 ml.) was evaporated under reduced pressure to leave a small amount (about 0.5 ml.) of a mobile, golden-brown liquid which was stored in the refrigerator. In later experiments, the eluate fractions were neutralised with sodium bicarbonate prior to ether extraction.

22. Ionophoretic and Chromatographic Behaviour of Compound B

Compound B prepared by ether extraction as described above was examined by ionophoresis in acetate buffer, pH 5 and formate buffer, pH 2, using No. 1 filter paper. The ionophoretograms were examined under ultraviolet light, but apart from the small movement due to electroendosmosis there was no migration of the substance in either case.

A chromatogram of compound B extracted from the acid eluate fractions on No. 1 paper was developed for 16 hours. Examination with ultraviolet light showed that the material was not quite homogeneous, for two spots were found moving quite close to the solvent front. The slower moving spot was much less intense than the faster one and their respective R_F values were 0.78 and 0.83. A photograph taken by transmitted ultraviolet light is reproduced in Figure 34 (p .113) and identical spots were produced on staining with aniline hydrogen phthalate. Samples of compound B extracted from neutralised eluate fractions were chromatographically homogeneous and had R_F values of 0.77.

Absorption at 284 mμ. in the eluate fractions furnished by ion-exchange chromatography of compound B commenced with fraction 86 and extended over about 20 fractions. This is the position of elution of compound B from the reaction mixture (see figure 33, p. 103).

23. Preparation of HMF

HMF was prepared from sucrose using the method described by Haworth and Jones.[181] Since the precise conditions which they specified could not conveniently be used, 200 g. of sucrose and 1.4 g. of oxalic acid were dissolved in 600 ml. of distilled water and heated in an autoclave at 5 to 6 atmospheres and a temperature of 130°C. for $2\frac{1}{2}$ hours and then allowed to cool slowly. Extraction of HMF from the filtered reaction mixture was effected in the manner used by Haworth and Jones, and the crude, dark brown product was distilled under vacuum. A light yellow oil (yield, 12 g.) distilled at 115°C. and a pressure of 0.12 mm. of mercury, and a slightly darker coloured fraction was obtained from the temperature range 118 to 122°C.

24. Ion-exchange Chromatography of HMF, Furfural, and Salicylaldehyde

A small quantity of HMF (0.1 ml.) was dissolved in 0.3N hydrochloric acid (10 ml.) and applied to the preparative ion-exchange column (p. 136). The absorption (ϵ_{284}) of eluate fractions was measured and was first observed in fraction 85 and began to diminish after fraction 100.

A solution of furfuraldehyde (0.2 ml.) in 0.3N hydrochloric acid (14 ml.) was passed through the ion-exchange column and the eluate fractions were scanned by measuring the absorption at 285 mμ.

Fractions 107 to 130 exhibited this absorption.

Salicylaldehyde (0.1 ml.) was dissolved in 0.3N hydrochloric acid and a portion of the solution (20 ml.) was applied to the column. An aliquot of the solution was diluted for measurement of the absorption curve (dilution = 11) and a maximum was observed at 255 mμ. The absorption of eluate fractions at this wavelength was therefore measured, but not until fraction 300 was any absorption observed and the absorption extended over about 25 fractions. The position of elution of this compound was confirmed to within 1% in two repeat experiments.

The relative elution positions of these three compounds is shown in figure 44 (p. 133), in which the absorption curve of salicylaldehyde is inset.

25. Separation of the Hydrolysis Products from Compound A

The aqueous solution of compound A which had been heated for 12 hours was fractionated by ion-exchange chromatography and the fractions were scanned by means of the orcinol-sulphuric acid reaction (which was used exactly as described by Svennerholm[177]), the Elson-Morgan reaction,[171] and measurement of the absorption at 284mμ.

Compounds giving a positive reaction in the orcinol test were observed in fractions 23 to 33 and the absorption curve of red-brown solutions was obtained and is included in figure 37 (p. 116).

Absorption at 284 mμ. was found in fractions 86 to 99. A very slight red coloration was obtained in the Elson-Morgan reaction from fractions 100 to 102, and a strong reaction was produced by fractions 145 to 158.

Fractions 23 to 33 were bulked, neutralised with di-n-octylmethylamine, and freeze-dried, and the material was designated A1.

One of the ultraviolet absorbing fractions was retained for measurement of the ultraviolet absorption curve and determination of distribution coefficients between the solution and both chloroform and ether as described for compound B (p.160). The remainder were bulked and extracted with ether using a continuous liquid-liquid extraction apparatus, but in repeats of the experiment, fractions were neutralised before extraction, or the solution extracted before separation on the column. The ether solutions were dried over anhydrous magnesium sulphate before evaporation of the ether to leave a mobile, pale yellow oil and this was A2. The distribution coefficients obtained for A2 between the hydrochloric acid and chloroform or ether were 0.28 and 0.23.

The three fractions (100 to 102) which gave the weak Elson-Morgan reaction were discarded because this was almost certainly due to unhydrolysed compound A. Fractions 145 to 158 were bulked and evaporated to low volume under reduced pressure, water being

frequently added in order to distil the hydrochloric acid, and a brown solution of A3 was obtained.

These three substances (A1, A2 and A3) were analysed chromatographically, the chromatograms being stained with alkaline silver nitrate. A1 contained two components, one of which had the same R_f value as mannose and the other, which was present in very much smaller quantity, was somewhat slower moving having R_M of 0.78 which is equal to that of glucose. A2 was chromatographically homogeneous when extracted from a neutral solution, and moved very close to the solvent front. The stain with silver nitrate was in the same position as the spot observed with ultraviolet light and its R_f value (0.78) is the same as that of compound B. A specimen extracted from an acid solution contained two compounds having R_f values of 0.84 and 0.77. The R_M value (0.54) of A3, which was chromatographically homogeneous, is the value obtained for glucosamine hydrochloride.

The solution of compound A which had been allowed to hydrolyse by standing for four weeks at room temperature was also separated on the preparative ion-exchange column. The fractions were scanned with the orcinol-sulphuric acid and Elson-Morgan reactions and positive reactions were obtained in the former from fractions 24 to 30, and in the latter from fractions 97 to 102 (a very weak reaction) and fractions 148 to 157.

Fractions 24 to 30 were bulked, neutralised, and freeze-dried and constituted A4. The fractions giving the weak reaction with Elson-Morgan reagent (97 to 102) were discarded and the fractions 148 to 157 were bulked and evaporated as described above to furnish A5.

Chromatographic examination of A4 and A5 showed that the former probably contained at least two components for a streak was obtained in the positions of mannose and glucose (R_M 1.0 to 0.75), and the latter was homogeneous and had R_M value of 0.54 which is that of glucosamine hydrochloride.

26. Hydrolysis of A2

A part of A2 (about 0.05 ml.) was dissolved in water (20 ml.) and the solution divided into two parts. One was diluted with an equal volume of water and the other with an equal volume of 4N hydrochloric acid. These two solutions were then refluxed and samples taken periodically and diluted for measurement of the absorption at 284 mμ and for Elson-Morgan determinations (on undiluted samples). The observations were the same as those on the hydrolysis of compound B (p. 159), i.e. that the aqueous solution remained unchanged, but heating with acid rapidly reduced the absorption and changed the shape of the curve. In neither case was any pink

coloration observed in the Elson-Morgan reaction. The absorption curves for the acid solution before and after hydrolysis are shown in figure 36 (p. 114).

27. Characterisation of A1 and A4

The freeze-dried materials, A1 (about 40 g.) and A4 (about 25 mg.), were separately dissolved in water (2 ml.) and each solution was examined by ionophoresis in borate buffer, pH 10. The ionophoretograms were stained with alkaline silver nitrate and that of A1 showed the presence of mannose (M_G, 0.52), a small spot attributable to glucose (M_G 1.0) and traces of substances having M_G values of 0.68 and 0.81. That of A4 had two spots of similar intensity having M_G values of 0.55 and 1.0 and these were attributed to mannose and glucose respectively. The results of paper chromatograms of the materials confirm these findings (p. 166).

The components of the two solutions were then separated by paper chromatography on 4 sheets of No. 1 filter paper. Guide strips were cut from the paper and stained with alkaline silver nitrate. Bands corresponding with these stains (a heavy one attributable to mannose and a weak one attributable to glucose) were cut from the sheets and eluted by pulping in distilled water. The extracts were filtered and the pulps washed with water, and the filtrates and washings from the respective materials were bulked and freeze-dried. Methanol

extracts of the freeze-dried residues were clarified by centrifugation and evaporated to a volume of about 0.1 ml.

The concentrated methanol extracts were refluxed for 30 minutes with p-nitraniline reagent (1.5 ml.) and allowed to cool. Yellow crystals were obtained fromthe two suspected mannose fractions on trituration of the cold solutions, but nothing was obtained from the other (glucose) fractions, even after concentrating the solutions.

The crystals were collected after centrifuging and were recrystallised from methanol. The yield from A1 was about 3 mg. and its m.pt. was 208°C which was undepressed on mixing with an authentic specimen of mannose-p-nitranilide. An even lower yield was obtained from A4 and there was sufficient material only for m.pt. determination (208°C).

The p-nitraniline reagent was freshly prepared by dissolving 0.9 g. of p-nitraniline in 20 ml. of methanol containing 0.02 ml. of concentrated hydrochloric acid.

28. Characterisation of A2 and Compound B

The yellow liquids obtained by evaporation of the ether solutions of compound B, and of A2 were dissolved in water (5 ml. and 2 ml., respectively) and a drop of ethanol was added to clarify the solutions. To these solutions, an equal volume of a solution of 2:4-dinitrophenylhydrazine in 2N hydrochloric acid was added and an orange-red precipitate was

produced in each case. These were centrifuged and recrystallised from aqueous ethanol, and the crystals were recovered by centrifuging and were dried in a vacuum desiccator over calcium chloride.

In the same manner, the 2:4-dinitrophenylhydrazone of a specimen of HMF was prepared and its m.pt. was 201°C (see also ref. 174).

The melting points of the derivatives[iv] prepared from compound B and A2 were determined; that of the compound B derivative was 200 to 202°C and was undepressed on admixture with HMF derivative, and that of the A2 derivative was 198 to 201°C, but there was insufficient material for a mixed melting point determination.

29. Characterisation of A3 and A5

An ionophoretogram carrying A3, A5, and glucosamine hydrochloride as a reference was stained with ninhydrin and single spots were obtained from A3 and A5 in the same position as that from the reference compound.

The concentrated aqueous solution of A3 was cooled to about -10°C and cold acetone was added to the solution until a faint, permanent precipitate formed[167]. The solution was then allowed to stand overnight in the refrigerator and more acetone was added, as before. Gradual addition of acetone was continued in this manner during a week, until no further crystallisation occurred. The crystals (yield,

88 mg.) were filtered and dried and a sample was submitted for infra-red analysis (potassium chloride disc). The remainder was accurately weighed and dissolved in distilled water (5 ml.) and the optical rotation of the solution was measured, using a 0.5 dm. tube, initially and at hourly intervals until equilibrium was established. The specific rotation was initially +86° and the equilibrium value +69° (c, 1.69, water). The infra-red spectrum was identical with that of α-D-glucosamine hydrochloride[182].

After measurement of the optical rotation, the solution was freeze-dried and redissolved in about 0.5 ml. of water for preparation of the N-carbobenzoxy derivative (yield, 30 mg.) following the procedure described by Chargaff and Bovarnick[203]. The melting point of the compound, after recrystallisation from 30% v/v methanol in water, was 211 to 213°C (decomp.) and this was undepressed on admixture with an authentic specimen.

30. Ultraviolet Absorption Curve of Laevulinic Acid

A solution of laevulinic acid (0.2 g.) in 0.3N hydrochloric acid (30 ml.) was prepared and its ultraviolet absorption spectrum was measured. The absorption curve is shown in figure 36 C and there is a broad peak with a maximum at 265 mμ.

31. Colour Tests for Amadori Rearrangement Compounds

Solutions of compound A, both of the freeze-dried material obtained after ion-exchange chromatography and the acid fractions from the ion-exchange resin, were made slightly alkaline with sodium hydroxide solution and separately treated with solutions (0.01% w/v) of methylene blue and 2:6-dichlorophenolindophenol[183]. There was no reduction in the intensity of the blue colour in either case, even after standing for several hours and similarly, there was no reduction of the colour by an alkaline solution of glucose p-nitranilide (N-p-nitrophenyl-glucosylamine). In a control test using a 0.1% w/v solution of ascorbic acid the blue colour was removed instantly from an alkaline solution of 2:6-dichlorophenolindophenol.

The same two colour tests were applied to samples withdrawn from aqueous solutions of compound A after various periods (up to several hours) of heating, but again there was no removal of the blue colour. A solution containing compounds A and C did decolorise either reagent before, during, or after heating at 100°C. for 12 hours.

A solution containing compounds A and C did not produce any colour in the cold, or on boiling for one minute, with a solution of resorcinol in hydrochloric acid (the Seliwanoff test[204]). A pink colour was obtained in the cold with solutions of com-

pounds B and A2, and with a solution of compound A which had been boiled for six hours. Control tests with solutions of known sugars showed that mannose and glucosamine hydrochloride gave no colour in the cold or on boiling for one minute; a pink colour developed rapidly on heating with fructose, and HMF gave a strong pink colour in the cold.

32. Heat Treatment of Mannose on Cellulose

Cellulose powder (8 g.) was slurried in an aqueous solution of mannose (1 g. in 40 ml.) and freeze-dried. The suspension was heated in an oven at 100°C for 1½ hours and eluted with distilled water (50 ml.). An almost colourless solution was obtained and its ultraviolet absorption spectrum was measured without dilution, and is shown in figure 39 B (p. 119).

33. Heat Treatment of Glucosamine Hydrochloride on Cellulose

A suspension of cellulose powder (8 g.) in an aqueous solution of glucosamine hydrochloride (1 g. in 40 ml.) was freeze-dried and the material so obtained was heated in an oven at 100°C for 1½ hours. Elution of the cellulose with water (50 ml.) gave a pale yellow solution which was diluted with an equal volume of water before measurement of its ultraviolet absorption spectrum. The absorption curve is illustrated in figure 39 B (p. 119).

34. The Effect of Heat on Solutions of Mannose and Glucosamine Hydrochloride

Solutions of mannose (6 mg./ml.), of glucosamine hydrochloride (6 mg./ml.), and of a mixture of these two sugars (3 mg. each /ml.) were prepared in distilled water. The three solutions (20 ml.) were then refluxed on a boiling water bath for 16 hours and aliquots (1 ml.) of each solution were taken initially and after periods of $\frac{1}{2}$, 1, 2, 3, 4, 5, and 16 hours, and were diluted with water (5 ml.) for measurement of the absorption at 284 mμ. The initial and final solutions were analysed by paper chromatography, but no difference was discernable between the corresponding solutions when chromatograms were stained with alkaline silver nitrate.

The solution of mannose remained colourless after 16 hours heating and no significant absorption developed during this period, but the solutions containing glucosamine hydrochloride were golden-brown in colour and the gradual development in absorption at 284 mμ. which was observed is shown as a plot of the absorption (per mg. of glucosamine hydrochloride per ml.) against time in figure 38 (p 118). The absorption curve of the solution of the mixture of mannose and glucosamine hydrochloride was identical with that of the solution of glucosamine hydrochloride, and the curve of the latter, measured after 5 hours heating, is shown in figure 39 A (p.119).

35. Autoradiography

The reaction on cellulose was carried out using C^{14}-labelled mannose (generally labelled). The radio-active mannose (0.01 mC.), mannose (2 g.), and glucosamine hydrochloride (2 g.) were dissolved in water (200 ml.) and cellulose powder (50 g.) was added. The slurry was freeze-dried, heated in an oven at 100°C. for $1\frac{1}{2}$ hours and eluted with water. A reference sample of the eluate was stored in the refrigerator and the remainder was freeze-dried. The freeze-dried material was dissolved in water (40 ml.) and the solution was analysed by ionophoresis and chromatography (16 hours development) before acidifying with 2N hydrochloric acid (7 ml.) in readiness for separation of the components by ion-exchange chromatography. Ionophoresis showed that the solution contained compound A, and paper chromatography indicated that compound B was also present.

Compound B was detected in the eluate fractions 86 to 95 which were bulked, carefully neutralised with sodium bicarbonate and extracted with ether (continuous extraction), and the ether solution was dried over magnesium sulphate and evaporated to low bulk (about 1 ml.) under reduced pressure. The Elson-Morgan reaction showed compound A to be present in fractions 97 to 105 which were therefore bulked, neutralised by extraction with di-n-octylmethylamine (p. 137), and freeze-dried.

The two preparations were analysed chromatographically, and compound A also by ionophoresis in borate buffer. The chromatogram of compound B was developed for 16 hours and that of compound A for 60 hours. The solution of compound A was refluxed for 12 hours and again analysed by chromatography and ionophoresis, but this time the chromatogram was developed for only 16 hours.

The chromatograms of the reaction mixture, compound B, and the solution of compound A after heating were examined and photographed with ultra-violet light. Those of compound B and the solution of compound A after heating were left in the dark in close contact with a broad strip of X-ray film (held firmly between glass plates, and the positions of the film on the chromatogram was carefully marked) for five weeks. In the same way, an autoradiograph of the ionophoretogram of compound A was set up.

When the films were developed, a dark spot appeared on that which had been in contact with the chromatogram of compound B. This spot coincided with that observed with ultraviolet light, and figure 40 (p.121) shows the autoradiograph alongside an ultraviolet light photograph, the latter carrying a reference sample of HMF. The spots had R_F values of 0.78, which is identical with that of HMF. A similar spot, but very much weaker, was observed on the autoradiograph of the solution of compound A which

had been heated, and other very weak spots were observed which had moved much more slowly on the chromatogram (R_F about 0.2 to 0.3). Spots on the autoradiograph of the ionophoretogram of compound A corresponded with those obtained on staining the ionophoretogram with alkaline silver nitrate, and comprised a strong spot attributable to compound A, a much weaker spot having M_G value of 1.0 (possibly glucose), and a faint, streaked spot between the two.

36. Formation of Compound C

A preparative experiment on cellulose powder (p.152) was inadvertantly heated at 120°C. for 3 hours. The material was eluted in the usual way and an extremely dark, reddish-brown solution was obtained which was freeze-dried, dissolved in 0.3N hydrochloric acid (400 ml.), extracted with ether for 48 hours to remove compound B, and then separated by ion-exchange chromatography. A number of very yellow solutions was found amongst the earlier eluate fractions and several of the first 80 fractions showed some absorption at 284 mμ., giving three small broad peaks in the fraction range 15 to 80, but there was virtually no ultraviolet absorption in the fractions beyond 85. An unusually large amount of compound Awas indicated by the Elson-Morgan reaction[171] in fractions 96 to 108 which were bulked. Two further aliquots of the reaction mixture were separated on the ion-exchange column and their Elson-Morgan positive fractions (96 - 108) were

combined with those from the first separation, and the solution was neutralised (di-n-octylmethylamine, p. 137) and freeze-dried.

The freeze-dried product was dissolved in a little water (about 5 ml.) and examined by ionophoresis and chromatography. On staining with alkaline silver nitrate, the ionophoretogram indicated that compound A, and small quantities of glucosamine hydrochloride and mannose were present; the spot due to compound A was unusually elongated. The chromatogram however, showed that in addition to these compounds, there was a large proportion of a new compound moving more slowly than compound A. The R_{GA} and R_M values of the new compound, which was named compound C, were 0.28 and 0.14, respectively. A second ionophoretic separation of the mixture was effected, but instead of the normal 600 volts for 4 hours, a potential difference of 700 volts was applied for 6 hours, and the spot previously attributed to compound A was then resolved into two spots. The slower had M_{GA} value of 0.58, equal to that of compound A, and the faster had M_{GA} of 0.69 and was attributed to compound C.

37. Preparation and Properties of Compound C

A small aliquot of the aqueous solution containing compounds A and C was heated on a boiling water bath for 6 hours and then examined by prolonged ionophoresis. There was no change in the intensity of the spot due to compound C, but that from compound A had almost disappeared.

The whole of the aqueous solution (see above) was then refluxed for 12 hours and samples were periodically withdrawn for chromatography and ionophoresis. Both chromatograms and ionophoretograms, which are illustrated in figure 41, showed that compound A was gradually hydrolysed whilst compound C remained unchanged. The initial pale yellow colour of the solution changed to a deep golden-brown and was accompanied by a progressive increase in the intensity of absorption at 284 mμ but no brown precipitate was formed.

After heating, the solution was separated by ion-exchange chromatography (p. 152). Fractions 95 to 103 gave a positive Elson-Morgan reaction and were therefore bulked, neutralised (di-n-octylmethylamine, p. 137), and freeze-dried. The freeze-dried material was dissolved in water (10 ml.), washed with chloroform, and examined by ionophoresis and chromatography, both of which showed the preparation of compound C to be homogeneous. A small portion of the solution was used to examine the reaction of compound C (on paper chromatograms) with a number of spray reagents and the remainder was freeze-dried and weighed (yield, 120 mg.).

Strips were cut from a paper chromatogram carrying six separate spots of compound C and severally sprayed with ninhydrin, Elson-Morgan reagent, alkaline silver nitrate, aniline hydrogen phthalate, alkaline triphenyltetrazolium chloride, and naphtho-

resorcinol. Positive reactions were obtained with all but naphthoresorcinol. The optical rotation of an aqueous solution of the freeze-dried product was measured and the specific rotation was +55° (c, 3.56 in water). A sample of the freeze-dried compound C was submitted for infra-red analysis. The main features of the spectrum were a peak due to hydrochloric acid, two or possibly three peaks due to N-H deformations, and the absence of a peak attributable to a carbonyl group.

38. Hydrolysis and Analysis of Compound C

Compound C (6 mg.) was dissolved in 2N hydrochloric acid (0.35 ml.) and heated for 3 hours in a bath of boiling water. A drop of the solution was then removed with a Pasteur pipette and neutralised with silver carbonate for chromatographic analysis: the remainder was diluted with distilled water to about 20 ml. for colorimetric analyses.

The chromatogram, which carried reference spots of mannose, glucosamine hydrochloride, and compound C, was stained with alkaline silver nitrate and it showed that compound C had been completely hydrolysed to mannose and glucosamine hydrochloride (figure 42, p. 126).

Colorimetric determinations of mannose and glucosamine hydrochloride were made on the diluted hydrolysate using the orcinol-sulphuric acid[177] and Elson-Morgan[171] reactions,

respectively. Similar determinations were also made on the solution of compound C before hydrolysis. The ratio by weight of mannose to glucosamine hydrochloride in the solution was, for compound C before hydrolysis, 64:66, and for compound C after hydrolysis, 64:72. The absorption curve of the colour produced in the orcinol reaction by the hydrolysate of compound C was measured and it had the same shape as the curve obtained from solutions of mannose in this reaction (figure 37, p. 116).

The hydrolysate of compound C was colourless and had virtually no absorption at 284 mμ (ϵ = 0.061).

39. Crystallisation of Compound C

The freeze-dried sample of compound C (90 mg.) was dissolved in a little water (0.5 ml.) and methanol (0.2 ml.) was added. Acetone was added to this solution until a faint permanent precipitate formed and the solution was allowed to stand overnight in the refrigerator. Very little crystallisation occurred during this time. A large excess of acetone (about 10 ml.) was added to the solution, producing a white turbidity which, on standing at 5°C for several hours, settled as a fine, hard, white, crystalline precipitate. This was scraped from the sides of the vessel and the acetone suspension was centrifuged. The residue was washed with acetone and again centrifuged. The two supernatant solutions were bulked and a little water was

added to clarify the solution which was then evaporated on a water bath to remove the acetone, and finally freeze-dried.

The crystalline, acetone-washed residue was scraped from the centrifuge tube and was found to be very hygroscopic. It was dried under vacuum at 60°C and then transferred to a vacuum desiccator, and submitted for elemental analysis. (Found; C, 36.46; H, 6.53; N, 4.05. $C_{12}H_{24}O_{10}NCl$ requires C, 37.19; H, 6.21; N, 3.62).

40. Oxidation of Compound C with Alkaline Hypoiodite[187]

Compound C (59 mg.) was dissolved in water (5.0 ml.). An aliquot of the solution (1.0 ml.) was pipetted into a stoppered flask and 0.1N iodine solution (1.0 ml.) and sodium carbonate-sodium bicarbonate buffer solution, pH 10.6, (2.0 ml.), were added. The stopper of the flask was moistened with 10% (w/v) potassium iodide solution and the contents were then allowed to stand at room temperature for 2 hours. A control experiment was set up using only the reagents. The two solutions were then diluted with water (20 ml.), and 2N sulphuric acid solution (1.5 ml.) was added. The solutions were titrated with 0.01N sodium thiosulphate solution using "thyodene" indicator. The molecular weight (M) of compound C was calculated using the equation given below and a value of 232 was obtained.

$$M \equiv \frac{2 \times \text{weight of sugar (mg.) in aliquot}}{\text{mls. } 0.01\underline{N} \text{ iodine absorbed}}$$

A brown precipitate was formed when the iodine solution was added to compound C. This and over-oxidation[188] may have given a low molecular weight and the experiment was therefore repeated in duplicate using cold solutions (5°C, the volumes being measured at room temperature) and the brown precipitate was again produced on mixing the iodine and compound C solutions. The control and experiments were allowed to stand in the refrigerator for 24 hours before the addition of acid and titration of the iodine. The duplicate experiments were in close agreement and a molecular weight of 208 was obtained.

41. Oxidation of Compound C with Periodate[205]

A solution of compound C (19.0 mg.) in 0.02M sodium metaperiodate (100 ml.) was prepared and the uptake, or absorption, of periodate by the compound was determined iodometrically after periods of 30 minutes, 2 hours, and 4 hours. Aliquots (10 ml.) of the solution were withdrawn at these intervals and sodium bicarbonate (about 1 g.) was added. Standard arsenious oxide solution (0.02M, 25 ml.), potassium iodide (about 0.5 g.) and more sodium bicarbonate (about 0.5 g.) were then added, in that order, and after standing for 10 minutes, the solution was titrated with 0.1N iodine solution using "thyodene"

indicator. Control experiments using mannose (11.7 mg. in 100 ml. 0.02M periodate solution) and glucosamine hydrochloride (10.0 mg. in 100 ml. 0.02M periodate solution) were carried out simultaneously with the oxidation of compound C. The solutions were kept in the dark throughout the experiment and control titrations were made on the sodium metaperiodate solution itself with each titration of the sugar solutions. Titration of the arsenious oxide solution with the iodine solution was used to check the normality of the latter, and to facilitate calculation of the number of moles of periodate consumed by the sugars.

After four hours, the formic acid released in each of the sugar solutions was determined by titration of the remaining solution (70 ml.) with 0.1N sodium hydroxide solution. Before titration, ethylene glycol (1 ml.) was added to each solution to decompose the excess periodate. The solutions, together with a control of an equal volume of the periodate solution (to which 1 ml. of ethylene glycol had been added) were titrated with the standard alkali, not less than 10 minutes after the addition of the glycol, using methyl red as the indicator.

The solution of compound C was found to consume 4.2 moles of periodate with the production of 2.1 moles of formic acid, per mole of compound C. The controls with mannose and

glucosamine hydrochloride consumed 4.8 and 4.5 moles of periodate with the formation of 3.8 and 3.4 moles of formic acid, per mole of the sugar, respectively.

Appendix

The most important single technique employed in this work was ion-exchange chromatography. Much tedious analytical work was involved, particularly in the earlier experiments, in the scanning of eluate fractions for the detection of the required materials. This would be greatly reduced if an automatic recording device could be connected to the fraction cutter to indicate the presence of fractions containing material other than the eluant. An apparatus based on refractive index changes has been used for this purpose[206] but is expensive and its construction is rather complex. Two simpler possibilities were therefore examined in collaboration with Dr. J. R. Majer.

The first attempt was based on the fall in viscosity of the solution permeating the column as material was eluted, for this would result in an increase in the rate of flow through the column and the time taken to collect fractions of a given volume would consequently be reduced. Provided that a constant head of eluant was used a stepwise fall in the time required to collect fractions would then be observed as the various components of a mixture were eluted from the column. A microswitch was fitted to the fraction collector and signals were transmitted to a moving pen galvanometer each time the siphon

balance arm of the fraction collector fell causing a peak to be described on a recording chart which was moving at a uniform speed (0.5 cm. per minute). The distance between peaks was measured on the charts recorded throughout several chromatographic separations of the mannose-glucosamine hydrochloride reaction mixture, but a large random variation was observed and no significant changes of the form sought, were found.

More encouraging indications were obtained when a differential refractive index method was examined. If the column eluate was passed through a cell containing a prism, one face of which was covered by a very fine graticule, and a beam of light incident upon the graticule passed through the cell, the emergent beams would be deflected when the refractive index of the contents of the prism changed, i.e. when the eluant passing through the prism was replaced by an eluate fraction containing additional (or less) solute. The deflection, which would be very small since dilute solutions would be involved, could most conveniently be detected by placing a second graticule over a photocell so that it would be exactly out of phase with the image of the first graticule when the cell and prism both contained the eluant. A movement of the image consequent upon the changing of the eluate within the prism (the contents of the

outer cell remaining unchanged) would then sensitise the photo-cell and give an impulse to a recorder. In this way, the position of useful eluate fractions could be automatically and immediately indicated during elution of the column.

Preliminary experiments using a solution of glucosamine hydrochloride in 0.3N hydrochloric acid, and the acid solution itself, gave encouraging results. The hydrochloric acid solution was placed in a cell carrying two optically flat faces set at an angle of about 70° to each other and one of the faces was covered by a graticule. The image of the graticule was focussed on the cross wires of a travelling microscope and with a path distance from the graticule to the microscope of about 10 cm., a measurable deflection of the image was observed when the acid was replaced by the solution containing glucosamine hydrochloride. Work is proceeding on the development of an apparatus suitable for the routine scanning of column eluates.

REFERENCES

REFERENCES

1. Caspersson, T.; Naturwissenschaften, 23, 527, (1935); 24, 108 (1936).

2. Miescher, F.; Hoppe-Seyler's Med. chem. Unters. (1871), 441, 502.

3. Levene, P.A. and Bass, L.W.; "The Nucleic Acids". The Chemical Catalog Co., New York, (1931).

4. Levene, P.A. and Tipson, R.S.; J. Biol. Chem., 109, 623, (1935).

5. Hotchkiss, R.D.; "The Nucleic Acids" Vol. II p. 436. Academic Press Inc., New York (1955).

6. Zamenhof, S.; Prog. Biophys. biophys. Chem., 6, 85, (1956).

7. Avery, O.T., Macleod, C.M. and McCarty, M.; J. exp. Med., 79, 137, (1944).

8. Brown, D.M. and Todd, A.R.; "The Nucleic Acids" Vol. I p. 409. Academic Press Inc., New York (1955).

9. Watson, J.D. and Crick, F.H.C.; Nature, 171, 737, (1953).

10. Wilkins, M.H.F., Stokes, A.R. and Wilson, H.E.; Nature, 171, 738 (1953) and 172, 759 (1953).

11. Bang, I.; Z. physiol. Chem., 27, 463, (1899).

12. Mirsky, A.E. and Pollister, A.W.; Biol. Symp., 10, 247, (1943).

13. Idem, J. Gen. Physiol., 30, 117, (1946).

14. Greenstein, J.P.; Adv. Protein Chem., 1, 209, (1944) (p. 211).

15. Wyatt, G.R.; Biochem. J., 46, 581, (1951).

16. Levene, P.A. and Jacobs, W.A.; J. Biol. Chem., 12, 411 (1912).

17. Dekker, C.A., Michelson, A.M. and Todd, A.R.; J. Chem. Soc., (1953), 947.

18. Brown, D.M. and Todd, A.R.; J. Chem. Soc., (1952), 52.

19. Signer, R., Caspersson, T. and Hammarsten, E.; Nature, 141, 122 (1938).

20. Chargaff, E.; "The Nucleic Acids" Vol. I, p. 369. Academic Press Inc. New York (1955).

21. Hammarsten, E.; Biochem. Z., 144, 383, (1924).

22. Gulland, J.M., Jordan, D.O. and Taylor, H.F.W.; J. Chem. Soc., (1947), 1131.

23. Cosgrove, D.J. and Jordan, D.O.; J. Chem. Soc., (1949), 1413.

24. Signer, R. and Schwander, H.; Helv. Chim. Acta, 32, 853 (1949).

25. Lee, W.A. and Peacocke, A.R.; J. Chem. Soc., (1951), 3361.

26. Shack, J. and Thompsett, J.M.; J. Biol. Chem., 197, 17, (1952).

27. Laland, S.G., et.al.; Biochim. Biophys. Acta, 14, 356, (1954).

28. Lawley, P.D.; Biochim. Biophys. Acta, 21, 481, (1956)

29. Greenstein, J.P. and Jenrette, W.V.; Cold Spring Harbor Symp. Quant. Biol., 9, 236, (1941).

30. Frampton, V.L.; J. Biol. Chem., 129, 233, (1939).

31. Creeth, J.M., Gulland, J.M. and Jordan, D.O.; J. Chem. Soc., (1947), 1141.

32. Jordan, D.O.; "The Nucleic Acids" Vol. I. p. 480. Academic Press Inc. New York, (1955).

33. Idem, ibid., p. 470.

34. Rayleigh, Lord; Phil. Mag., 41, 447, (1871).

35. Debye, P.; J. appl. Phys., 15, 338, (1944).

36. Idem, J. Phys. Colloid Chem., 51, 18, (1947).

37. Zimm, B.H. and Doty, P.; J. Chem. Phys., 12, 203, (1944).

38. Atherton, E.; Hexag. Dig., 12, 3, (1952).

39. Brice, B.A., Halwer, M. and Speiser, R.; J. Optical Soc. America, 40, 768, (1950).

40. Zimm, B.H.; J. chem. Phys., 16, 1093, (1948); 16, 1099, (1948).

41. Doty, P. and Bunce, B.; J. A. C. S., 74, 5029, (1952).

42. Steiner, R.F.; Trans. Farad. Soc., 48, 1185, (1952).

43. Reichmann, M.E., et al.; J. A. C. S., 76, 3047, (1954).

44. Rowen, J.W.; Biochim. Biophys. Acta, 10, 391, (1953).

45. Crampton, C.F., Lipshitz, R. and Chargaff, E.; J. Biol. Chem., 211, 125, (1954).

46. Brown, G. L. and Watson, M.; Nature, 172, 339, (1953).

47. Pauling, L. and Corey, R.B.; Arch. Biochem. Biophys., 65, 164, (1956).

48. Watson, J.D. and Crick, F.H.C.; Nature, 171, 964, (1953).

49. Lee, W.A. and Peacocke, A.R.; J. Chem. Soc., (1951), 3374.

50. Cox, R.A. and Peacocke, A.R.; J. Chem. Soc., (1956), 2646.

51. Preston, B.N. and Peacocke, A.R.; in the press.

52. Cox, R.A., et al.; Nature, 176, 919, (1955).

53. Thomas, R.; Biochim. Biophys. Acta, 14, 231, (1954).

54. Toennies, G. and Bakay, B.; Nature, 176, 696, (1955).

55. Bernstein, M.H. and Mazia, D.; Biochim. Biophys. Acta, 10, 600, (1953).

56. Tristam, G.R.; Nature, 160, 637, (1947).

57. Davison, P.F., Conway, B.E. and Butler, J.A.V.; Prog. Biophys. biophys. Chem., 4, 159, (1954).

58. Butler, J.A.V., et al.; Biochim. Biophys. Acta, 13, 224 (1954).

59. Crampton, C.F., Moore, S. and Stein, W.H.; J. Biol. Chem., 215, 787, (1955).

60. Brunish, R., Fairley, D. and Luck, J.M.; Nature, 168, 82, (1951).

61. Luck, J.M., Cook, H.A., and Eldredge, N.; XIII Int. Congress P.A. Chem. Abstracts (1953), 87. (Ref.57)

62. Davison, P.F. and Butler, J.A.V.; Biochim. Biophys. Acta, 15, 439, (1954).

63. Stern, K.G. and Davis, S.; Fed. Proc. 5, 156, (1946).

64. Shooter, K.V., Davison, P.F., and Butler, J.A.V.; Biochim. Biophys. Acta, 13, 192, (1954).

65. Bang, I.; Betr. chem. Physiol. Pathol., 5, 319 (1904).

66. Miescher, F.; "Die histochemischen und physiologischen Arbeiten", Vol. II, p. 71 (Leipzig, 1897).

67. Stern, K.G.; Exp. Cell Res. Suppt., 1, 97, (1949).

68. Frick, G.; Biochim. Biophys. Acta, 8, 202, (1952).

69. Fleming, M. and Jordan, D.O.; Disc. Farad. Soc., 13, 217, (1953).

70. Butler, J.A.V., Davison, P.F. and James, D.W.F.; Proc. Biochem. Soc., 54, xxi, (1953).

71. Feughelman, M., et al.; Nature, 175, 834, (1955).

72. Marko, A.M. and Butler, G.C.; J. Biol. Chem., 190, 165 (1951).

73. Jones, A.S. and Marsh, G.E.; Biochim. Biophys. Acta., 14, 559, (1954).

74. Sevag, M.G., Lackman, D.B., and Smolens, J.; J. Biol. Chem., 124, 425, (1938).

75. Stedman, E. and Stedman, E.; Phil. Trans. Roy. Soc. B. 235, 565, (1951).

76. Sakaguchi, S.; J. Biochem., Tokyo, 5, 13, (1925).

77. Dische, Z.; "The Nucleic Acids", Vol. I, p. 287. Academic Press Inc., New York, 1955

78. Jones, A.S., Lee, W.A. and Peacocke, A.R.; J. Chem. Soc., (1951), 623.

79. Kolthoff, I.M. and Stenger, V.A.; Ind. Eng. Chem., Analyt. Ed. 7, 79, (1935).

80. Belcher, R. and Bhatty, M.K.; Mikrochim. Acta., 7-8, 1183 (1956).

81. Brand, E. and Kassel, B.; J. Biol. Chem., 145, 359 (1942).

82. Levy, A.L. and Chung, D.; Anal. Chem., 25, 396, (1953).

83. Vengreley, R., Knobloch, A., and Matsudaira, H.; Nature, 181, 343, (1958).

84. Rosenberg, H., Ennor, A.H., and Morrison, J.F.; Biochem. J., 63, 153, (1956).

85. Foster, A.B.; Chem. and Ind., (1952), 1050.

86. Britton, H.T.S.; "Hydrogen Ions" Vol. I., p. 359. Chapman and Hall Ltd., London, 1955

87. Idem, ibid.; p. 357.

88. Doty, P. and Zubay, G.; J.A.C.S., 78, 6207, (1956).

89. Crampton, C.F.; Lipshitz, R., and Chargaff, E.; J. Biol. Chem., 206, 499 (1954).

90. Chargaff, E. and Saidel, H.F.; J. Biol. Chem., 177, 417, (1949).

91. Chargaff, E. "The Nucleic Acids", Vol. I, p. 314. Academic Press Inc., New York, 1955.

92. Idem, ibid., p. 336.

93. Chargaff, E. and Lipshitz, R.; J.A.C.S., 75, 3658 (1953).

94. Durrum, E.L.; J.A.C.S., 72, 2943, (1950).

95. Tiselius, A.; Disc. Farad. Soc., 13, 29, (1953).

96. Luck, J.M., et al.; Arch. Biochim. Biophys., 65, 449 (1956).

97. Springall, H.D.; "The Structural Chemistry of Proteins", p. 223, Butterworths Scientific Publications, London, 1954.

98. Sanger, F.; Biochem. J., 39, 507, (1945).

99. Cox, R.A. and Peacocke, A.R.; J. Chem. Soc., (1956), 2499.

100. Idem, ibid.; (1957), 4724.

101. Idem; J. Polymer Sci., 23, 765 (1957).

102. Chargaff, E. and Zamenhof, S.; J. Biol. Chem., 173, 327 (1948).

103. Beaven, G.H., Holiday, E.R. and Johnson, E.A.; "The Nucleic Acids" Vol. I., p. 514. Academic Press Inc., New York 1955.

104. Shack, J., Jenkins, R.J. and Thompsett, J.M.; J. Biol. Chem., 203, 373, (1953).

105. Cavalieri, L.F.; J.A.C.S., 74, 1244, (1952).

106. Peacocke, A.R.; The Chemical Society, Special Publication No. 8, 139 (1957).

107. Blout, E.R. and Asadourian, A.; Biochim. Biophys. Acta, 13, 161 (1954).

108. Dewar, M.J.S.; "The Electronic Theory of Organic Chemistry" p. 316. Oxford University Press, 1949.

109. Putzeys, P. and Brosteaux, J.; Trans. Farad. Soc., 31, 1314 (1935).

110. Stacey, K.A.; "Light Scattering in Physical Chemistry", chap. 2 Butterworths Scientific Publications, London, 1956.

111. Doty, P. and Steiner, R.F.; J. Chem. Phys., 28, 1211, (1950).

112. Benoit, H.; J. Polymer Sci.; 11, 507, (1953).

113. Bawn, C.E.H.; "The Chemistry of High Polymers", p. 144. Butterworths Scientific Publications, London 1948.

114. Preston, B.N.; Ph.D. Thesis, University of Birmingham, 1957.

115. Doty, P. and Edsall, J.T.; Adv. Protein Chem., 6, 35, (1950).

116. Mie, G.; Ann. Physik., 25, 377 (1908).

117. Peacocke, A.R. and Preston, B.N.; Personal communication.

118. Hall, C.E.; J. Biophys. Biochem. Cytology, 2, 625, (1956).

119. Watkins, Winifred M.; Nature, 181, 117 (1958).

120. Zillikin, F., et al.; J. Biol. Chem., 208, 299, (1954).

121. Wallenfels, K.; Ann. Chem., 579, 113, (1952).

122. Alessandrini, A., et al; J. Biol. Chem., 220, 71 (1956).

123. Bayly, R.J., Bourne, E.J., and Stacey, M.; Nature, 168, 510, (1951).

124. Idem, ibid.; 169, 876, (1952).

125. Mitts, Eleanor and Hixon, R.M.; J.A.C.S., 66, 483 (1944).

126. Schiff, H.; Ann. Chem. Pharm., 140, 123, (1866)

127. Amadori, M.; Atti reale accad. nazl. Lincei [6], 2, 237, (1925).

128. Sokokin, B.; Ber., 19, 513, (1886).

129. Ellis, G.P. and Honeyman, J.; Adv. Carbohydrate Chem., 10, 95, (1955).

130. Barry, C.P. and Honeyman, J.; J. Chem. Soc., (1952), 4147.

131. Erickson, J.G.; J.A.C.S., 75, 2784, (1953).

132. Sjollema, B.; Rec. trav. chim., 18, 292, (1899).

133. Irvine, J.C., Thompson, R.T., and Garnett, C.S.; J. Chem. Soc., (1913), 238.

134. Hough, L., Jones, J.K.N., and Richards, E.L.; J. Chem. Soc., (1952), 3854.

135. Smith, L.I. and Anderson, R.H.; J. Org. Chem., 16, 963, (1951).

136. Barclay, J.L., Foster, A.B., and Overend, W.G.; J. Chem. Soc.,(1956), 476.

137. Perkow, W., Kuhner, P., and Weygand, F.; Chem. Ber., 84, 594, (1951).

138. Barclay, J.L., Foster, A.B., and Overend, W.G.; J. Chem. Soc.,(1955), 1541.

139. Anderson, R.H.; J. Org. Chem., 19, 1238, (1954).

140. Wolfrom, M.L., Scheutz, R.D., and Cavalieri, L.F.; J.A.C.S., 71, 3518, (1949).

141. Cavalieri, L.F. and Wolfrom, M.L.; ibid., 68, 2022, (1946).

142. Kuhn, R. and Dansi, H.; Ber., 69, 1745, (1936).

143. Kuhn, R. and Weygand, F.; ibid., 70, 769, (1937).

144. Weygand, F.; ibid., 73, 1259, (1940).

145. Hodge, J.E.; Adv. Carbohydrate Chem., 10, 169, (1955).

146. Hodge, J.E. and Rist, C.E.; J.A.C.S., 75, 316, (1953).

147. Isbell, H.S.; Ann. Rev. Biochem., 12, 206, (1943).

148. Hodge, J.E.; J. Agric. Food Chem., 1, 928, (1953).

149. Wolfrom, M.L., Cavalieri, L.F., and Cavalieri, Doris K.; J.A.C.S., 69, 2411, (1947).

150. Gottschalk, A.; Biochem. J., 52, 455, (1952).

151. Abrams, A., Lowy, P., and Borsook, H.; J.A.C.S., 77, 4794, (1955).

152. Anet, E.F.L.J.; Chem. and Ind., (1958), 1438.

153. Newth, F.H.; Adv. Carbohydrate Chem., 6, 83, (1951).

154. Petualy, F. and Kunssberg, U.; Monatsch., 83, 80, (1952).

155. Nodzu, R.; Bull. Chem. Soc. Japan, 10, 122, (1935).

156. Harley-Mason, J.; Lecture, University of Birmingham, 1958. [See also Biochem. J., 64, 27P., (1956)].

157. Baddiley, J. et al.; J. Chem. Soc., (1943), 383, and subsequent papers in the same series.

158. Kuhn, R.; Ber., 68, 1201, (1935).

159. Bergmann, M. and Zervas, L.; ibid., 65B, 1201, (1932).

160. Danehy, J.P. and Pigman, W.W.; Adv. Food Research, 3, 260, (1950).

161. Haq, S. and Whelan, W.J.; J. Chem. Soc., (1956), 4543.

162. Walker, P.G.; Biochem. J., 67, 22P, (1957).

163. Dent, C.E.; ibid, 41, 240, (1947).

164. Helfrich, B. and Kosche, W.; Ber. deutsch. chem. Ges., 59(1), 69, (1926).

165. Whistler, R.L. and Durso, F.; J.A.C.S., 72, 677, (1950).

166. Roseman, S. and Ludoweig, J.; J.A.C.S., 76, 301, (1954).

167. Gardell, S.; Methods of Biochem. Anal., 6, 289, (1958).

168. Moore, S. and Stein, W.H.; Cold Sp. Harb. Symp. quant. Biol. 14, 179, (1950).

169. Crumpton, M.J.; Biochem. J., 69, 25p, (1958), and 72, 479, (1959).

170. Elson, L.A. and Morgan, W.T.J.; ibid, 27, 1824, (1933).

171. Svennerholm, L.; Acta. Soc. Med. Upsal., 61, 287, (1956).

172. Yemm, E.W. and Cocking, E.C.; Analyst, 80, 209, (1955).

173. Lester-Smith, E. and Page, J.E.; J. Soc. Chem. Ind. (Transactions), 67, 48, (1948).

174. Wolfrom, M.L., Schuetz, R.D., and Cavalieri, L.F.; J.A.C.S., 70, 514, (1948).

175. Bethge, P.O.; Svensk Papperstidning, 61, 565, (1958).

176. Hewitt, L.F.; Biochem. J., 31, 360, (1937).

177. Svennerholm, L.; J. Neurochem., 1, 42, (1956).

178. Kiermayer, J.; Chem. Ztg., 19, 1003, (1895).

179. van Ekenstein, W.A. and Blanksma, J.J.; Chem. Weekblad., 7, 387 (1910).

180. Reichstein, T. and Zschokke, H.; Helv. Chim. Acta, 15, 251, (1932)

181. Haworth, W.N. and Jones, W.G.M.; J. Chem. Soc., (1944), 467.

182. Stephens, R.; Ph.D. Thesis, University of Birmingham, (1954).

183. Anet, E.F.L.J.; Austral. J. Chem., 10, 193, (1957).

184. Micheel, F. and Frowein, A.; Chem. Ber., 90, 1599, (1957) and 92, 304, (1959).

185. Cifonelli, J.A. and Dorfman, A.; J. Biol. Chem., 231, 11, (1958).

186. Bell, D.J.; "Modern Methods of Plant Analysis", (Paech, K. and Tracey, M.V.), Vol. II, p. 9. Springer-Verlag, Berlin, 1955.

187. Hirst, E.L., Hough, L., and Jones, J.K.N.; J. Chem. Soc., (1949), 929.

188. Jeanloz, R.W. and Forchielli, E.; J. Biol. Chem., 188, 361, (1951).

189. Editorial Report on Nomenclature; J. Chem. Soc., (1952), 5119.

190. Moggridge, R.C.G. and Neuberger, A.; ibid., (1938), 745.

191. Foster, A.B. and Stacey, M.; Adv. Carbohydrate Chem., 7, 271, (1952).

192. Dunlop, A.P. and Peters, F.N.; "The Furans", p. 378. Reinhold Publishing Corporation, New York. 1953.

193. Höpner, T.; Das Papier, 1, 102, (1947). Chem. Abs. 42, 3701, (1948).

194. Consden, R., Gordon, A.H., and Martin, A.J.P.; Biochem. J., 38, 224, (1944).

195. Partridge, S.M.; ibid, 42, 238, (1948).

196. Partridge, S.M.; Nature, 164, 443, (1949).

197. Trevelyan, W.E., Proctor, D.P., and Harrison, J.S.; ibid, 166, 444, (1950).

198. Wallenfels, K.; Naturwiss., 37, 491, (1950).

199. Gross, D.; Nature, 178, 29, (1956).

200. Britton, H.T.S.; "Hydrogen Ions" Vol. I, p.361. Chapman and Hall Ltd., London, 1955.

201. Idem. ibid, p. 355.

202. Idem. ibid, p. 362.

203. Chargaff, E. and Bovarnick, M.; J. Biol. Chem., 118, 421, (1937).

204. Pigman, W.W. and Goeppe, R.M. "Chemistry of the Carbohydrates", p. 135. Academic Press Inc., New York, 1948.

205. Dyer, J.R.; Methods of Biochem. Anal., 3, 111, (1954).

206. Trenner, N.R., Warren, C.W., and Jones, S.L.; Anal. Chem., 25, 1685, (1953).