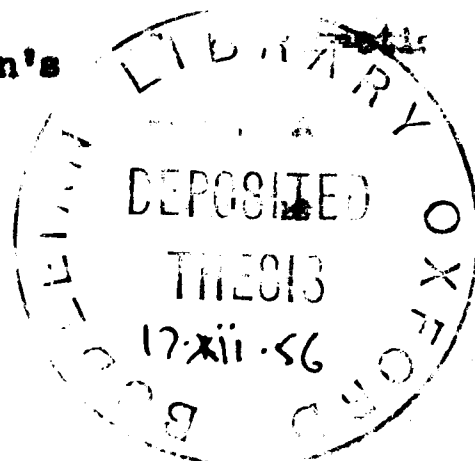


ABSTRACT OF THE THESIS

"BIOCHEMICAL STUDIES ON THE MATERIAL OF INTERCELLULAR SPACES"

submitted to the Board of Biological
Sciences for the degree of Doctor of
Philosophy by J. H. Fessler, The Queen's
College. Trinity Term, 1956.



The thesis is based on some physical investigations of the properties and organization of some components of inter-cellular space. The work was divided into two parts: (1) an investigation of the contribution of hyaluronic acid and collagen to the structure of Wharton's jelly found in human umbilical cord, and (2) an investigation of neutral salt soluble collagen.

(1) Investigation of the contribution of hyaluronic acid and collagen to the structure of Wharton's jelly found in human umbilical cord.

After a study of the swelling of the interstitial tissue of the cord in various media, and the effect of swelling on

the extractability of hyaluronic acid, a method was adopted for extracting the material. Care was taken to obtain fresh cords and to avoid degradation during extraction. The Wharton's jelly was dissected out, removing blood vessels and as far as possible the fibrous tissue associated with them. The Wharton's jelly was finely minced and the mince was treated with small volumes of solvent (water and buffer solutions) and, after being left to stand, was cleared by centrifugation. The conditions of extraction were studied and the stability of the extract on storage. A number of successive extractions were required to remove the bulk of the polysaccharide, and more severe treatment could yield still further extracts.

Sedimentation and viscosity studies on polysaccharide extracted by mild methods showed that its physicochemical properties resemble those of the hyaluronic acid of the synovial fluid of human knee joints fairly closely. An investigation of these synovial fluids was made in collaboration with Drs. J. E. Stanier and A. G. Ogston.

The structure of Wharton's jelly was investigated by visible and electron microscopy. This suggested that there is a network of unbranched collagen fibres, many of them very fine (down to 100A diameter), which lie freely in the jelly. Similar fibres were found in cords which had not reached full term. Material was examined after digestion with hyaluronidase

and with collagenase. Electron micrographs of hyaluronic acid suggested the possibility of a fibrous structure; but doubts were cast upon this interpretation by studies of the appearance of "pearl-strings" in random distributions of small glass spheres.

These findings led to a hypothesis, of the structure of intercellular spaces, and specifically of the parts played by water, hyaluronic acid, and collagen fibres. Theoretical considerations show that a combination of a fibrous network and an incompressible fluid can provide an important contribution to the mechanical strength of the tissue, provided that the incompressible fluid, water, is not permitted to move freely through the network. Experimentally it was shown that hyaluronic acids offer a considerable resistance to water moving through them, and calculation showed that a collagen network could in turn offer sufficient resistance to a solution of hyaluronic acid, to hinder the movement of water. Sedimentation and viscosity studies on native and degraded hyaluronic acids showed the importance of a high degree of polymerisation in producing this effect, and suggested a mode of action of hyaluronidase as it is employed in clinical practice.

Further investigations, by means of model systems, were obstructed both by the lack of a suitable fibre-forming system

and by the difficulty in determining the proportions of collagen and hyaluronic acid in tissues

(2) Investigation of neutral salt soluble collagen.

This was first undertaken as a simple investigation of the physico-chemical properties of neutral salt soluble collagen in solution. The study was hampered by the ease with which solutions formed precipitates under the conditions of investigation. An investigation of this precipitate formation was therefore undertaken, which showed that the precipitates are fibrous and have the typical cross striations of collagen as examined in the electron microscope. Above a certain temperature, determined by the other conditions such as pH, fibres were rapidly precipitated from the solution, and with care the process could be partly reversed by cooling.

By these means it was shown that the original material consists of three components: A, which reversibly precipitates as fibres on warming and re-dissolves on cooling; B, which does not precipitate; and C which precipitates but does not readily re-dissolve on cooling. Polarimetric studies showed a method of distinguishing between native material and gelatin formed from it. A method was devised for separating the three components of neutral salt soluble collagen.

Sedimentation, viscosity, flow birefringence and ultraviolet absorption studies were made on solutions of the unfractionated material, of the separate fractions, and of a control solution which had undergone the fractionation procedure but whose fractions had not been separated. These studies indicated that all the materials are similar and consist of elongated particles having a "molecular weight" of 5×10^5 . All the fractions except A were found to be heterogeneous. Electron microscope studies were also made on these solutions, and these suggested that Fraction C consists of only partly disintegrated fibres.

BIOCHEMICAL STUDIES ON THE MATERIAL OF
INTERCELLULAR SPACES

Thesis submitted to the Board of Biological Sciences
for the degree of Doctor of Philosophy

by

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The Queen's College,

Oxford

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PART A

GENERAL

INTRODUCTION

A.1.1.

Extracellular space may be divided into that space contained by the circulatory and lymphatic systems, and the remainder, called interstitial space. Two aspects of the composition of the interstitial space aroused my particular interest: (1) while the space contains 75% of the extracellular water of a mammal, only negligible amounts of free fluid can be found on dissecting a healthy animal, and (2) most of the mechanically structural parts of the body occur in interstitial space.

Fluid injected into the space, for example subcutaneously, is normally prevented from spreading rapidly. ²⁸ Duren-Reynolds discovered a factor called spreading factor, which allows both injected fluid to permeate tissues quickly, and previously held fluid to be moved from one part to another. Spreading factors found in streptococci, seminal fluid, snake venom and other sources, have the common enzymatic property of degrading the group of substances known as hyaluronic acids. These are polysaccharides, to be described in detail later, found in connective tissue in particular in synovial fluid and in umbilical cord. Various studies ^{25, 111} indicate that the hyaluronic acids to a large extent determine the rheological properties of interstitial medium in many locations.

Ogston & Stanier,^{89, 90, 91, 95} using bovine synovial fluid, isolated and studied hyaluronic acids in such a form that the rheological properties of the materials were unchanged as judged by their techniques. They extended their experiments to human and sheep joint fluids and I joined them in this venture.

Wharton's jelly, in umbilical cord, contains hyaluronic acids in the gel state, and I extended the methods to these substances. It seemed that water retained by the hyaluronic acids could act as a structural component of the animal body, resisting compression in certain localities, and a hypothesis is formulated to this effect. An integral part of this hypothesis is the interaction of hyaluronic acids with fibrous elements. Electron microscopy of Wharton's jelly showed fine collagen fibres, which had previously only been demonstrated after harsh treatment.

It was hoped that the hypothesis which had been formulated could be further investigated with the help of model experiments. It soon became clear, however, that on the one hand it would be difficult to draw up a representative analysis of Wharton's jelly so that a model of similar composition might be devised, and on the other hand there were no suitable microscopic fibres available to

incorporate into such a model.

Just at that time Professor A. Neuburger and Dr. D. S. Jackson invited me to make measurements on a "soluble collagen". The study of this material was taken up, partly for its own interest and partly to investigate its fibre-forming capacity. The properties of the collagen itself proved to be of such interest that the work on it was continued.

While it is certain that fibroblasts are involved in the production of collagen fibres the detailed steps of their formation are unknown. Certain soluble substances^{45, 49, 52, 55, 56, 86, 98, 99, 140} of the composition of collagen have been isolated, and it is fairly certain^{49, 56, 99} that at least some of these substances are precursors of collagen fibres.

The soluble collagen which I investigated was isolated by Dr. D. S. Jackson⁵⁵, using a modification of the procedure of Harkness et al.⁴⁹. At first an erratic formation of precipitates interfered with my studies, and while attempting to avoid this obstacle I found that the material spontaneously changed into fibres on warming (9° - 39°C). Further investigation enabled me to use this

property to separate the material into three fractions which were studied. The work also involved a number of subsidiary experiments, such as some detailed checks of the isolation procedure.

Hyaluronidase is used clinically, and an experiment was made in vitro, which indicates the mode of action of hyaluronidase when used in this fashion in vivo.

A.1.2. Arrangement of thesis:

The thesis is divided into three parts, separated by coloured cards:

- A - General: comprising this preface and a description of apparatus, such as ultracentrifuges and viscometers, and their use; the method of calculation of molecular parameters, and also a glossary of special terms and symbols used.
- B - Hyaluronic Acid: comprising a description of the work done on the structure of Wharton's jelly and the state of hyaluronic acid within it.
- C - Soluble Collagen: comprising a description of the work done on neutral salt soluble collagen.

Parts B and C have been arranged in chapters, each chapter comprising a group of related investigations. The varied and special experimental techniques used would have made a single experimental section rather unwieldy. Experimental techniques other than those described in Part A are therefore described in appropriate chapters, and if a technique applies to matters dealt with in several chapters, it is fully described once and suitable cross references are given.

Each chapter has an introduction, experimental part, discussion of results and conclusion.

Tables and figures are located at appropriate points in the text; photographs are placed at the end of parts B and C.

Three publications have been made in connection with this work and reprints are attached.

A.2.1.

GLOSSARY OF SPECIAL TERMS USED

1. Acid Soluble Collagen:- material extracted with buffer of acid pH. from mammalian tissue out of which all neutral salt soluble collagen has been removed; the material, after suitable purification belonging to the collagen group by analysis.
2. (1) Fraction A:- that part of a solution of neutral salt soluble collagen which, when warmed to 37°C, forms a gel which rapidly dissolves again on standing near 0°C.
 (2) Fraction B:- that part of a solution of neutral salt soluble collagen which does not form a gel on warming to 37°C.
 (3) Fraction C:- as fraction A, except that the gel does not dissolve rapidly on standing near 0°C.
3. Gel Ratio:- the function:

$$100 - \frac{\left(\begin{array}{l} \text{(refractive increment of supernatant solution.)} \\ \text{(refractive increment of solution from which} \\ \text{this supernatant was prepared)} \end{array} \right)}{\left(\begin{array}{l} \text{(refractive increment of supernatant solution.)} \\ \text{(refractive increment of solution from which} \\ \text{this supernatant was prepared)} \end{array} \right)} \times 100$$
4. Heat Precipitate (also Heat Gel):- the precipitate or gel formed by suitable solutions of collagen on increasing their temperature to above that of the cold room (0 - 8°C).

5. Neutral Salt Soluble Collagen:- material extracted from mammalian tissue in buffer of neutral pH, and after purification having an amino acid composition belonging to the collagen group. (Unless otherwise stated, or used as a generic term, the source was rabbit skin).
6. Precipitation Time:- the minimum time taken for a definite precipitate to appear on warming a solution of neutral salt soluble collagen to a given temperature, using the experimental conditions of (C.4.1.)
7. Ultra Filtrate Residue (U.F.R.):- the material remaining behind on a suitable glass filter, on filtering a solution containing hyaluronic acid. Unless otherwise stated the term is applied to the complex of hyaluronic acid and protein isolated by the method of Ogston & Stanier⁸⁹ from bovine synovial fluid (E.8.2.4.)

A.2.2

GLOSSARY OF SYMBOLS USED

- a** semi-axis of rotation of a spheroid particle
- b** equatorial semi-axis of a spheroid particle
- c** concentration, unless otherwise stated in g./100 ml.
- D** translational diffusion coefficient
- E** activation energy
- F** force
- f** molar frictional coefficient
- f'** molar frictional coefficient for spherical particles
having the same volume as a given particle
- f''** frictional coefficient, characteristic of a given
sedimenting system
- G** shear rate, velocity gradient
- H** radio active counts per hour emitted from a gel made
from unit quantity (10 mg.) of parent
- J** ellipticity, the axial ratio of a hydrated particle
(= axis of rotation/equatorial axis)
- k** the α factor in porous plug treatment
- K'** a constant ($= Ng'(\rho_p - \rho)$)
- k'** an apparent constant associated with the distribution
of radio active ^{32}P in certain extracts of neutral
salt soluble collagen
- k_v** rate constant of a reaction
- l** distance

M	molecular weight
N	Avogadro's number
P	pressure
R	gas constant
r	radius
S	surface area per unit volume of particles in porous plug treatment ($\text{cm}^2/\text{ml.}$)
s	sedimentation coefficient (general term)
s_{20}	sedimentation coefficient corrected to water as solvent at 20°C
s_0	s_{20} extrapolated to zero concentration
S	the Svedberg unit = 10^{13} sec.
T	absolute temperature
t	time; in particular "precipitation time"
V	volume
V'	effective specific volume of hydrated particle (ml./g.)
$\bar{V}$	partial specific volume of solute
v_1	relative velocity of solute and solvent in sedi- mentation
v_2	mean linear velocity of flow
$X(J)$	function of J used to obtain S, the surface area per unit volume of particles in porous plug treatment
λ	tortuosity factor in porous plug treatment

- $d(J, \chi)$ Scheraga function of ellipticity J and angle of isocline χ .
- $[\alpha]_D$ specific optical rotation for D lines of sodium light
- ϵ fraction of volume occupied by solvent
- $\epsilon(\lambda)$ molar absorption coefficient for light of given wavelength
- η viscosity, in particular absolute viscosity of solvent
- η_R relative viscosity
- $(\eta_R)_G$ relative viscosity at shear rate G , as measured in the Couette apparatus
- $(\eta_R)_0$ or η_0 relative viscosity extrapolated to zero shear rate
- $(\eta_R)_{50}$ or η_{50} relative viscosity at 50 sec^{-1}
- $(\eta_R)_{\text{Ostwald}}$ relative viscosity measured in a given Ostwald viscometer
- $[\eta]_c$ intrinsic viscosity, $\lim_{c \rightarrow 0}$ (specific viscosity)
- $\left[\frac{\eta_0 - 1}{c}\right]$ specific viscosity
- Θ rotary diffusion coefficient
- Θ_b rotary diffusion coefficient about equatorial axis b of a spheroid
- Θ' angle between the normal to the surface of a particle and the direction of sedimentation in porous plug treatment

$\xi(J)$	the Simha function relating ellipticity J to intrinsic viscosity
ρ	density of solvent
ρ_p	density of one sedimenting particle
ϕ	volume fraction of solute
ϕ'	volume of one sedimenting particle
χ	angle of isocline
$\psi(J)$	the Perrin function ($= f/f^1$)
ω	angular velocity (radians per sec.)

A.3.

APPENDIX

1. Ultracentrifuges
2. Couette viscometer
3. Ostwald viscometers
4. Flow birefringence apparatus
5. Refractometer
6. Ultraviolet spectrophotometer
7. Polarimeter

A.3.1. Ultracentrifuges

One preparative and two analytical machines were used. The preparative ultracentrifuge was a Spinco model L¹²⁷. This was used according to standard instructions, and in the text are quoted the particular rotors and speeds used together with the times of centrifuging at full speed.

The analytical ultracentrifuges used were: (1) the Svedberg oil turbine ultracentrifuge installed at Oxford and (2) a Spinco model E ultracentrifuge used at the National Institute for Medical Research, Mill Hill. The machines were standard models as described by Svedberg & Pedersen¹²⁹ and the Specialised Instrument Corporation, California, respectively, fitted with Philpott-Svensson¹⁰⁵ Schlieren optical systems; an absorption system employing an Ilford optical wedge was also used in some of the experiments with coloured solutions. The Oxford machine had been modified² in that: (1) to the diagonal edge of the Schlieren optical system had been added a bar of drawn glass and (2) the mean rotor speed was measured by means of a revolution counter driven by a phonic motor. The counting device was designed and constructed by Dr. R. Cecil, checked against a crystal clock and shown by Dr. R. L. Baldwin.

to give the mean rotor speed to within a maximum error of 0.1% under standard conditions of use.

The illuminating system of the Oxford machine was improved by replacing the existing slit source with a system of lenses, so that the arc of the lamp itself acted as the effective source.

Method of use: - the machines were operated according to standard procedure, the Oxford machine was run at 26 - 28°C, the Mill Hill one at 4 - 9°C. The magnification factor of the Spinco machine was determined (2.12 ± 0.005) and found to be uniform over the optical field used.

Sedimentation coefficients S_{20} , corrected to water as solvent at 20°C, were calculated by the method of Cecil & Ogston¹⁷. (The correction of ⁿBlachere & Pegibet⁹ for adiabatic stretching of the Spinco analytical rotor was applied).

A.3.2.1. Conette viscometer

The principle of the apparatus is that the fluid which is being investigated is sheared in the annular space between a cylindrical pot and a co-axial cylindrical core. The pot can be rotated at various known speeds; the central core is fitted with a galvanometer mirror and is suspended from a torsion wire. The torque transmitted from

the steadily rotating pot to the central core is a function of the viscosity of the fluid under the particular conditions. When a Newtonian fluid is used the torque transmitted to the central core is proportional to the product of the viscosity of the fluid and the mean velocity gradient. Shear rate is the rate of change of peripheral velocity with radial distance, measured through the fluid, and can be calculated from the dimensions of the apparatus and the speed of rotation used.

The apparatus of Ogston & Stanier⁹⁵ was used with the following modifications:

- (1) A velodyne motor was used to give a drive of "continuous, variable speed.
- (2) The semi-circular scale was replaced by a 12" perspex ruler marked to 0.1", ground opaque, and mounted at an arc of 75 cm. radius and at that distance from the torsion wire. (1" thereby equivalent to 1° of arc).
- (3) The torsion head was rebuilt by Mr. J. T. Cox to incorporate a circle divided in degrees to better than $\pm \frac{1}{20}$ h. degree, fitted with a 0.1° vernier and magnifying glass, and two sets of centring screws.

The viscometer was used as previously described^{32, 95}, except that to obtain a reading the torsion head was rotated until the mirror attached to the suspended bob threw the image of the galvanometer lamp onto some part of the scale. The reading of the scale was added to that of the torsion head.

Care was taken to ensure that the central bob hung co-axially with the centre of the pot and tests with a Newtonian fluid (glycerol-water mixture) then showed the response of the torsion wire to be linear to $\pm 1\%$ over 360° of twist. The apparatus was further checked by determining the absolute viscosity of water at 25°C , with the 10 ml. attachment the value obtained was within 0.8%, with the 3 ml. attachment within 1.1% of the international value.

Cooling water at $4 - 5^\circ\text{C}$ could be supplied to the water jacket of the viscometer from the refrigerated bath of a Hilger electrophoresis apparatus. The galvanometer mirror was treated with the proprietary demisting fluid "Fog-off".³⁵

A.3.2.2. Measurement viscosities and their definition

The apparatus was calibrated each day with the appropriate buffer the deflections being noted for five

or six set speeds. Proportionality between deflection and shear rate (speed of revolution) was checked and the measurements were repeated till the proportionality constant was independent of time, indicating that a steady temperature had been reached.

The solutions to be investigated had been brought to approximately the same temperature and degassed if necessary. Each was introduced into the clean and dry pot in turn, and at least 10 minutes were allowed for temperature equilibration before commencing measurements on a solution. Care was taken that no air bubbles were trapped. Speeds were measured with a stroboscope or by noting the time, of the order of 30 - 60 seconds, for a given number of revolutions; a stop watch reading to 0.1 second was used.

At a given shear rate G :

Angular deflection of torsion wire with fluid

$(\eta_r)_G$

Angular deflection of torsion wire with buffer = relative viscosity

$(1/\eta_r)_G$ was plotted against shear rate (G) and extrapolated to zero shear rate to give the limiting value $(1/\eta_r)_0$ or $1/\eta_0$.

Measurements were repeated for solutions of different concentration c (g/100 ml.) of solute. By means of a plot of the logarithm of the specific viscosity $[(\eta_0 - 1)/c]$

versus c the limiting value $\lim_{c \rightarrow 0} [(\eta_0 - 1)/c]$ was determined by extrapolation. This limiting value is called the intrinsic viscosity and is denoted by $[\eta]_c$.

For a Newtonian fluid η_r is independent of shear rate, but in the fluids investigated η_r varied with shear rate. A measure of this variation is the ratio $(\eta_r)_0/(\eta_r)_{50}$ or (abbreviated to (η_0/η_{50})), where η_{50} is the relative viscosity at a shear rate of 50 sec^{-1} . For comparing this variation for different solutes and at different concentrations a plot of $\log_{10} (\eta_0/\eta_{50})$ against $\log_{10}(\eta_0)$ was found convenient³⁴.

A.3.3. Ostwald viscometers

Standard Ostwald viscometers were used, immersed in a thermostat. The viscometers were cleaned with nitric acid-alcohol mixtures, water and re-distilled acetone. One viscometer had a flow time of 11.8 seconds for 2 ml. of water at 25°C , the other a flow time of 30.5 seconds for 1 ml. at 37°C .

A.3.4. Flow birefringence apparatus

The Couette apparatus could be modified for birefringence experiments, as described by Ogston & Stanier⁹⁵

The apparatus was redesigned so that the sample volume was reduced to 3 ml. and also so that higher shear rates (up to 1000 sec^{-1}) could be applied. Drive was by means of a continuously variable speed 'velodyne' motor, modified to rotate both clock-and anti clockwise. Two large Nicol prisms, kindly lent by Dr. H. Kuhn of the Clarendon Laboratory, were used in the optical system.

Method of use:- The sample was introduced through a special filler opening by means of a hypodermic syringe. For experiments at high speeds, the perspex ring forming the top of the cell was sealed at its outer edge by means of a little silicon 'housing putty'¹⁴, and this effectively prevented centrifugal leakage of fluid.

The motor was set to a given, calibrated, speed, the cross of isocline observed and the cross wire of the protractor superimposed upon it. The angle reading of the protractor was noted and the experiment repeated with the machine rotating in the opposite sense. Half the difference of the two protractor readings was taken as the angle of isocline. Duplicate values were taken and the experiment then repeated at a number of different speeds. The whole procedure was repeated for a set of solutions of different concentrations.

A.3.5. Refractometer

The differential refractometer of Cecil & Ogston¹⁸ was used. The volume of solution required for a measurement could be reduced to 0.25 ml. by occluding a space which was not traversed by light, with a small polythene plug.

Solutions were exhaustively dialysed against three changes of buffer before measurement. The temperature of solutions was brought to within 1°C of that of the refractometer before introducing them into it, and then 10 minutes were allowed for temperature equilibration.

The instrument is accurate to about 2×10^{-6} units¹⁸ of refractive index, corresponding to a concentration of protein of 10^{-3} g/100 ml.

A.3.6. Spectrophotometer

A Hilger "Uvispek" machine was used according to Standard procedure. A correction for scatter was made from a reading at which there was no apparent absorption, usually at 350 or 300 mμ.

For estimation of tyrosine the molar absorption coefficient ϵ (mol) of 1.34×10^3 at 275 mμ was used, as given by Neaven & Holiday⁶.

A.3.7. Polarimeter

A Hilger polarimeter reading to 0.01 degree of arc was used with sodium light. Solutions were placed in a Hilger jacketed polarimeter cell of 10 cm. path length. Thermostating water was circulated by means of a pump from a thermostat ($\pm 0.25^{\circ}\text{C}$) and the temperature was measured with a small thermometer dipping directly into the solution. The windows of the cell were treated with the demisting preparation "Fog-off".³⁵

Readings were taken by approaching the extinction point from alternate sides, two successive readings forming a pair of which the mean value was taken. At least six pairs of readings were taken to determine a single point. The angular scale was read to 0.005° . Within a set of six pairs the greatest difference between means was 0.02° - 0.06° , the angles being of the order of 0.40° . When equilibrium had been attained the mean values of sets of six pairs did not differ by more than $\pm 0.005^{\circ}$.

Buffer solutions acted as controls.

A.4.1.

BUFFERS

All buffers were made up from Analar reagents in glass distilled water, and were saturated with toluene. Merthiolate⁷⁷ was added to final dilution of 1:34,000 w/v to all solutions which had to be kept for several days.

A.4.2. Standard buffer for hyaluronic acid experiments⁸⁹

NaCl, 0.2M; Na_2HPO_4 , 0.0077M; KH_2PO_4 , 0.0023M;
pH 7.3.

A.4.3. Phosphate buffers for soluble collagen experiments

M/15 KH_2PO_4 and Na_2HPO_4 in standard combinations to give buffers of pH 7.0, 7.4, and other values as required.

A.4.4. Versene buffers for soluble collagen experiments

NaCl 0.2M, disodium versenate[disodium ethylene diamine tetraacetic acid], not Analar, but best available grade) 0.62 g per litre, adjusted with 2N NaOH to about pH 6.8; pH measured with glass electrode.

A.4.5. Citrate buffer for soluble collagen experiments

Citric acid M/15, NaCl 0.033M, NaOH 0.033M pH 3.7.

A.4.6. Acetate buffer for soluble collagen experiments

M/15 CH_3COOH and $\text{CH}_3\text{COO Na}$ in standard combinations,
usually to pH 3.6 as checked by glass electrode.¹⁴⁴

A.5.1.

CALCULATION OF MOLECULAR PARAMETERS

Various theories have been evolved for relating physical measurements made on solutions of macromolecules to parameters of certain simple models of these molecules. It is possible to decide which of various equivalent shapes fits in best with the measurements, and the shapes which can be handled mathematically are the spheroid, rod, and random coil. Three parameters are normally allotted to each model, and correspondingly three independent measurements are required to determine them.

The measurements made in this work were: (1) variation of sedimentation coefficient with concentration ($d(1/s)/dc$) (2) sedimentation coefficient extrapolated to zero concentration (S_0), (3) intrinsic viscosity $[\eta]_c$, (4) rotary diffusion coefficient (Θ). Translational diffusion coefficients are measured with some difficulty for the type of material used, and only one determination was made (by Dr. J. R. Hall).

The theories which could be applied were limited by the measurements which had been made. There were used: (a) Ogston's plug theory^{33, 92}, which required the previous measurements (1), (2) and (3); (b) a combination of the

Simha -Perrin 103, 125 treatment with the computations of Scheraga, Edsall & Gedd¹¹⁸ for flow birefringence, using (2), (3) and (4).

The model used in both treatments is a hydrated spheroid of effective specific volume V' and axial ratio J ($=$ axis of rotation/equatorial axis), containing a weight M of the material (in terms of molecular weight units).

A.5.2. Treatment (a): plug theory ^{33, 92.}

Particles sedimenting through solvent in the ultracentrifuge are analogous to a porous plug through which liquid moves. Sullivan & Hertel¹²⁸ assumed that a plug consisted of identical parallel cylindrical channels having a volume equal to that of the fluid and a surface area equal to the total surface of solid in the plug. They introduced a shape factor (k) and a tortuosity factor (ζ) to correct for the channels being neither cylindrical nor parallel and straight. Applying the treatment to sedimentation:

$$S = \frac{1}{\eta S^2} \cdot \frac{\zeta}{k} \cdot \frac{c(1-\bar{v}_p)}{100} \cdot \frac{\epsilon^3}{(1-\epsilon)^2} \cdot \frac{1}{\epsilon} \quad (1)$$

where: η = viscosity of solvent

S = surface area per unit volume of particles

c = concentration of solute (g/100 ml.)

- $\bar{V}$ = partial specific volume of solute
 ρ = density of solvent
 ϵ = fraction of volume occupied by the solvent.
 $\bar{z}$ = tortuosity factor equal to the mean value of $\sin^2 \theta'$, where θ' is the angle between the normal to the surface of the particle and the direction of sedimentation.

Since in sedimentation the sedimenting particles cause a reverse flow of solvent a final term $1/\epsilon$ is introduced. ϵ is given by $(1 - c\bar{V}/100)$ and if this is small compared with 1, equation (1) becomes:

$$\frac{1}{s} = \eta S^2 \cdot \frac{k}{\bar{z}} \cdot \frac{(\bar{V}')^2}{100(1 - \bar{V}\rho)} \cdot c \quad \text{--- (2)}$$

This equation will predict that when the concentration is zero the sedimentation coefficient is infinite, and to correct for this a term $1/s_0$ is added to the right hand side of (2). This will represent the resistance to sedimentation of particles when the solution is so dilute that they sediment singly, their interaction to form a plug having disappeared. Thus (2) becomes:

$$\frac{1}{s} = \eta S^2 \cdot \frac{k}{\bar{z}} \cdot \frac{(\bar{V}')^2}{100(1 - \bar{V}\rho)} \cdot c + \frac{1}{s_0} \quad \text{--- (3)}$$

To obtain M , V' and J : differentiating (3):-

$$\frac{d(1/S)}{dc} = \frac{\eta}{100(1-\bar{v}_p)} \cdot \frac{k}{f} \cdot S^2 (V')^2 \quad (4)$$

S can be expressed in terms of M , V' and J . Thus:-

$$S = \frac{3}{\sqrt{2}} \left[\frac{4\pi N}{3} \right]^{\frac{1}{3}} (M V')^{-\frac{1}{3}} \cdot X(J) \quad (5)$$

where: N = Avogadro's number

$X(J)$ = a function of J

Substituting (5) in (4):

$$\frac{d(1/S)}{dc} = \frac{1}{(1-\bar{v}_p)} \cdot \frac{9}{200} \cdot \left[\frac{4\pi N}{3} \right]^{\frac{2}{3}} \cdot \frac{k}{f} \cdot M^{-\frac{2}{3}} (V')^{\frac{4}{3}} \cdot X^2(J) \quad (6)$$

S_0 and $[\eta]_c$ are also obtained in terms of M , V' and J .

Thus for S_0 Svedberg and Pedersen¹²⁹ give:

$$S_0 = M(1-\bar{v}_p) / f \quad (7)$$

Where f is the molar frictional coefficient. The frictional coefficient for spherical particles of the same volume (f') is given by Stokes' law as:

$$f' = 6\pi\eta N \left(3MV' / 4\pi N \right)^{\frac{1}{3}} \quad (8)$$

and

$$f/f' = \psi(J) \quad (9)$$

where $\psi(J)$ is the Perrin function¹⁰³ of the ellipticity J .

Combining (7), (8), (9).

$$S_0 = \frac{1}{(1-\bar{v}_p)} \cdot \frac{M^{1/2} (4\pi N/z)^{1/2}}{6\pi\eta N(v')^{1/2} \psi(J)} \quad (10)$$

From Sinha's treatment of viscosity:

$$v' = 100 [\eta]_c \left| \xi(J) \right| \quad (11)$$

where $\xi(J)$ is a function of the ellipticity (J) and has been tabulated by Sinha¹²⁵.

Combining (6), (10) and (11):

$$\frac{\bar{z}}{k} \cdot \frac{s_0 [d(1/s)/dc]}{[\eta]_c} = \frac{x^2(J)}{\xi(J) \cdot \psi(J)} \quad (12)$$

Ogston⁹² has tabulated values of the right hand side of this equation for various values of J . If $d(1/s)/dc$, S_0 and $[\eta]_c$ are known and estimates can be given of $\bar{z}$ and k then J can be calculated. The choice of values for $\bar{z}$ and k presents some uncertainty. $\bar{z}$ has not been computed for spheroids, but since it has the same value $(2/3)$ for spheres

and for randomly orientated rods, Ogston suggests that the use of this value for spheroids is unlikely to produce serious error. There is more doubt in the choice of a value for k . Ogston used both 1, the value obtained in studies on porous plugs, and 1.8, a mean of values for flow through single channels. The value of 1.8 appeared to give more satisfactory results and this figure was used in the subsequent calculations.

Procedure:- J is found from equation (12) and then V' is obtained from equation (11) M is directly calculated from

$$M = 7.65 \cdot 10^6 \cdot (\eta)^{3/2} \cdot [\eta]_c^{1/2} \left\{ \frac{s_0}{(1-\bar{v}_p)} \right\} \cdot \psi^{3/2}(J) \cdot \xi^{-1/2}(J). \quad (13)$$

which is a combination of (11) and (10).

4.5.3. Treatment (b) - combination of Sinha, Perrin & Scheraga treatments

Equation (13) was derived by combining the treatments of Sinha and Perrin with the classical one of Sedwerg. The unknown in it is ellipticity J and in plug theory $(d(Vs)/dc)$ is used to determine it. In this treatment

the rotary diffusion coefficient Θ , determined from flow birefringence measurements, is used for the same purpose.

For a prolate ellipsoid in which $J > 5$, the following equation holds for Brownian rotary diffusion¹¹⁸.

$$\Theta = (3RT/16N\pi\eta a^3)(2\text{Lnc}(2J) - 1) \quad (14)$$

where $\Theta = \Theta_b$, the rotary diffusion coefficient about the equatorial axis b , a is the length of the semi-major axis, R is the Gas Constant and T the absolute temperature.

Simply from geometrical considerations for a prolate ellipsoid we also have:

$$a^3 = V' J^2 M (3/4\pi N) \quad (15)$$

Substituting (15) in (14):

$$\Theta = \frac{RT}{4\eta} \cdot \frac{(2\text{Lnc}(2J) - 1)}{J^2} \cdot \frac{1}{V' M} \quad (16)$$

From Scheraga et al's¹¹⁸ treatment of flow birefringence we also have:

$$\Theta = \frac{G}{2(J \cdot \chi)} \quad (17)$$

where $\alpha(J, \chi)$ is a function of the ellipticity J and the observed angle of isocline χ when the solution is sheared at a rate G (i.e. has a velocity gradient G). Scheraga et al.¹¹⁸ tabulate $\alpha(J, \chi)$ in terms of J and χ .

If there exists appreciable particle-particle interaction χ is influenced by concentration. This occurs with neutral salt soluble collagen and therefore χ values were obtained at a series of concentrations and extrapolated to zero concentration. The extrapolated values were used in equation (17).

¹⁰³
Perrin and Sinha¹²⁵ give tabulated values of $\psi(J)$ and $\xi(J)$ in terms of J .

Procedure:- From $[\eta]_c$ and S_0 , a set of values of M are calculated corresponding to a range of values of J by means of equation (13). Similarly V' is determined for a set of values of J by means of equation (11). Employing equation (16) a set of values of Θ can now be calculated for different values of J . A corresponding set of values of Θ is calculated from the birefringence measurements by use of (17). The best value of Θ is obtained by comparison, and hence J , V' and M .

PART IILYANTRONIC IN

B.1. GENERAL INTRODUCTION - TO UMBILICAL CORD, YAMINAL
and HYALURONIC ACIDS

B.1.1. Umbilical Cord

The umbilical cord originates as the mesodermal component of the amniotic stalk, which separates from this to form the body stalk. The body stalk is at first attached to the caudal end of the embryonic plate, but reaches a more central position as the caudal end grows. As the amnion, filled with amniotic fluid, enlarges to obliterate the extra-embryonic coelom, it comes to cover the body stalk on all sides, with remnants of the yolk-sac apparatus trapped between them. The body stalk lengthens, partly by addition of tissue from the body wall of the embryo, and as umbilical cord connects this with the chorion and its villi which are forming a placenta. Vascular spaces develop early to form the umbilical vessels, consisting of a pair of arteries and a vein, a fellow vein degenerating at an early stage. The vessels are spirally twisted about each other and lie in what is known as Wharton's jelly. This consists of protein and various polysaccharides. One of these contains sulphur, but the principal component is the substance

hyaluronic acid⁸⁰, shown by Meyer⁸⁵ to contain, at least principally, N-acetyl-2 amino D glucose and glucuronic acid, in the proportion of 1:1.

B.1.2. Synovial fluid

Synovial fluid is a pale yellow, clear viscous fluid containing protein, hyaluronic acid, and a small number of cells⁴. All the plasma proteins except fibrinogen are represented, and the electrolyte composition may be regarded as a plasma dialysate after allowing for the Donnan Equilibrium³.

The volume and viscous properties of synovial fluids vary widely. 0.4 - 1.1 ml.^{4, 114}, may be obtained from the human knee joint and relative viscosities from 2 to 1,400 have been reported^{109, 114}.

B.1.3. Hyaluronic Acid

These viscous properties of synovial fluid are due almost entirely to hyaluronic acid⁸¹. This has also been isolated from vitreous^{36, 78, 79} and aqueous humor, skin^{82, 88} especially in myxodema^{100, 137}, cock's comb¹², granulation tissue, tumours^{83, 107} and bacteria such as streptococci⁶⁵.

The isolation of purified hyaluronic acid has been done using precipitation as mucin by alcohol or acetone^{10, 48, 70, 71, 75, 81, 84, 123} by cold acetone¹¹³, and by fractional precipitation with ammonium sulphate in the presence of pyridine or alcohol^{1, 47, 58}. Protein has been removed by the method of Savag¹²⁴ or with proteolytic enzymes.

In the course of these isolations there occurs a decrease in viscosity⁸⁹ and a possible loss of polysaccharide fractions⁴⁷. The chemical composition of the products is however reasonably constant, with a very low sulphur content, total N 3.3 - 4.5%, acetyl 11.7 - 12.7 and ash 7.7.^{12, 84, 78} Figures for uronic acid and hexosamine contents have varied with the preparation, but in the best preparations correspond to a polymer composed of equal amounts of glucuronic acid and acetyl glucosamine.

Acidic polysaccharides, such as hyaluronic acid and chondroitin sulphuric acid, form insoluble salts, mucin clots, when mixed with protein at pH 4 - 5. It has been repeatedly shown that there is no soluble, physiological entity corresponding to mucins^{8, 24, 51, 80, 89}. The formation of mucin clots can be used for purification, detection and estimation.

Ox hyaluronic acid has been prepared by ultra-filtration, when its viscous properties remain unaltered^{89, 91}. It is then associated with some 25% of protein, which cannot be removed without altering the viscous behaviour, and the preparation has been called ultrafiltrate residue (U.F.R.), with an analysis of: acetyl glucosamine 30.6, glucuronic acid 35.9% and ash 6.8.

Hyaluronic acid is readily altered from its native state and this, together with the rather non-ideal solutions which it gives, may to some extent explain the disagreement found over the molecular dimensions and physico-chemical constants. (That is: physico-chemical constants are open to the uncertainties incurred in extrapolating measurement made on non-ideal solutions at finite concentrations to infinite dilution where ideal conditions are presumed to exist.)

B.1.4. Molecular dimensions of the hyaluronic acids

Blix & Snellman¹⁰ from sedimentation, viscosity and double refraction of flow estimated a molecular weight 2.5×10^5 and a particle length of 4000A. Preparations treated with alkali gave a reduced molecular weight.

Material from human umbilical cord was allocated a molecular weight of $4-7 \times 10^5$ by osmotic pressure studies²¹ and 5.2×10^5 by sedimentation and diffusion⁶², by Jensen et al. Light scattering measurements by Laurent & Cergoley⁶⁸ gave a molecular weight of $2.8-4.3 \times 10^6$ and a root mean square radius of 2,200A and they concluded that while no model fitted the observations, that of a partially rigid coil was the most likely. Blumberg & Oster¹¹ obtained a molecular weight of 8×10^6 , also from light scattering measurements, and decided on a highly hydrated spherical molecule of 2,100A diameter containing 0.27% by weight of anhydrous material. (Blumberg & Oster's measurements give a molecular weight of 4.8×10^6 when recalculated as in (B.7.4.)). Jensen et al.'s values of 5×10^5 for the molecular weight may be in error because of both their drastic method of preparation and the uncertainties attached to the osmotic pressure and diffusion measurements.

Investigators seem to agree that hyaluronic acid obtained from ox vitreous humour is of lower molecular weight than that obtained from umbilical cord. This finding of Blix & Snellman¹⁰ has recently been confirmed by Laurent⁶⁷ and by Rowen et al.¹¹⁶. Laurent found a

molecular weight of $3.4-5 \times 10^5$ by light scattering, for material containing only 3% of protein, and while it was obtained by a mild method fractionation could readily have occurred, the smaller particles being measured. The light scattering envelope indicated that the material was not homogeneous and Laurent suggests a random coil model which probably lengthens and is of increased hydration as the ionic strength is decreased. Viscosity measurements definitely contra-indicated a stiff, rod molecule. Rowen et al.¹¹⁶ found a molecular weight of 1.27×10^6 and again a light scattering envelope which does not fit any model. Their own graphs indicate best agreement with a coil of radius of gyration 1,200A at high ionic strength, yet determined to fit the results into an elongated model, they assume a rod shape and obtain a length of 2,900A. This becomes 6,700A at zero ionic strength, and there is evidence that the particle changes shape with decreasing ionic strength. This was supported by streaming birefringence measurements which give approximate lengths of 5,000A in water and 2,500A at ionic strength 0.3. These measurements also showed the material to be polydisperse, yet by applying an equation of Peterlin¹⁰⁴ they found a particle half way between a stiff rod and a flexible molecule.

Ogston & Stanier³⁴, 89, 90, 91 working on their preparation from synovial fluid gave a molecular weight of 10^6 from sedimentation and diffusion, which was later modified to be of the order of 10^7 , the particle having an axial ratio of about 10 and the large effective hydrodynamic volume of 500. Other estimates⁵⁸ of molecular weights have been of "several millions".

Jensen⁶⁰ has assumed that hyaluronic acids from such widely different sources as shark endolymph and human umbilical cord are members of one group of linear polymers and has, not very reassuringly, supported this by applying an extended Staudinger relationship for viscosity and molecular weight.

Different investigators have used material from different sources, purified it by different methods, applied different techniques, used different methods of calculation even for the same technique^{11, 116} not to speak of individual preferences for various assumptions made. Differences in results are thus natural, yet most results fit into the not very well defined model of a chain polymer which may or may not be branched, and takes up the formation of a partially rigid coil, entraining much solvent and behaving as a polyelectrolyte so that extension occurs at lower ionic strengths. The particle weight is in the range $0.5-5.0 \times 10^6$ and

probably all preparations are far from monodisperse, not to speak of contamination with protein or chondroitin-sulphuric acids.

Such macromolecules ought to be visible under the electron microscope. Gross⁴⁰ showed how the appearance could vary with method of preparation from globules of various sizes to "rivulets" when the solution had been applied to a tilted support. Recently Rowen et al¹¹⁶ obtained similar rivulets but identified them as fibres, though with increasing dilution they obtained more small globules. They did also find some more definite and large fibrous aggregates, yet they imply that these are the product of an aggregation which occurs when they allowed their solutions to stand. After mild action of hyaluronidase only separate globules were apparent. Jensen & Carlsen⁶¹ obtained an aggregate which they identify with fibres, though in the published photographs these just appear as small, yet definite globules.

There does then appear to be some tendency to linear aggregation. The dehydrated molecule on the electron microscope grid cannot be expected to resemble the model deduced to exist in solution.

B.2. INTRODUCTION TO THE STRUCTURE OF WHARTON'S JELLY and the
STATE OF HYALURONIC ACID IN IT

The principal aim was to find the state, and possibly some of the functions of hyaluronic acid in Wharton's jelly.

The physical properties of this hyaluronic acid were investigated. It is agreed by most workers in this field^{113, 115} that many of the physical properties of hyaluronic acids are so different from those of most substances found in association with them, that the corresponding physical properties of a mixture are chiefly determined by the hyaluronic acid component; for example, the viscosity of synovial fluid is practically entirely determined by the hyaluronic acid contained in it^{3, 89, 91} and conversely the sedimentation coefficient of hyaluronic acid in synovial fluid is only little affected by small amounts of protein⁶³.

Hyaluronic acids are known to be readily degraded during their isolation from mixtures as judged by such physical methods as viscous behaviour^{89, 91, 94}. No attempts were therefore made at chemical purifications, and instead care was taken not to damage the material during extraction.

First the microscopic nature of Wharton's jelly was briefly investigated. The optical microscope showed in which regions of umbilical cord fibrous tissue was least likely to be encountered and these regions were then used in the subsequent studies. The electron microscope showed that even in these regions there were numerous, fine collagen fibres and that these were present during the functional period of umbilical cord and not only at the end of gestation.

A mild method of extraction of hyaluronic acids from umbilical cord was then developed, and some physical properties of the extract were studied. By comparison with similar measurements made on human synovial fluid, it was concluded that the hyaluronic acids from the two sources are closely similar.

Various other studies on hyaluronic acids were made, in particular, the swelling behaviour of umbilical cords, the retention of hyaluronic acids on sintered glass filters and the action of the enzyme hyaluronidase.

A hypothesis of the state of hyaluronic acid in connective tissue in general and in Wharton's jelly in particular, was then formulated. The state of hyaluronic acids in umbilical cord was inferred to be that of polymers

entraining fluid and itself entangled in a fibrous network.

A probable function of the material is to reduce kinking and compression of the umbilical blood vessels in vivo.

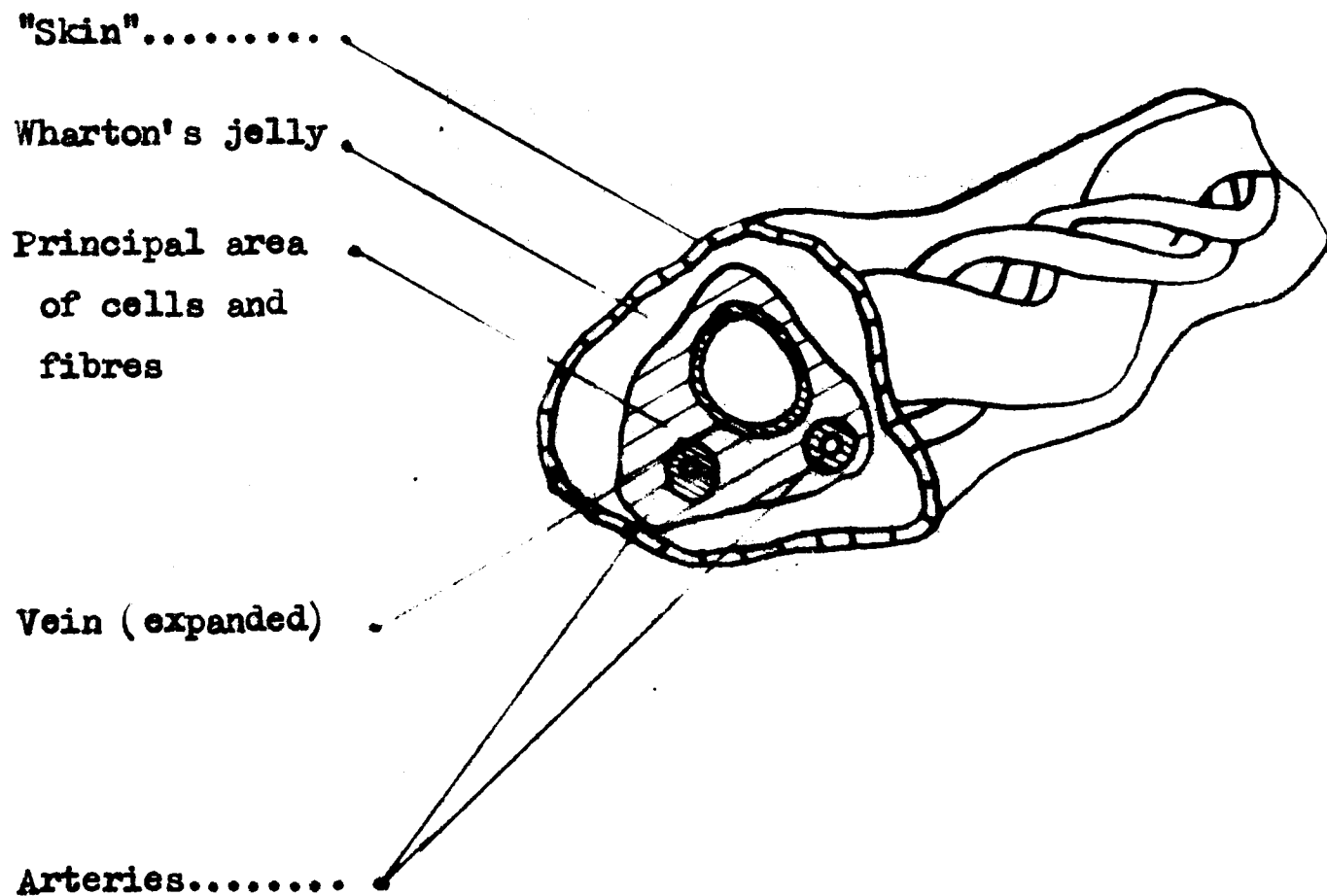
From this a mechanical function of hyaluronic acid-water systems in connective tissue is postulated.

The description is divided into chapters in the following order:-

- a) Histological investigations
- b) Swelling studies on whole umbilical cords
- c) The extraction of hyaluronic acids from umbilical cord.
- d) Physical measurements on extracts of umbilical cords.
- e) Physical measurements on human synovial fluids
- f) The action of hyaluronidase as it is employed in clinical practice
- g) Various experiments with hyaluronic acids, coupled with a theoretical treatment of ultra-filtration
- h) General discussion.

Fig. 1.

Diagram of the structure of umbilical cord.



[The lumen of the arteries are relatively larger during
life; the walls of the arteries contract during birth.]

B.3.

HISTOLOGICAL INVESTIGATIONS

B.3.1.

General

Anatomically human umbilical cord consists of a large vein, and sometimes traces of a second, small degenerate vein, and of two small arterics. The vessels are enmeshed by fibrous tissue which is permeated by a mucoid mass of varying consistency called Wharton's jelly. The whole is enclosed in a few layers of closely packed cells, which form a kind of thin but quite tough "skin".

Dissection and brief histological investigation

(B.3.3.) showed that there is much more cellular and fibrous material associated with the blood vessels than is commonly stated¹¹⁷, and that this material spreads outwards in decreasing density, towards the periphery of the cord. The zone of fibrous material seemed to have no clear border and electron microscope studies indicated that there was no region of Wharton's jelly quite free from fibres.

B.3.2. Umbilical cords

Human umbilical cords were collected from the Maternity Department, Radcliffe Infirmary, Oxford and some from the sister institution at Cambridge. Inevitably cords were soaked in various, diluted biological fluids during parturition. Such exposure varied widely and

depended on the nursing staff. Within one hour the cords were severed from the placenta and after any extraneous blood had been removed they were stored at 0°C and used within 18 hours.

Cords which had been soaked excessively in blood, water, etc. could be distinguished by their swollen and often blood-stained appearance, and were discarded.

B.3.3. Investigations with the optical microscope

Normal and water swollen cords were used and either observed directly in thick spread preparations or fixed in formal-Zenker fluid and then stained with haematoxyline and eosin, and with toluidine blue. My own investigations were kindly confirmed and extended by Mr. E. H. Leach, Physiological Laboratory, Oxford.

Findings:- the "jelly" was inhomogeneous, particularly so around the blood vessels. Mast cells and fibroblasts were present, and some cells were disintegrated, particularly in the swollen cords. The vessels themselves showed a normal, strong metachromasia, and the general space did so also, but more weakly. The general metachromasia was reduced on swelling the cords, but not so the metachromasia of the vessels.

Spaces and fibre-like precipitates were present throughout, but it was difficult to distinguish true fibres from artefacts produced by fixation.

Maximow & Bloom⁷² and others discuss apparent lines and connections stretching through Wharton's jelly, and to a certain extent between fibroblasts. Standard methods of fixation and staining give, however, only poor sections of a general, metachromatically stained expanse, that alternates between obscuring and suggesting structures. Electron microscopy was therefore tried as a different approach.

B.3.4. Electron microscopy

B.3.4.1. Method

Regions of umbilical cord were selected for all experiments which were distant from the blood vessels and the fibrous regions associated with them.

Standard copper grids for electron microscopy were covered with a thin nitro-cellulose film.

A cube of about 3 mm side was taken from the umbilical cord, chopped with a razor blade, mildly disintegrated, in a glass homogeniser, and after fixing in 1% formal-saline (0.9% NaCl) samples were placed on grids. Alternately the grids were simply touched against the side of an incision

in a cord, the sample allowed to dry, and then fixed in 1% phosphotungstic acid for 1 minute.

After several washes with water many grids were shadowed in vacuo with gold-palladium at an angle of 30°.

For the examination of solutions of hyaluronic acid a small drop was placed on a grid and after most of the fluid had been removed with filter paper the material was fixed with phosphotungstate or formal saline, washed and shadowed as before.

B.3.4.2. Findings

Electron microscopy was used in two ways: (a) to examine the composition of Wharton's jelly and (b) to study the appearance of hyaluronic acid.

B.3.4.2a Composition of Wharton's Jelly

Many fibres were found and they were embedded in a general matrix. In (Phot.1) are shown three views of small pieces of Wharton's jelly which had been mildly homogenised (B.3.4.1.). The repeat units visible on many of the fibres indicate them to be collagen fibres. This was supported by experiments in which Wharton's jelly, placed on grids, was digested with collagenase ²³ plus hyaluronidase at 37°C - nothing remained after 12 hours.

Similar fibres were found on just touching an electron microscope grid against the side of an incision in an umbilical cord (B.3.4.1.). The photographs shown in (Phot.2) were obtained in this way from the cord of a 24 week old foetus. Such fibres were frequently seen and some were as long as 100 μ . Similar fibres were seen in mature cords, but wider ones also occurred in the older cords. No systematic networks or anastomoses were observed.

The general conclusion was drawn that Wharton's jelly is permeated by a criss-cross work of fine fibres, which have different widths, and many are about 400 $\text{\AA}$ wide. Also that at least many of these fibres are collagenous and that they are part of the functional structure of the cord and are not simply produced towards the end of gestation.

Fine collagen fibres have previously been demonstrated in umbilical cord, as by Randall¹¹⁰, but only after severe treatment, such as ultrasonic disintegration. Here, they were shown to lie freely in the Wharton's jelly, as they could not otherwise have been obtained by merely touching the sides of an incision in a cord.

B.3.4.2b The appearance of hyaluronic acids

In (Phot.3) is shown the typical appearance of an extract of umbilical cord (B.3.4.1.) under the electron

microscope. Some small, fibril-like formations are seen on closer inspection. The material had been clarified on the preparative ultracentrifuge (Rotor 40, 39,000 r.p.m. for 1 hour). Incubation with collagenase did not alter the appearance of the material and hyaluronidase was also without effect, except under severe conditions.

A series of experiments with the hyaluronic acids of synovial fluid, together with some studies using random distributions of spheres, led to the following conclusions. While formations similar to fibrils or 'pearl-strings' may represent the true state of hyaluronic acids, the available evidence is insufficient proof for the existence of such structures.

Synovial fluid as collected from the Slaughterhouse (B.8.2.3.) contained various fibres as seen under the electron microscope. Most of these were removed by ordinary centrifuging (International centrifuge; head 267, 2,000 r.p.m. for 30 minutes) and all were removed by the preparative ultracentrifuge (Rotor 40, 39,000 r.p.m. for 20 minutes). A typical specimen, after centrifuging, is shown in (Phot.4) 'Pearl-strings' are present and the general appearance is much like that of umbilical cord extract (Phot.3), except that there is a greater tendency for dense clumps to be formed. The appearance depends upon the concentration of material upon the electron microscope grid.

By means of filtration ultrafiltrate residue (U.F.R.) was made (B.8.2.4.) from the synovial fluid which had been clarified in the preparative ultracentrifuge. The U.F.R. was taken up in standard phosphate buffer pH 7.0 and exhaustively dialysed against this buffer. The final solution was again spun in the preparative ultracentrifuge (Rotor 40, 39,000 r.p.m. for 1 hour) and the precipitate examined under the electron microscope (Phot.5). One or two definite, long 'pearl-strings' are apparent which appear twisted together, and it would be difficult to ascribe such a formation to chance.

The dialysed and centrifuged solution was treated with excess hyaluronidase (Benger's Hyalase) at 37°C (about 500 Benger's units to 5 ml. solution). Samples were withdrawn at intervals: (Phot.6) shows material after 30 mins incubation, (Phot.7) shows a sample removed after 3 1/2 hours. Comparing these two photographs it becomes clear that the concentration of material upon the grid has a greater influence upon the appearance of the material than has the action of hyaluronidase. Even so, when looking at a number of samples one obtains the impression that hyaluronidase may reduce the tendency to the formation of long, single line, pearl strings. (Phot.8) shows a typical appearance after the action of hyaluronidase, using a fairly even distribution of material upon the electron microscope grid.

Shorter 'pearl-strings' can readily be formed by the chance arrangement of individual spheres, as was shown in the following experiment. (It should be noted that units of the order of size which is the limit of resolution of a given electron microscope appear globular, independent of any shape that might be demonstrated by an instrument of higher resolving power).

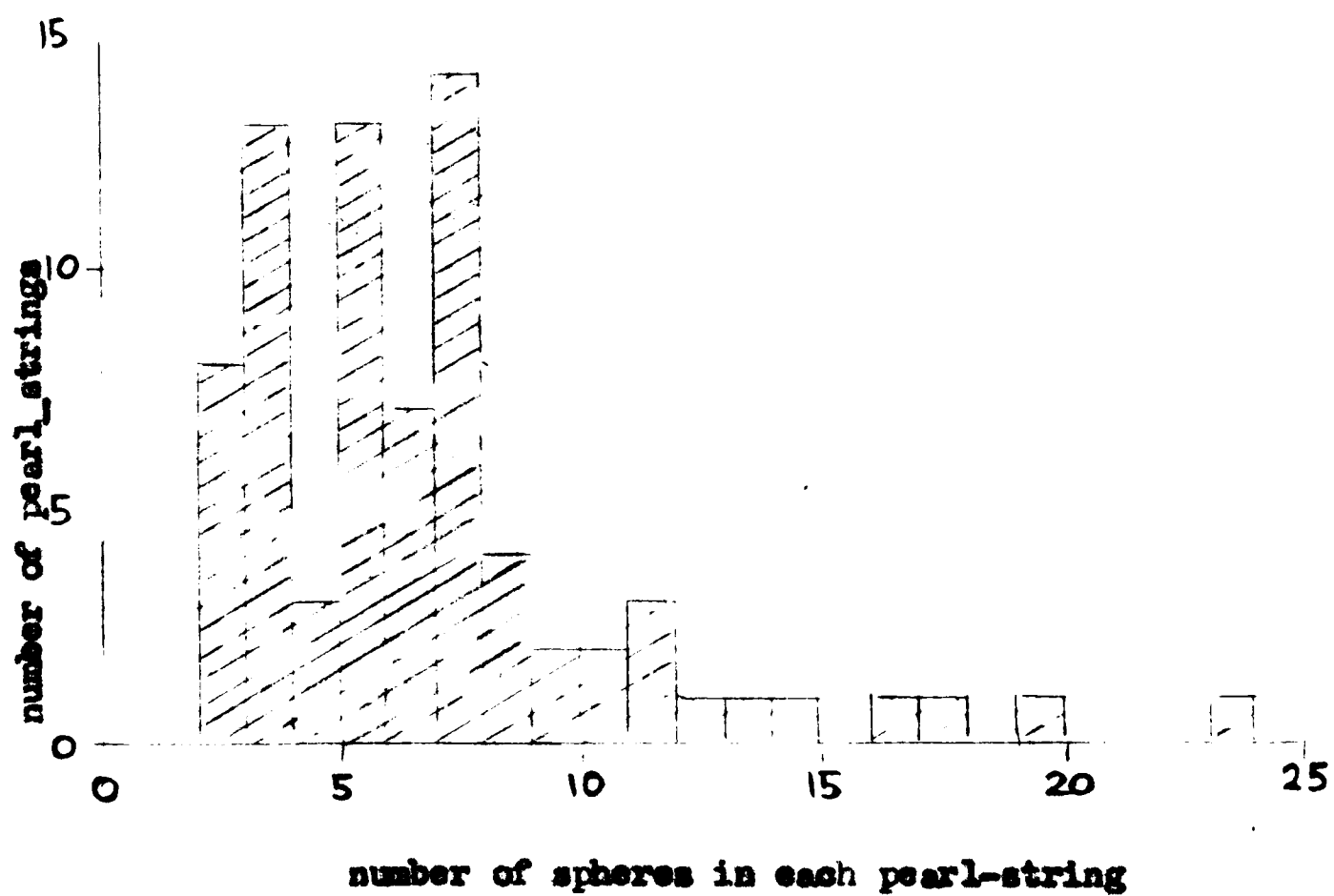
B.3.3. 'Pearl-string' like formation as apparent in electron micrographs

Filaments, similar to strings of pearls, were apparent in some of the electron micrographs (shot. 3,4,5). They were difficult to interpret and could correspond to (1) a definite structure in the material, (2) an artefact of preparation, in particular due to structure of supporting film or presence of salts, (3) an optical illusion: a grouping together of randomly placed particles by the observer.

The experiment to be described was made to investigate (3) and it was concluded that (3) could account for the observations. Although this of course does not imply that (1) and/or (2) may not also have occurred, no conclusion based on (1) can be made.

Fig. 2.

Histogram: frequency of occurrence of pearl-strings
of different lengths.



Glass spheres of about 0.1 mm diameter (Ballotini, No.14, English Glass Co., Ltd.) were scattered over a horizontal glass plate and projected in silhouette onto a screen. A projector such as is used for reading microphotostats was found convenient. Such pearl-string formations as were readily apparent were noted and the number of spheres making up each one was recorded. The experiment was repeated a number of times with fresh scatterings of spheres. The experiment was stopped when it had become clear that the eye readily aligns spheres into 'pearl-string' formations. The results are given as a histogram in (Fig.2) and in particular four to eight spheres are readily grouped into a string, though as many as 24 were observed in one undulating row. There were very few scatterings in which no 'pearl-strings' were seen and they occurred particularly near the periphery of denser clusters of spheres, where presumably fairly regular packing can occur. Statistical analysis was likely to be difficult and would only have been of secondary interest, so that no attempt was made to collect the necessarily extensive information for such work.

The 'shadowing' used in electron microscopical technique could intensify the illusion of pearl strings,

especially when applied to uneven, multilayer deposits.

Reardon¹⁰¹, placed hemispheres of varying diameters on a rectangular grid, according to a set of random numbers. He also found that several hemispheres would often touch, though it remained for the observer to decide between 'strings' and aggregates.

Fig. 3.

The swelling of umbilical cords kept in various fluids;
expressed as percentage of the initial weight.

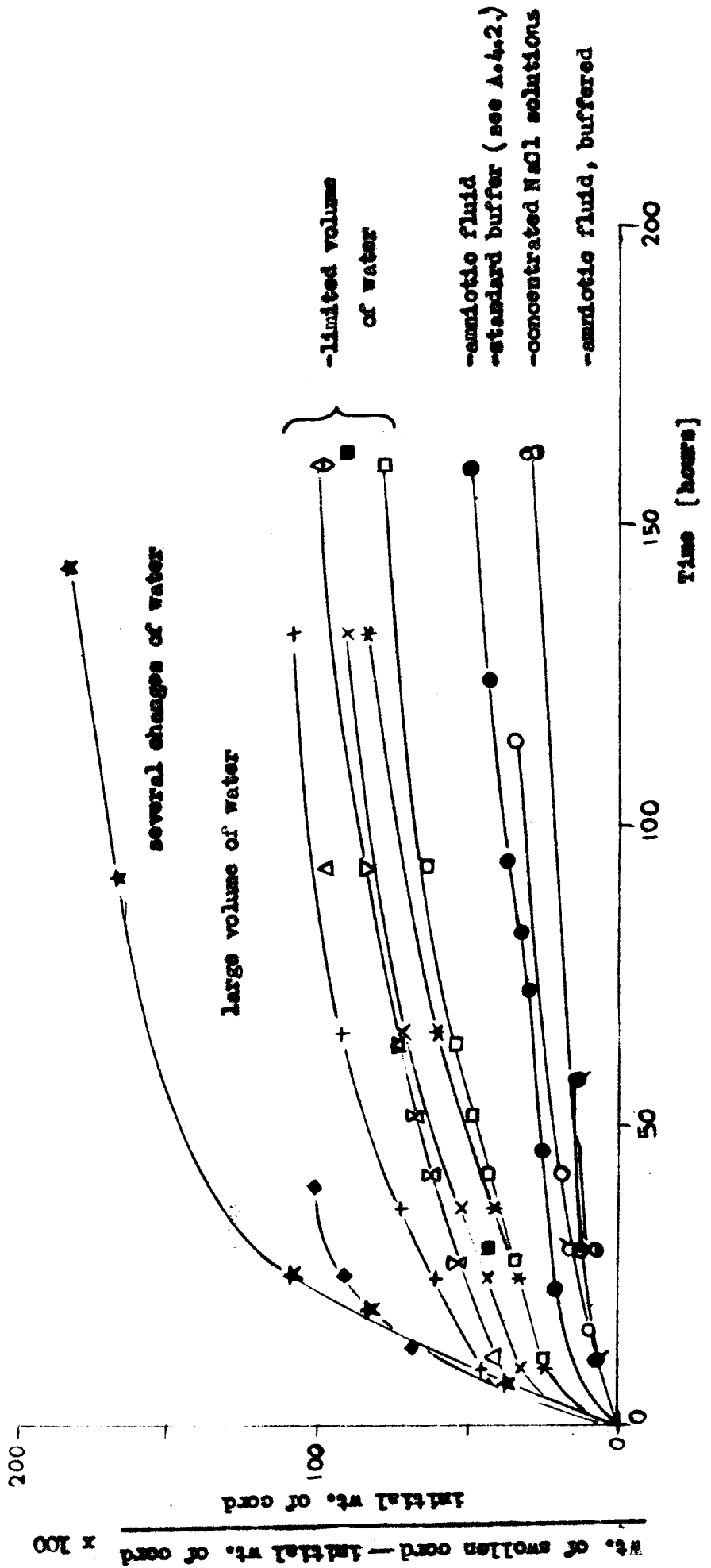
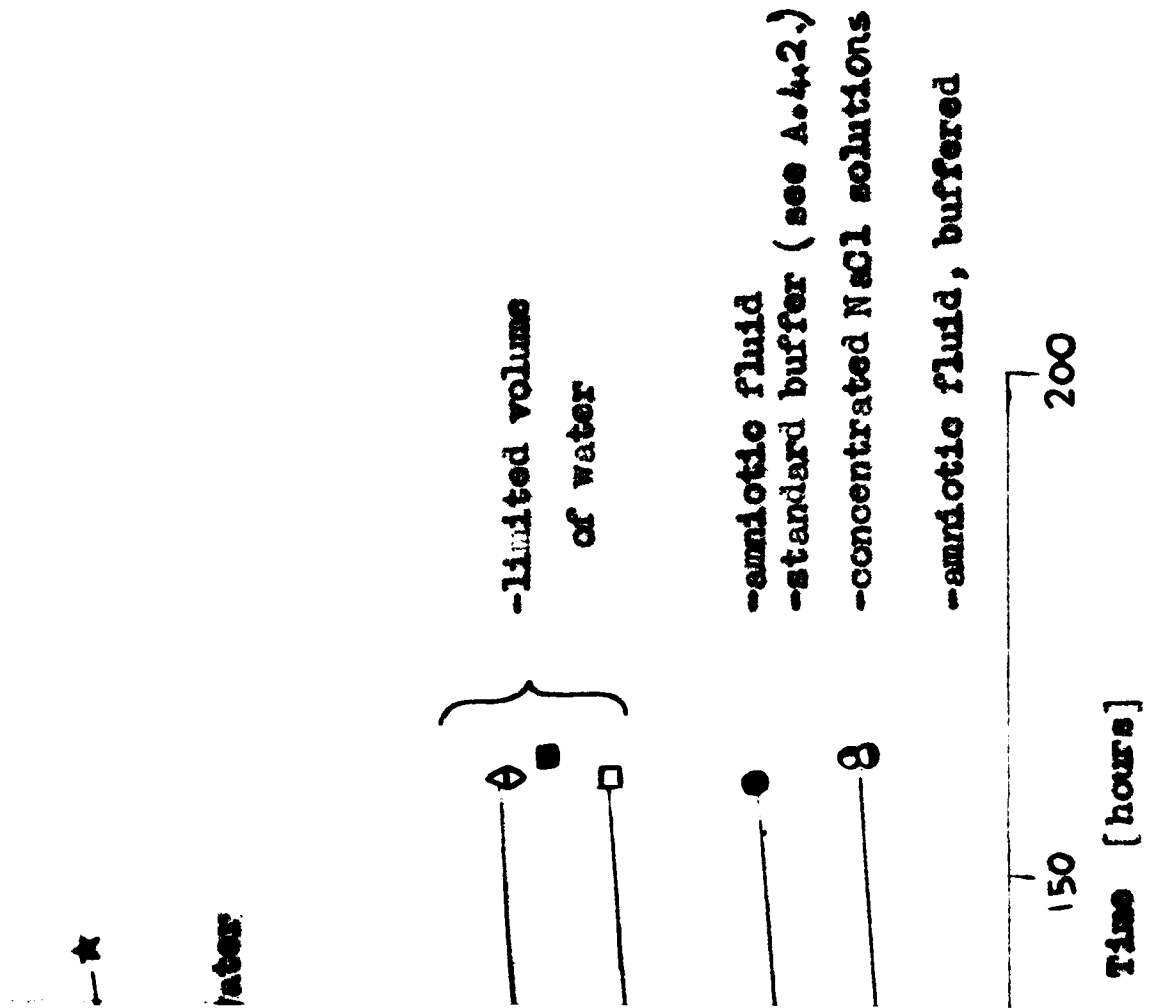


Fig. 3.

The swelling of umbilical cords kept in various fluids;
expressed as percentage of the initial weight.



B.4.

SWELLING STUDIES OF UMBILICAL CORDS

The ease with which umbilical cords swell is so apparent, that a small investigation was made of this behaviour.

B.4.1. Method:

A suitable piece of cleaned cord was tied with string at both ends, a short length of string joining the two ends. The loop of umbilical cord thus formed was quickly blotted, weighed, and then freely suspended from a glass rod lying across the rim of a beaker. A measured volume of fluid was placed in the beaker and conditions so arranged that while the out ends of the cord were kept above the level of the fluid, as great a part as possible of the cord was immersed.

The beaker was covered and kept in the refrigerator. After various time intervals the cord was removed, quickly blotted and weighed, and then returned to the beaker.

As a measure of swelling after time t there was taken:

$$\left[\frac{\text{Wt at time } t - \text{initial wt.}}{\text{initial wt.}} \right] \times 100$$

B.4.2. Results

Rapid swelling occurred in a large volume (800 ml.) of water, especially when the bathing fluid was renewed (Fig.3). Swelling was slower, and did not proceed as far in

a limited volume of water (30 - 150 ml.). Provided that the "skin" of the umbilical cord remained undamaged, and that blood and bacterial action were absent, no protein or polysaccharide could be demonstrated in the fluid. Probably salts escape into the solution, and when sodium chloride was added to the bathing fluid swelling was decreased, but could not be reversed or abolished. The pH of bathing solutions was 7.4 - 7.7, and swelling was much the same in saline or buffered solution. Limited swelling occurred in amniotic fluid, and minimal swelling (13% of initial weight) occurred in amniotic fluid when care had been taken to maintain the natural bicarbonate - CO₂ buffer at pH 7.4.

Excessive swelling of a cord would sometimes cause it to split open in a number of places and some protein would then escape into solution, though the rigid structure of the swollen material remained unaltered.

The confinement of hyaluronic acids and proteins can only in part be due to the "skin" of umbilical cord. A dissected piece of cord or Wharton's jelly always has a bodily structure of its own, and while an applied drop of fluid may dissolve out a little of the material, the piece will also swell and a stationary state is soon reached as was shown in the extraction experiments. Indeed complete leaching out of material was shown to be very difficult and handling the

material left one with the impression that some internal, confining structure was present. On evaporating an extract down it never passed through a gel state, but just changed from a fluid of increasing viscosity to dry material. It seemed that the extract alone could not form a gel, and that some other component, such as could be supplied by some internal, confining structure of the cord, was also required.

It was concluded that the macromolecular components of intact umbilical cord cannot escape into the surrounding bathing fluid when swelling occurs, and that under near-physiological conditions there is little tendency to swell.

B.5. THE EXTRACTION OF HYALURONIC ACIDS FROM UMBILICAL CORD

B.5.1. Introduction

The hyaluronic acids of umbilical cords have been extracted and studied by many^{1, 10, 36, 46, 47, 48, 58, 59, 71, 74, 75, 83, 84, 100} but the material was exposed to conditions, such as precipitation with glacial acetic acid, which have been shown by Ogston & Stanier⁹¹ to affect the physical behaviour of the product. In the study to be described, care was taken to avoid all conditions which might have caused a degradation of the material.

(For a description of the morphology of umbilical cord see (B.3.1.) and the diagram (Fig.1.))

B.5.2. Collection of umbilical cords (see also B.3.2.) was arranged to prevent changes in the material. Thus while others conveniently accumulated a store of cords in acetone, the material used here was externally cleaned and placed in glass jars at 0°C as soon as could conveniently be done after birth. The cords were used within 18 hours and often much earlier. Deep freeze storage was tried, but interfered with subsequent dissection. Mechanical maltreatment, such as squeezing or forcing water through the blood vessels, was avoided and the ends of cords were usually tied after gentle removal of most blood.

Cords vary widely in many ways such as length, thickness, degree of twisting of the blood vessels and content of jelly. Some types can be recognised, but as far as possible no discrimination was made and all material was used. Cords were found to swell rapidly in water (compare B.4.) so that it was never certain what part of the weight of a cord was water imbibed subsequently to parturition. Grossly swollen or blood stained cords were discarded. The behaviour of Wharton's jelly on swelling varied between two extremes, those in which incision in a swollen cord allowed a mass of slimy fluid to escape and those in which a similar cut would display a swollen, tough pad with hardly moist borders.

B.5.3. Dissection of umbilical cords (see also Fig.1.)

Blood was cleared from the vein by gentle stroking followed by rapid, though gentle rinsing with distilled water by means of a Pasteur pipette. The ends of the cord were tied, the whole quickly rinsed with distilled water and then blotted dry.

Two small scalpel incisions were made on opposite sides of an artery and parallel to its axis. A connection was made between the two incisions, central to the artery, which was then tied in two places and severed. Lifting one of the ends so made to the vessel, the artery was dissected out along

the whole length of the cord, care being taken to remove adjoining sheet-like structures with the vessel. If a vessel became damaged, it was clamped and any exuding blood at once removed. Both arteries were dissected out in this fashion.

The tied ends of the cord were now cut off and a coloured glass rod gently pushed into the vein, along its whole length. The rod was mounted in a clamp and the extraneous material adhering to the vessel - and constituting the Wharton's jelly - was dissected off, leaving any sheet-like structures behind with the vein as far as possible.

The Wharton's jelly so collected was contaminated with the covering of "skin" of the cord, but this is only a few layers of cells and was not removed.

The material was either cut up with scissors or minced in a 'Latapie' mincer and then weighed. Several grams of material were obtained from a cord.

Throughout care was taken to avoid contamination with blood and while the material was kept clear of gross fluid, it was occasionally just moistened with distilled water to prevent drying. Blood stained portions were as far as possible discarded.

In a few cases the cord was first swollen in water and then pieces of jelly were obtained by blunt dissection.

TABLE 1Preparation of umbilical cord extracts:-

the effect of increasing time of extraction on
clot weight:

1 part of minced Wharton's jelly plus 3 of water:

Clot made from 1 ml. of extract.

<u>Extraction time</u>	<u>Clot weights</u>	<u>Mean clot weight</u>
2 hours	2.81 mg.)	2.9 mg.
	2.99 mg.)	
3½ hours	3.21 mg.)	3.2 mg.
	3.21 mg.)	
20 hours	3.4 mg.)	3.35 mg.
	3.31 mg.)	

B.5.4. Extraction of Wharton's jelly

To the minced material one to five times its weight of water or buffer was added and the whole was stirred in the cold room in a closed glass jar without allowing frothing, to occur. After varying times, of the order of hours, the mixture was centrifuged in the Spinco ultracentrifuge (Rotor 40, 39,000 r.p.m. for 20 minutes). The supernatant was kept, while the remaining material could be extracted several times.

Reasonable care was taken to avoid microbial contamination and all experiments on an extract were made within a few days of its preparation.

If required, the extract was dialysed against several changes of standard buffer.

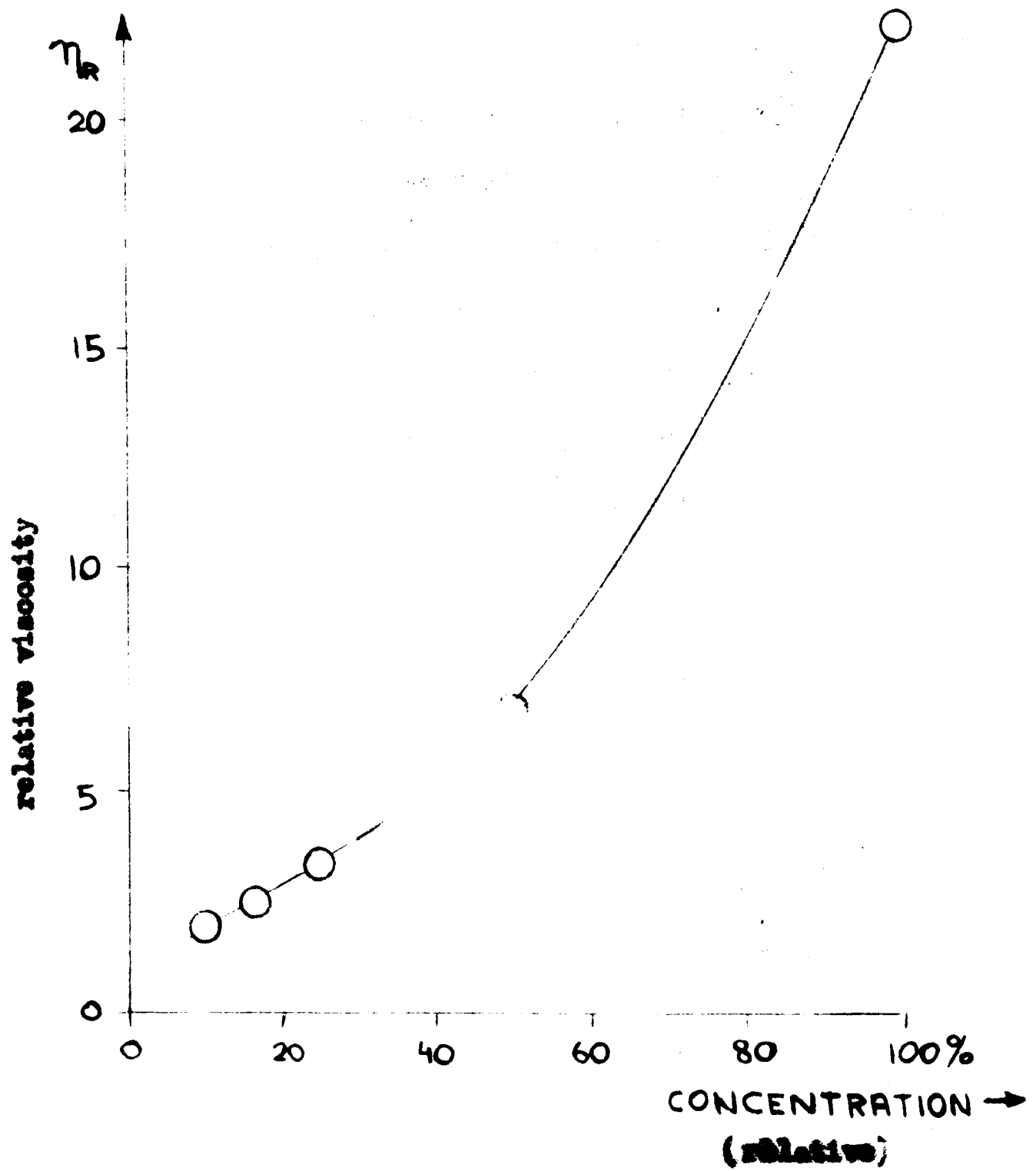
An extract could also be obtained by directly ultracentrifuging pieces of Wharton's jelly cut from a swollen cord.

B.5.5. General

Use of a Latapie mincer was found to be the most convenient way of cutting up the Wharton's jelly. There was little difference in extracting with saline or water. Water caused more swelling than solution of the material, but as ultracentrifugation was necessary this did not matter, the extract being squeezed out of the swollen material.

Fig. 4-

Emulsion card extract: variation of relative viscosity
with dilution of a sample.



Two parts of water per part of mince gave a near homogeneous paste and usually 4 - 10 parts of weight of fluid were therefore added to the mince. Little was gained by extracting for more than 6 hours. The relative viscosity η_R measured in an Ostwald viscometer was shown to be a fair index of the concentration of material in the extracts (Fig.4) This index only increased from 5.7 to 6.8 on increasing the extraction time from 7 to 36 hours, and then gained a plateau value at 62 hours of $\eta_R = 6.9$ (Fig.5)

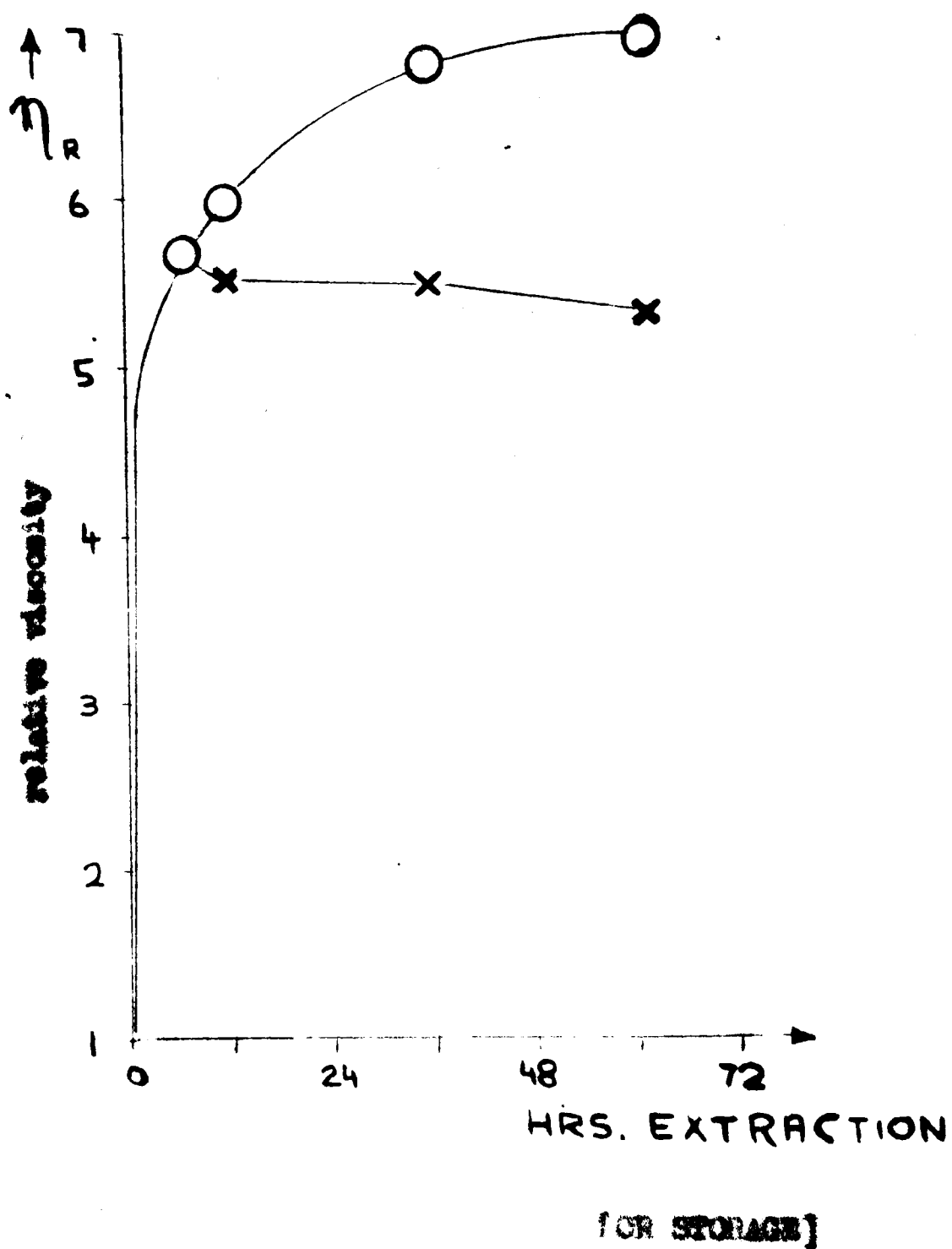
The water extracts contained enough protein to form excellent clots on adding acetic acid. Following the course of extraction by means of clot weights (B.6.2.) similarly indicated a few hours' stirring to be adequate (Tab.1.)

Next using η_R (Ostwald) as an index it was shown that the extracts are reasonably stable for at least 36 hours in the cold room (Fig.5.). The absence of any inherent hyaluronidase activity was found by incubating at 39°C, when the relative viscosity changed from 22.2 to 22.05 after 75 minutes; the small change could be due to bacterial contamination. Merthiolate⁷⁷ was added to extracts after it had been shown that at 1:34,000 it does not affect the viscous behaviour of synovial fluid hyaluronic acid (B.5.7.) The direct ultra-centrifuging of pieces of swollen cord gave a concentrated extract in one case (extract a Q\), but was not successful

Fig. 5.

Umbilical cord extract: relative viscosity as a measure of extraction and as an index of stability during storage.

- - η_R of samples extracted for different lengths of time.
x - η_R of samples kept in the refrigerator for different lengths of time.



with another cord. This seemed to depend on some inherent quality in the umbilical cords.

B.5.6. Nature of the extract

How representative are such extracts of material in the umbilical cord? The plateau concentrations reached with increasing extraction time (Fig.5.) were reminiscent of the swelling studies (B.4.) on whole cord, as if some apparent equilibrium were reached between extract and swollen material. Three successive extractions were made, the minced and swollen jelly being "squeezed" by ultracentrifuging (Rotor 40, 20,000 r.p.m. for 30 minutes) at the end of each extraction. Little material was removed in the third extract, but after homogenizing the remnant in a Potter homogenizer, stirring for some hours and ultracentrifuging, a solution containing ample polysaccharide was obtained, that is: addition of sulphosalicylic acid showed no evidence of protein while 2 volumes of acetone gave a voluminous precipitate; again acetic acid only gave a clot after the addition of protein. From this it was concluded that some more vigorous mode of division than the Latapie mincer was required and studied.

TABLE 2

Effect of various treatments on the relative viscosity
of bovine synovial fluid, at different shear rates.

Treatment	η_0	η_{50}
None	7.7	6.0
Freezing and fragmenting	7.4	5.5
Razor blade homogeniser	6.75	5.32
None	6.4	4.0
Potter homogeniser	3.1	2.34

$\eta_0 = \eta_R$ extrapolated to zero shear rate.

$\eta_{50} = \eta_R$ at 50 sec^{-1}

For intermediate shear rates the relative viscosity of the native fluid was also always greater than that of a fluid exposed to any of the treatments.

A vigorous form of extraction could damage the material. To investigate this studies were made with synovial fluid, as it had been shown that that material which had been extracted from umbilical cord by mild methods, closely resembled that from joint fluid. The following were tried: treatment with the Potter homogenizer, a revolving razor blade homogenizer and, fragmenting by impact material frozen to -170°C . Variation of η_s with shear rate, in the Couette apparatus, showed that all these treatments affected synovial fluid to decreasing extent (Tab.2).

Thus to extract all the material from cord would require methods which would themselves damage it, and the subject was therefore pursued no further.

B.5.7. The use of "Merthiolate"⁷⁷ to counter bacterial action in solutions

Bacterial action was a recurrent problem in work with synovial fluid and became acute when the fluid was separated into polysaccharide, protein and other constituents. That part of synovial fluid which passes through dialyzing membrane constitutes an excellent medium for bacterial growth.

A search for an effective antiseptic, which would not interfere with the measurements to be made, was carried out.

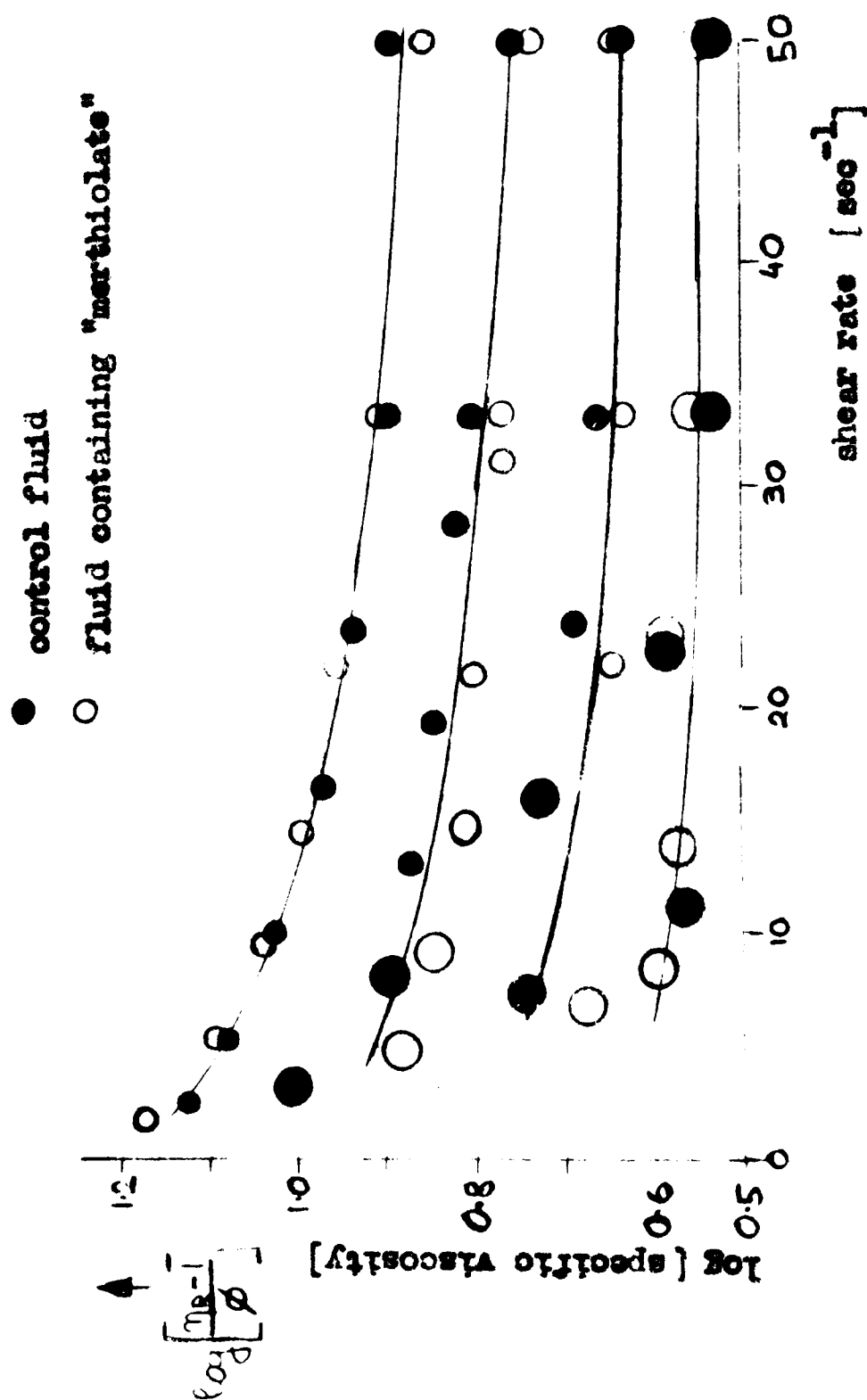
Small samples of synovial fluid were incubated at 25°C overnight, after adding a mixture of the following: synovial fluid and minced umbilical cord which had been allowed to deteriorate, in a closed vessel, to an advanced stage; scrapings from the slaughterhouse floor and dirt from the laboratory dust pan; puddle water and mud. As inhibitors of the growth of microorganisms there were tried singly and in various combinations, penicillin, streptomycin, chloromycetin; merthiolate⁷⁷, acriflavine, toluene, thymol, chloroxylenol, teepol, lyzol, and iodine in KI. The samples were inspected after 14 hours and after five days and the following conclusions drawn:

- (1) Best antiseptic action was shown by merthiolate or merthiolate together with the three antibiotics.
- (2) A mixture of the three antibiotics had a good action, but only for a limited time.
- (3) Some long term action only was shown by toluene, and to a lesser extent by teepol and thymol, perhaps also by chloroxylenol.
- (4) The other agents were inactive or cause precipitation.

By a similar experiment it was found that the final concentration of merthiolate must be greater than 1:100,000, that dilutions of 1:10,000 and 1:30,000 had much the same

Fig. 6.

Effect of "merthiolate" (1:34,000 w/v) on the variation of specific viscosity with shear rate of bovine synovial fluid.



ϕ = relative volume fraction; for the most concentrated solution:

$$\phi = 1.$$

effect and that 1:3,000 was deleterious as judged by change in η_r (Ostwald).

Ogston & Stanier⁹¹ have established the variation of relative viscosity with shear rate and concentration as a test for the native state of hyaluronic acids in synovial fluids. A sample of bovine synovial fluid made 1:34,000 w/v with respect to merthiolate was similarly investigated in the Couette viscosimeter (A.3.2.) and compared with a control fluid. Shear rates from 50 to 3 sec^{-1} were applied to four sets of dilutions (Fig.6)

Within experimental error merthiolate did not affect the behaviour of the most concentrated and dilute fluids, while at intermediate concentrations it lowered some relative viscosities by just more than an estimated experimental error of $\pm 2\%$. It was concluded that merthiolate, at this concentration, had little, if any effect on the viscous behaviour of synovial fluids and was therefore added to a number of solutions used. In a trial sample kept in the cold room merthiolate prevented bacterial growth for 4 months. Fresh stock solutions of merthiolate are essential.

B.6. PHYSICAL MEASUREMENTS OF EXTRACTS OF UMBILICAL CORDS

B.6.1. Experimental

Extracts of umbilical cords were made as described in (B.5.2,3 & 4). Five dilutions were made of each extract such that the final concentrations of buffer were that of standard buffer (A.4.2.), and viscosity (A.5.2.) and sedimentation measurements (A.3.1.) were made on each solution.

The concentration of each extract was determined by the method of mucin clots as follows.

B.6.2. Preparation and weighing of mucin clots⁸⁹

0.5-3 ml. of the fluid to be investigated was exhaustively dialysed against water. The dialysate was quantitatively transferred to a standard flask and made up to 5 ml. with water. Depending upon its concentration, about 1 ml. of the solution was pipetted or weighed into a small test tube. A little concentrated protein solution, prepared from bovine synovial fluid, was added for estimations of U.F.R. The volume was made up to 2 ml. with water. 0.06 - 0.09 ml. of 10% acetic acid were slowly added while gently stirring with a glass rod. The mucin clot which formed was collected and shaped on the rod, and

then allowed to synerese for a few minutes. The clot was then transferred on the glass rod to a test tube filled with 1% acetic acid and given a five minute wash. After a second, similar wash, the clot was stripped off into a tared crucible and dried at 105°C to constant weight.

The crucibles were cooled in a desiccator containing phosphorus pentoxide. Weighing was done in small weighing bottles with well-fitting lids, on a standard Sartorius balance marked to 0.1 mg and read to one tenth of this.

Checks were made (1) that all the hyaluronic acid in the sample taken for making a clot was in fact precipitated and (2) that no excess protein was adsorbed on the clot.

(1) This was simply checked by adding protein solution to the liquid left after removal of the mucin clot. No further clot formed, and further addition of acetic acid also gave no precipitation.

(2) If a large excess of protein was present when the mucin clot was formed, an appreciable weight of protein was carried over on the clot. In the estimation of U.F.R., clots were therefore made from a series of solutions to which different amounts of protein (isolated from synovial fluid) had been added. A range of solutions

TABLE 3

Sedimentation coefficients, intrinsic viscosities, and molecular dimensions of hyaluronic acids

Material and sample	$10^{13} \times S_0$	$10^{13} \times d(1/S_{20})/dc$	$[\eta]_c$	$10^{-6} \times M$	v^1	J
Human knee fluid	5.4	1.5	22	1.2	68	18
Va	5.2	1.3	36	1.3	120	30
Pe	9.5	2.3	30	3.7	220	10
Human umbilical cord extract						
aQ			54	4.5	235	14
aL	10.0	2.55	35	4.1	274	10
aP			22	3.6	310	6
Sheep ankle fluid*	11.5	2.6	35	5.5	320	8
Ox ankle fluid*	14.7	3.3	35	1.0	520	8

* taken from: Fessler, Ogston & Stanier³⁴.

② additional estimate $S_0 = 10$
 $(\frac{1}{S}) = 2.8$

was found in which clot weights were independent of the concentration of protein in the initial solution, and these clot weights were taken as being representative. Under these conditions the amount of refracting material removed from the solution as mucin clot, was approximately in agreement with that calculated from the clot weight combined with the specific refractive increment for mucin (1.64×10^{-3}) given by Stanier⁹¹ and confirmed by Johnson⁶³.

For purposes of calculation the composition of mucin determined by Stanier⁹¹ was assumed to hold, i.e. that 45% by weight of the mucin clot is hyaluronic acid complex.

B.6.3. Results

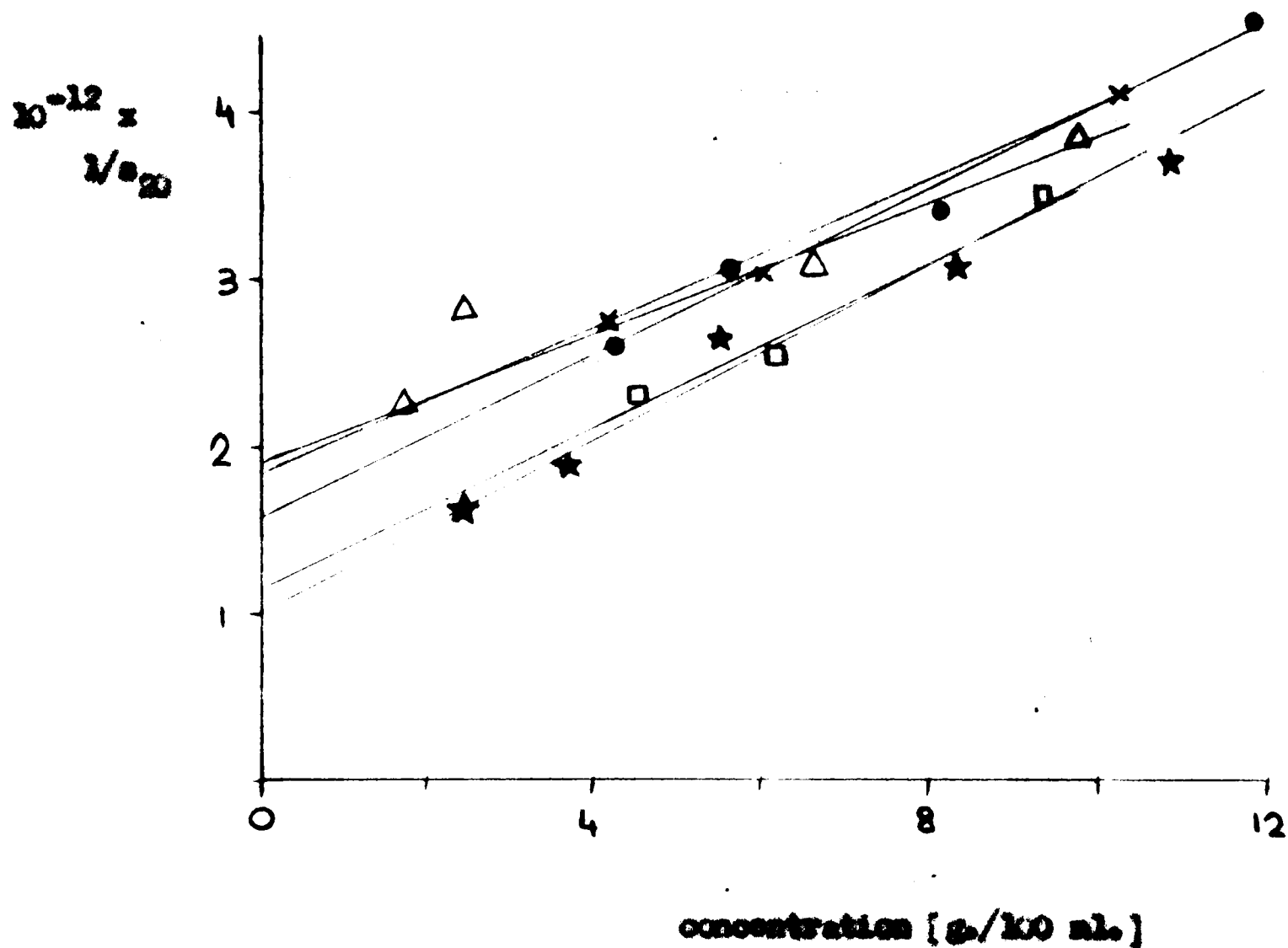
These are given in (Tab.3), (Fig.7), (Fig.8). Some representative samples of the plots of variation of $1/\eta_R$ (and η_R) with shear rate, used for obtaining the values of η_0 and η_{50} are shown in (Fig.9). A picture of the schlieren diagram obtained with the ultracentrifuge is given in (Phot.9).

Molecular parameters were calculated with the help of 'plug theory' (A.5.2.) and the following assumptions were made:

- (1) η = viscosity of solvent = 10^{-2} (viscosity of water near 20°C)
- (2) ρ = density of solvent = 1 (density of water near 20°C)

Fig. 7.

Hyaluronic acids: variation of reciprocal of corrected
sedimentation coefficients with concentration



[Concentration calculated for hyaluronic acid complex
 taking this to form 45% of the weight of mucin elot⁹¹]

Human synovial fluids:	sample No	×
	Va	Δ
	La	□
	Da	●

Human umbilical cord extract #2 ★

(3) $\bar{V}$ = partial specific volume = 0.69 (from Ogston & Stanier⁸⁹
of hyaluronic acid complex [determined for ox hyal-
uronic acids])

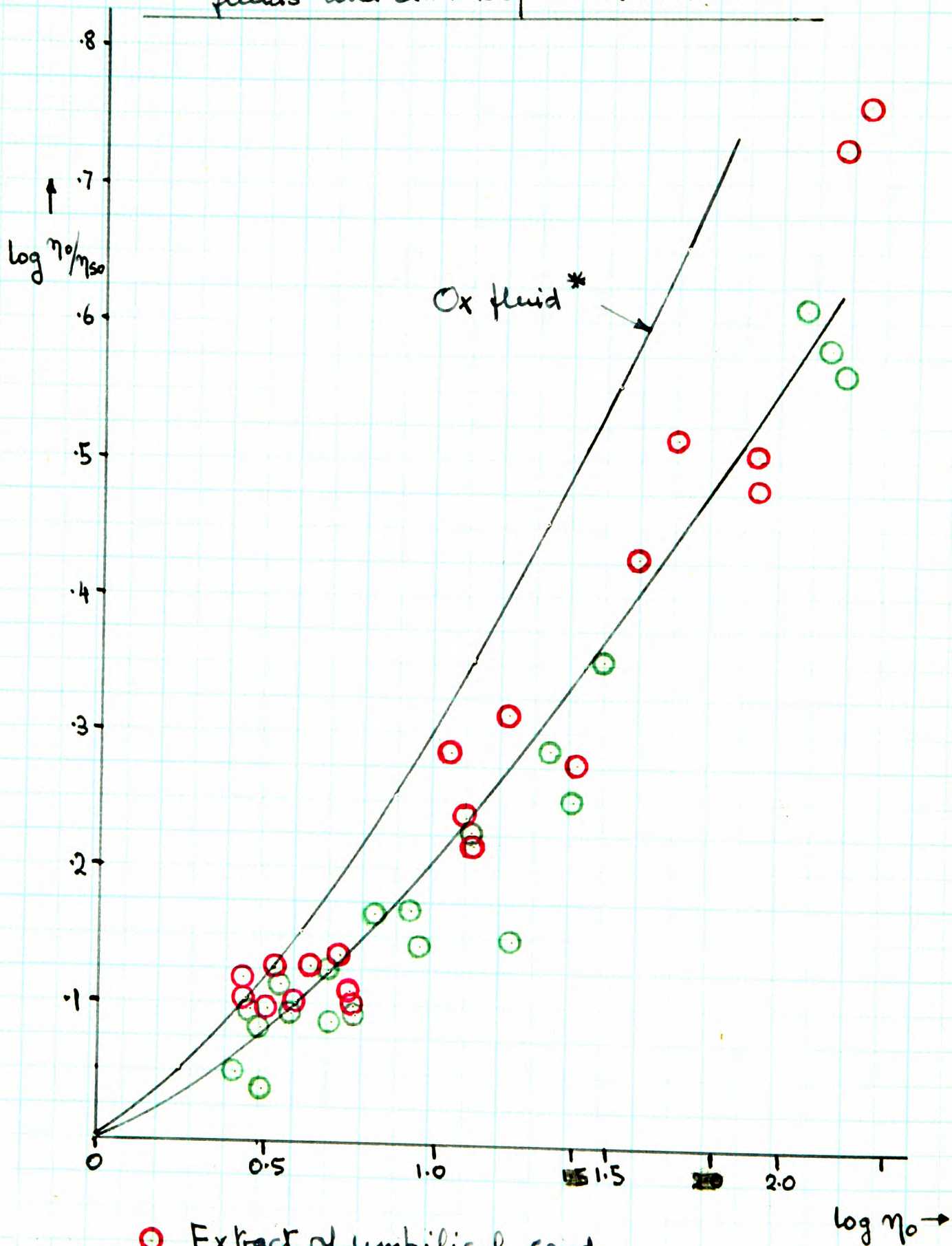
The parameters which were obtained are given in
(tab.3)

B.6.4. Discussion

These results are discussed together with the work
on human synovial fluids in (B.7.4.)

Fig. 8

$\log \eta_0 / \eta_{50}$ v. $\log \eta_0$ for human synovial fluids and extracts of umbilical cords



○ Extract of umbilical cord.

○ Synovial fluid.

* Taken from reference 91

B.7. PHYSICAL MEASUREMENTS OF HUMAN SYNOVIAL FLUIDS**B.7.1. Introduction**

These measurements were made by me as part of a larger investigation on synovial fluids made in conjunction with Dr. A. G. Ogston and Dr. J. S. Stanier. The results obtained were closely similar to those obtained with extracts of human umbilical cords.

B.7.2. Experimental

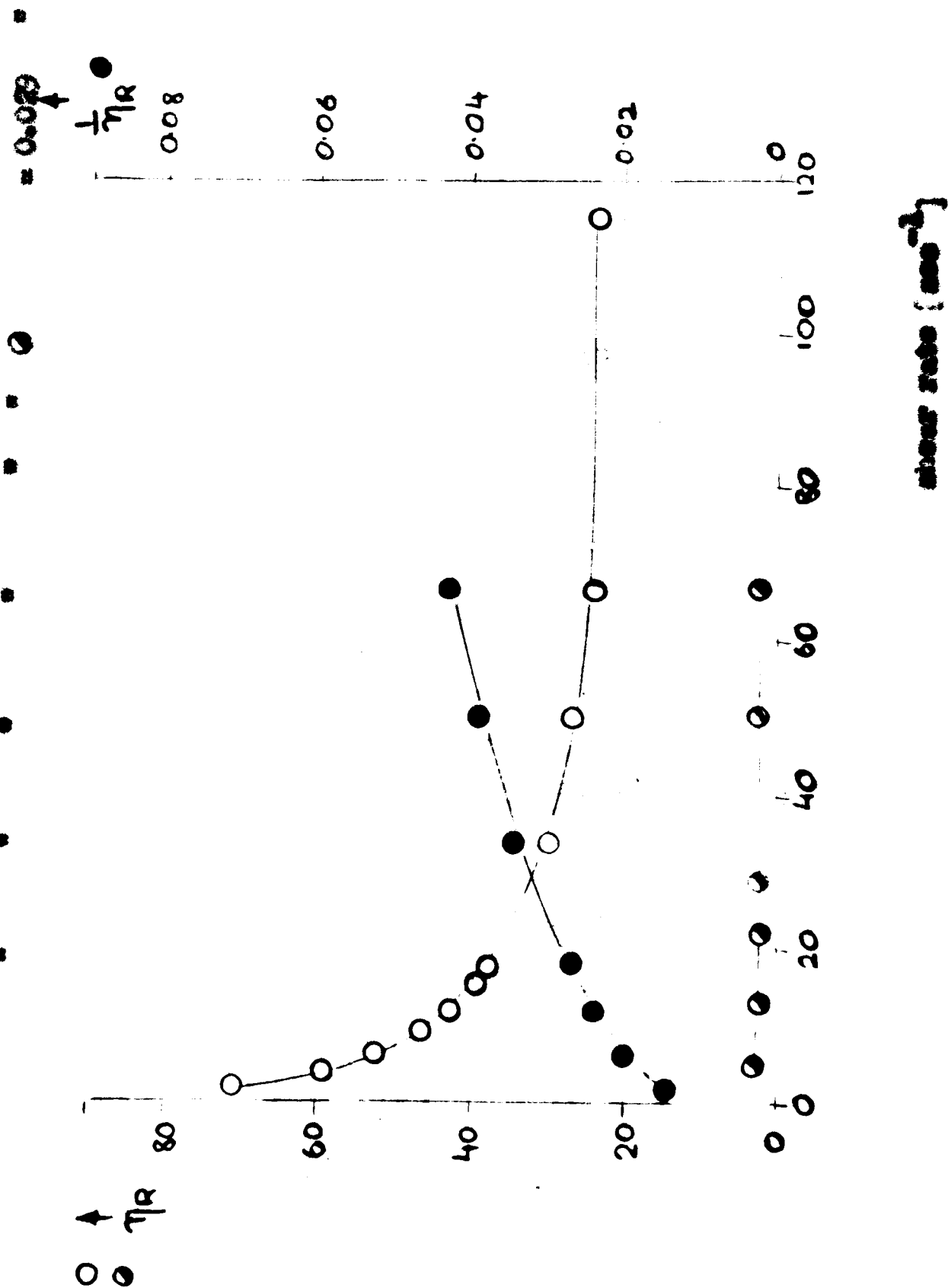
Human synovial fluids were kindly supplied in sterile condition by Mr. R. G. Taylor of the Wingfield-Morris Orthopaedic Hospital, Oxford, where they had been collected at operations from cases of moderate trauma of the knee joint free from any obvious disease. If a clot, presumably of fibrin, formed this was centrifuged off. Volumes were of the order of 1 ml.

The concentrations of hyaluronic acid complex in each fluid was estimated as mucin clot, by the method described in (B.6.2.). Four or five dilutions were made with standard buffer (A.4.2.) from each fluid and sedimentation and viscosity measurements were made on each solution (A.3.1. and A.3.2.)

Fig. 9.

Umbilical cord extracts: examples of the variation of
relative viscosity η_R with shear rate.

Extract no. Concentration of hyaluronate acid complex in % and $\odot = 0.220$ g./100 ml.



B.7.3. Results

Four fluids were investigated. In one case (fluid B) there was not sufficient material for a reliable estimation of concentration to be made and molecular parameters were therefore not calculated for this sample, although the measurements fitted in well with those obtained on the others.

The results are shown in (Tab.3), (Fig.7) and (Fig.8). Molecular parameters were calculated by means of 'plug theory' (A.5.2.) with the same assumptions as in (B.6.3.) and the results are given in (Tab.3). A picture of the schlieren diagram obtained with the ultracentrifuge is given in (Phot.10).

B.7.4. Discussion

A characteristic property of hyaluronic acids is the variation of the relative viscosity of their solutions with rate of shearing. Ogston & Stanier^{91, 94} have shown that on progressively degrading hyaluronic acids this is the first characteristic property to indicate that a change has occurred.

To compare this property for different hyaluronic acids $\log(\eta_0/\eta_\infty)$ was plotted against $\log \eta_0$ (see A.3.2.2. for nomenclature) as shown in (Fig.8). The results for human synovial fluid and human umbilical cord extract fall

within such the same region, indicating that they are similar. Some of the points for umbilical cord extracts lie perhaps a little nearer to the curve for ox synovial fluid than the other points do. This is in agreement with the other evidence, given later, that while the hyaluronic acids from the two human sources are closely similar, the properties of umbilical cord hyaluronic acid lie nearer to those of bovine synovial hyaluronic acid than do those of human synovial hyaluronic acid.

The calculation of molecular parameters is only semi-quantitative, not much better than to an order of magnitude. There are several reasons for this.

First, the material was impure. It could not be purified by ultrafiltration, as is discussed later in (B.9.4.), and from the work of Ogston & Stanier^{89, 91, 94} it was feared that damage would be done to the material if other methods were applied.

Secondly, considerable particle-particle interaction in solution exists. This is demonstrated in the shape of the sedimentation schlieren diagrams (phot9 and 10) which for an ideal solute have the shape of a Gaussian curve. Now the theories used for calculating the various molecular parameters are based on ideal conditions of solution. It was therefore necessary to extrapolate all measurements,

made at finite concentrations, to infinite dilution where ideal conditions are presumed to exist. Such extrapolations necessarily introduce uncertainties.

Thirdly, plug theory can only give an approximate hydrodynamic treatment (in particular the choice of the parameters ζ and k in equation (12) is somewhat arbitrary).

In view of this, the assumptions made are reasonable: i.e. that human hyaluronic acids are sufficiently akin to bovine ones, for the values of partial specific volume and mucin composition obtained for bovine material⁸⁹ to be used in these calculations. Similarly, no accuracy would have been gained by taking better values for solvent density and viscosity than those for water at about 20°C.

The molecular weight of 4×10^6 for material from umbilical cord (see (Tab.3)) is in agreement with the values obtained by Laurent & Gergely⁶⁸ of $2.8-4.3 \times 10^6$. The estimation of Blumberg & Oster¹⁴ gave a molecular weight of 8×10^6 . This was, however, based on a specific refractive increment of 0.0018, which is different from the value usually found for polysaccharides. (Dr. Blumberg, in discussion, agreed with this). If one uses the value of 0.0014 found by Ogston & Stanier⁸⁹ for the specific refractive increment, Blumberg & Oster's measurements give a molecular weight of 4.8×10^6 , in good agreement with the values given by

Laurent & Csergely⁶⁸ and those found in this investigation. Values given by Jensen and collaborators^{21, 62} are lower than the previous ones by a power of 10, but probably the material used by the Scandinavian investigators was degraded during the severe procedures of isolation.

The hydrated volume of 270 ml/g, indicates hardly more than very large hydration which the other investigators also found, and which Malmberg & Oster¹¹ estimate at 370 ml/g. from light scattering measurements. The axial ratio of the order of 10 indicates that the material is not in that configuration of a random coil which a freely rotating linear polymer would occupy, on the other hand it is consistent with the model of a partly rigid coil to which most investigators now incline^{62, 67, 68}.

The molecular weight of 1.4×10^6 obtained for hyaluronic acid present in normal synovial fluid from traumatised human knee joints, is in agreement with values obtained by others, e.g. $1.2-21 \times 10^6$ ⁶³ and 1.2 and 2.6×10^6 ⁶⁴. The hydration is again large and the particle is not spherical.

On this basis it is concluded that hyaluronic acids from human umbilical cord and human synovial fluid are closely similar.

When the molecular parameters found for human hyaluronic acids are compared with those obtained for sheep and ox hyaluronic acids, it seems likely that they all belong to the same group of substances. The sheep and ox particles have slightly higher molecular weights and hydrations, and the umbilical material is nearer to them in these properties than is that from human synovial fluid. All the substances have a highly anomalous viscosity behaviour in solution (i.e. the solutions are non-Newtonian liquids), and in this respect also the hyaluronic acids from the two human sources investigated are clearly similar.

B.8 THE ACTION OF HYALURONIDASE AS IT IS EMPLOYED IN CLINICAL PRACTICE

B.8.1. Introduction

Clinically hyaluronidase is chiefly used to promote the spreading of injected fluid. It is known²⁵ that there is polysaccharide material in connective tissues and that hyaluronidase facilitates the flow of a Ringer's fluid through isolated fascia.

It seemed to me that such action of hyaluronidase could be of two kinds, and that it should be possible from ultracentrifuge and viscosity studies to decide which was the likely one to occur in vivo. It is a problem of relative resistances. When intercellular polysaccharide offers a resistance to the bulk flow of liquids through tissues two types of frictional interaction must occur: one between aqueous solution and polysaccharide, the other between polysaccharide and structural components, such as fibres, of the tissues.

The sedimentation coefficient, S_{20} , measured in the ultracentrifuge is a direct measure of the frictional interaction of solvent and sedimenting material - for which extracted polysaccharide could be used. The relative viscosity, η_r , as measured in an Ostwald viscometer, is a

direct measure of the frictional interaction between the confining capillary and the system: extracted polysaccharide plus solvent. Studying the effect of hyaluronidase on S_{20} and η_r might then give an indication whether hyaluronidase acts in vivo by altering the polysaccharide in such a way that injected fluid can flow through it, or whether the enzyme enables polysaccharide to be pushed through the tissues with the injected fluid. The second case is indicated from the following experiment.

B.8.2. Experimental

B.8.2.1. Outline of materials and methods used

Ultrafiltrate residue (U.F.R.) was made from bovine synovial fluid (B.2.4.). A concentrated solution of U.F.R. was incubated at 37°C with a minimal concentration of hyaluronidase in an Ostwald viscometer. When the relative viscosity (η_r) had reached a desired value, the enzymatic action was reduced to a small rate by chilling and diluting the mixture, and sedimentation coefficients were determined at several dilutions. The experiment was repeated for five values of η_r .

The concentration of the solution of U.F.R. used was determined by the method of mucin clot weights (B.6.2.)

B.8.2.2. Reasons for choice of material and methods

B.8.2.2a Enzyme

A testicular hyaluronidase specifically prepared for clinical application was used, namely Bengner's Hyalase⁵⁴.

B.8.2.2b Substrate

Ready sources were umbilical cord and synovial fluid. An anti-hyaluronidase was found in umbilical cord by spreading tests, at least an enzyme concentration 100 times that employed clinically would have had to be used. Others¹⁵¹ have subsequently experienced similar difficulties. Specially concentrated U.F.R. was therefore used.

B.8.2.2c Control of enzyme action

The action of the enzyme had to be stopped at any stage by a method which would neither modify the polysaccharide nor interfere in the sedimentation experiments. Heparin seemed the most promising inhibitor⁷⁵, but a well purified, commercial sample⁵⁰ only gave 40% inhibition of the rate of change of η_R with time, under the necessary conditions, and also reduced the sedimentation coefficients of both native and hyaluronidase degraded U.F.R.

The method used was therefore developed. Using 0.03 Benger units per ml., $3\frac{1}{2}$ hours were required at 37°C to reduce η_r from ca 470 to 63. The rate of decrease of η_r with time itself rapidly decreases, and the reaction rate of testicular hyaluronidase is in addition at least halved by a reduction in temperature of 10°C ²⁷ such as is required for sedimentation runs at ca 27°C . Again at 0°C , ten times the strength of enzyme used, i.e. 0.3 Benger units per ml. reduced η_r from 11 to 8 during 17 hours. 0.003 Benger units per ml. were found to have only a weak action at 37°C and 0.001 Benger units per ml. none. The concentration of Hyalase used for clinical infusions is 1 unit per ml.

Some reduction in viscosity must have occurred during the sedimentation experiments, but as S_{20} was found to be independent of η_r over the applicable range studied ($\eta_r = \text{ca. } 470 - 7$) such change did not matter.

B.8.2.3. Collection and storage of synovial fluids

Bovine synovial fluids were collected from the hind limbs of cattle 15 - 30 minutes after slaughtering. A lumbar puncture needle was introduced into the sac-like dilation of the "ankle" (astragalotibial) joint immediately anterior to the Achilles tendon and the fluid withdrawn into a syringe⁸⁹. 5 - 20 ml. of fluid were obtained from each joint.

Fatty material was removed from the pooled fluids by straining through muslin. Debris and cells were at first removed by ordinary centrifuging (International centrifuge Rotor 267; 200 ml. buckets, 30 minutes at 1,860 r.p.m.)⁸⁹ but in later preparations a Spinco ultracentrifuge was used (Rotor 20; 19,000 r.p.m. for 30 minutes) and contamination with microorganisms thereby materially reduced.

Normally fluids were used at once, but when short storage was necessary either "merthiolate"⁷⁷ was added to a final dilution 1 in 34,000 w/v. (see B.5.7.) or the fluids were saturated with toluene. This was achieved by soaking a small dialysis bag in water, then filling it with toluene and placing it in the fluid. Denaturation of protein at a water-toluene interface was thereby prevented. As far as possible all work was done at cold room temperature.

B.8.2.4. Preparation of ultrafiltrate residue (U.F.R.)

Synovial fluid was filtered through No.5 sintered glass filters (average pore diameter 4.1μ)⁸⁹. Unless otherwise stated the pressure at the filtrate side of the filter was reduced to water pump vacuum. Filtration was complete after 8 - 24 hours and the residue was then dissolved in water, and after adding concentrated saline to 0.2M NaCl the filtration was repeated. After 2 - 10 saline washes had thus been applied, a similar number of water washes were carried out. Finally the material was taken up in water or buffer and centrifuged to remove any debris. All solutions were either saturated with toluene or made 1:34,000 w/v. with respect to merthiolate. The preparation was done in the cold room.

A concentrated solution of U.F.R. for the hyaluronidase experiments was prepared as follows:- A dilute solution of U.F.R. in standard buffer (A.4.2.) was ultra-centrifuged (Rotor 40, 39,000 r.p.m. for 40 minutes) and the supernatant carefully collected and then concentrated on a thoroughly cleaned sintered glass filter. Finally the concentrated solution was dialysed against standard buffer. A marble enclosed in the dialysis sac made it possible to mix the rather viscous solution.

B.8.2.5. Isolation of protein from synovial fluid

The first filtrate from the filtration of bovine synovial fluid through sintered glass Grade 5 filters was placed in a dialysis sac. The inside of the sac was connected to the atmosphere through a funnel containing some of the fluid. The sac was covered with a well fitting silk sleeve and placed in a flask which was evacuated. Concentrated protein solution remained in the sac at the end of the ultrafiltration, which was carried out in the cold room.

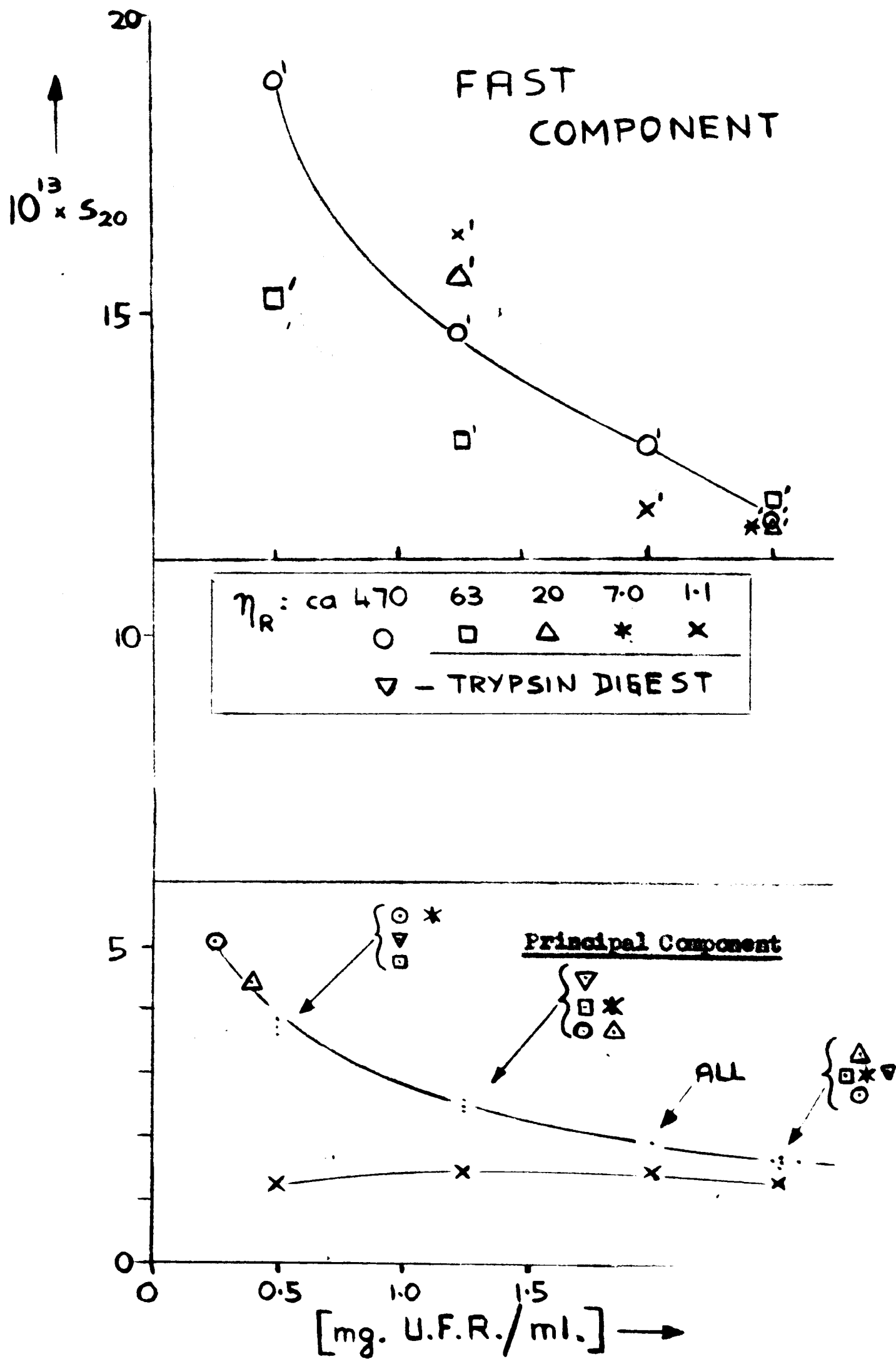
B.8.2.6. Incubation of concentrated U.F.R. with hyalase

Incubation was done in a waterbath at 37°C in an Ostwald viscometer whose flow time for 1 ml. of water was 30.6 seconds. 1 ml. of concentrated U.F.R. (B.8.2.4.) was placed in the dry viscometer and 0.01 ml. of enzyme solution added. The solution was mixed by gentle sucking and blowing from the capillary end of the viscometer, and when bubbles had risen to the surface in the wide limb of the tube, flow times were determined from time to time. It had been ascertained that a few bubbles in the wide end of the viscometer did not affect the measured flow times (within 1%) provided that the liquid flowing through the capillary was kept quite clear of bubbles.

where ϕ' = volume of one particle
 ρ_p = density of particle

Fig. 10.

The effect of hyaluronidase and trypsin digestion on the variation of sedimentation coefficient with concentration of bovine ultra filtrate residue (U.F.R.); relative viscosity η_r is used as a measure of the action of hyaluronidase.



B.8.2.6.

The final concentration of enzyme in the fluid was 0.03 Benger units per ml. unless complete degradation was required when 20 Benger units per ml. were used.

When the flow time had reached the required value the viscometer was cooled in ice, dilutions with standard buffer were rapidly made by weight and the solutions kept in ice. Sedimentation runs were done as soon as possible (within a few hours) and were kept as short as possible. Some dilutions were made roughly by volume and the concentrations were later determined refractometrically.

B.8.3. Results

These are given in (Fig.10). The relative viscosity was degraded from ca. 470 to :63, 19.8, 7.0 and 1.1.

B.8.4. Theoretical

The force F acting on N particles sedimenting, due to centrifugal acceleration, in a uniform medium is:

$$F = N \phi' (p_p - p) \omega^2 x \quad (18)$$

where ϕ' = volume of one particle

p_p = density of particle

ρ = density of solvent

ω = angular velocity

x = distance from centre of rotation.

Introducing a frictional coefficient f'' between solvent and particles and characteristic of the system:

$$f'' \cdot dx/dt = N\phi'(p_p - p) \omega^2 x \quad (19)$$

$$f'' = (N\phi'/s)(p_p - p) \quad (20)$$

where $S = dx/dt / \omega^2 x$ = sedimentation coefficient.

If we assume that an enzymatic cleavage of a particle does not affect p_p , which will hold to a first approximation for limited enzymatic action, and provided all particles sediment with the same sedimentation coefficient, then equation (20) becomes for a given concentration of total material:

$$f'' = K'/s \quad (21)$$

where $K' = N\phi'(p_p - p)$ = constant.

Under the specified conditions a comparison of observed values of S can thus be used to infer relationships between corresponding values of frictional coefficient f'' .

B.8.5. Discussion

Unless enzymatic degradation had proceeded very far ($\eta_R = 1.1$), the material continued to sediment together, (Phot.11).

The sedimentation coefficient of degraded material was never higher than that of the native material (Fig.10) so that by equation (21), the frictional coefficient f'' was not decreased. This may not apply for the most degraded material of $\eta_r = 1.1$. For the other materials the sedimentation coefficients, at a given concentration, were not even changed. Thus in the concentration range studied hyaluronidase did not decrease the frictional interaction of solvent and polysaccharide, while the relative viscosity was reduced from ca 470 to 7.

From this it is concluded that at least under these conditions hyaluronidase admixed with an infusion fluid would change polysaccharide so that it would be pushed through the tissue spaces with the fluid, rather than that the enzyme would enable the fluid to permeate the carbohydrate more readily.

This finding is reasonable from another view point. The initial hydrolytic action of hyaluronidase is small, while the relative viscosity of hyaluronic acid solutions is rapidly reduced. The breaking of a small number of bonds is more likely to affect a particle-particle interaction, than to alter the permeability of a particle to solvent.

How well do the conditions in vitro represent those that can be presumed to exist in vivo?

Interstitial polysaccharide will include sulphur containing carbohydrate, such as is not found in synovial fluid. Hyaluronidases affect such material to varying extent⁵⁷, but the main field of action of the Bengner's preparation is on sulphur free hyaluronic acid.

The concentration of material in vivo is unknown. It would be difficult to estimate the validity of any method used to investigate this, indeed the meaning of the term concentration becomes ill defined when applied to a system which is known to be unhomogeneous in phase and distribution of components. Again conditions will be changed at the advancing front of an injected fluid.

Over the experimental range studied the conclusions are valid independently of the proportion of water to solids. The most concentrated material flowed only with difficulty, approaching the gel state. Dangerous as it is to extrapolate, the measurements do not show any trend that might indicate the conclusions to be invalid outside the range studied. While prolonged action of hyaluronidase may be expected to lead to quite different conditions, the minimal effective action might be presumed to be the most important one clinically.

TABLE 4

Effect of sodium chloride on formation of mucin clot

1 ml. of bovine synovial fluid diluted to 4 ml. with saline and water; all solutions were clear. Then added 0.2 ml. of 10% acetic acid [pH of solutions by Johnson test paper: 3.6-4.6]

<u>Molarity NaCl added</u>	<u>Observation</u>
4/5th saturated	voluminous white ppt.
1.5M	strong cloudiness
1.0M	cloudy
0.5M	strongly opaque
0.25M	opaque
0.175M	small clot
0.1M	clot
0.0	clot

B.9 VARIOUS INVESTIGATIONS ON HYALURONIC ACIDS

B.9.1. The influence of ionic strength on the formation of mucin clots, and the formation of mucin clots by heparin

It was not possible to prepare U.F.R. from human material (B.9.4.), and Ogston & Stanier⁸⁹ had shown that usual methods of removing protein, such as Sevag's¹²³ procedure, altered the physical properties of the hyaluronic acids. Another method, using heparin⁵⁰ was therefore devised, but this was not successful; at the same time more precise conditions for the good formation of mucin clots were delineated.

The formation of mucin clots on adding acetic acid to a mixture of protein and hyaluronic acids (B.6.2.) is a convenient way of estimating the latter⁸⁹, but is, like other methods of estimating these substances, open to various uncertainties and lack of reproducibility^{34, 63}.

In qualitative experiments I found that clot formation is interfered with by the presence of: NaCl, NH_4Cl , CaCl_2 , choline chloride, $[\text{Co}(\text{NH}_3)_6]^{3+}\text{Cl}_3$, sodium citrate and potassium ferro- and ferricyanide.

Adding NaCl to bovine synovial fluid (see (Tab.4.)) showed that:

- (1) clot formation is best in the absence of any salts.

- (2) clot formation is maximally inhibited at 0.25M NaCl
- (3) a further precipitate is obtained at higher salt strengths; a similar precipitate was formed by protein isolated from synovial fluid (B.8.2.5.)

Fluids were therefore dialysed against water before making mucin clots for analysis.

Heparin also forms clots with the protein of synovial fluid, and this process was shown not to be inhibited in 0.25-0.5 M NaCl. To remove protein from umbilical cord extract this was made 0.5M to NaCl, then heparin and acetic acid added and the precipitate removed. Tests with an Ostwald viscometer showed, however, that the relative viscosity of the hyaluronic acid had been reduced from 3.8 to 2.3 and the method was therefore not used.

B.9.2. Various enzyme experiments and the nature of "Fast Component"

Ogston & Stanier found "fast component" a substance often present in U.F.R. which, like hyaluronic acid has a hypersharp peak in the schlieren diagram of the ultracentrifuge⁹⁰, and sediments with a coefficient of the order of 15 S. (Svedberg units).

In the experiments with hyaluronidase and U.F.R. it was noted that "fast component" did not disappear. Sometimes a second fast component called "fast 2" was observed.

Sedimentation coefficients of "fast components" varied considerably with concentration. The results are given in (Fig.10). From these findings it was concluded that "fast component" may not be a hyaluronic acid.

Incubation of U.F.R. with trypsin showed the hyaluronic acid peak to be unaffected, but the peak of "fast component" was made smaller and a little wider, and then made nearly to disappear.

U.F.R. from which some protein had been removed by Sevag's procedure¹²⁴, (there remained 6% protein as compared with the usual 25-30%), showed no "fast component" in the ultracentrifuge.

From this it is concluded that "fast component" represents a substance which is, at least chiefly, a protein.

If the hypersharp peak of hyaluronic acid in the ultracentrifuge could be resolved, measurements of the degree of heterogeneity of the material could be made in the ultracentrifuge. The peak is hypersharp because of the high dependence of the sedimentation coefficient on concentration, which in turn is due to considerable particle-particle interaction. The means which were, unsuccessfully, tried to lessen this interaction were: high concentrations of various salts, coupled with an acid pH, addition of urea, and of heparin, and the action of trypsin.

B.9.3. A theoretical approach to the formation of an ultrafiltrate residue (U.F.R.)

Consider a viscous solution of a polymer flowing through a capillary of radius r under an applied pressure gradient $\left(\frac{dP}{dl}\right)$. By Poiseuille's law:

$$\text{Volume flow } \frac{dV}{dt} = \frac{\pi r^4}{8\eta} \cdot \frac{dP}{dl} \quad (22)$$

where η is the absolute viscosity of the solution. If r is decreased the resistance to flow is increased. A situation may be reached where the resistance of the capillary to bulk flow of solution is greater than the resistance of the polymer to solvent flowing through it: filtration of solvent from polymer will then occur.

From (22):

Mean linear velocity of solution with respect to capillary: $v_2 = \frac{(\text{Volume flow})}{\text{area}}$

$$\begin{aligned} &= \frac{1}{\pi r^2} \cdot \frac{\pi r^4}{8\eta} \cdot \frac{dP}{dl} \\ \text{or } \frac{dP}{dl} &= v_2 \frac{8\eta}{r^2} \quad (23) \end{aligned}$$

Consider the same solution of polymer, of concentration c g./100 ml., sedimenting in the ultracentrifuge.

Weight of solute in a cube of 1 cm. side of solution

$$\begin{aligned} &= \frac{dP}{dl} \\ &= \frac{c}{100} (1 - \bar{V}_p) \omega^2 x \quad (24) \end{aligned}$$

where: $\bar{V}$ = partial specific volume of solute

ρ = density of solvent

ω = angular velocity

x = mean distance from centre of rotation.

Let the solute be observed to move with a velocity $\frac{dx}{dt}$

$v_1 = \frac{dx}{dt}$ then by definition:

$$dx/dt = s\omega^2 x$$

$$\text{or } \omega^2 x = \frac{dx/dt}{s} = v_1/s \quad (25)$$

where s = sedimentation coefficient of the solute.

Combining (24) and (25):

$$\frac{dP}{dr} = \frac{c}{100} (1 - \bar{V}\rho) v_1/s \quad (26)$$

Comparing (23) and (26)

$$v_2 \cdot 8\eta/r^2 = v_1 \cdot \frac{c}{100} \cdot \frac{(1 - \bar{V}\rho)}{s}$$

$$\text{or } v_1/v_2 = \frac{1}{r^2} \cdot \frac{8\eta \cdot s \cdot 100}{c(1 - \bar{V}\rho)} \quad (27)$$

The greater the ratio $\frac{v_1}{v_2}$, which we call the filtration ratio, the more polymer will v_2 be left behind the solvent, i.e. filtered.

As a trial value let $\left(\frac{v_1}{v_2}\right) = 1$, then substituting representative values for bovine synovial fluid³⁴ we have from (27):

$$r^2 = v_2/v_1 \cdot \frac{800 \cdot \eta \cdot s}{c(1 - \bar{V}\rho)}$$

$$= \frac{800 \cdot 0.5 \cdot 2 \cdot 10^{13}}{0.12(1 - 0.7)}$$

$$\text{or } r = 5 \cdot 10^{-5} \text{ cm} = 0.5 \mu \quad (28)$$

On comparing this value with the pore diameter of ca 1μ required to prepare U.F.R. from bovine synovial fluid⁸⁹ the agreement is very reasonable in view of the conditions and the approximations used. The value of η for hyaluronic acids is known to increase steeply (Fig.9) with concentration and decreasing shear rate, so that once started, filtration will progressively increase.

The chief approximations used to derive equation (27) are that : (1) Poiseuille's law can be applied to the flow of a non-Newtonian fluid through a porous disc and (2) that the mean linear flow through a capillary equals $\frac{(\text{volume flow})}{\text{area}}$ which clearly does not hold for a non-Newtonian fluid (a non-Newtonian fluid is one in which the viscosity varies with the rate of shearing). More detailed treatment involves algebraic complications, which the unknown nature of the porous disc does not justify.

B.9.4. Applications of the theoretical treatment of the formation of ultrafiltrate residue (U.F.R.)

Attempts at making U.F.R. from human synovial fluid and from umbilical cord extracts failed. If the appropriate data are placed in equation (27) pore diameters of much the same order of magnitude as for bovine synovial fluid are calculated (e.g. for umbilical extract $r = 0.6\mu$).

This would indicate that (1) the derivation of equation (27)

is not sufficiently accurate, but also (2) that conditions for the successful manufacture of U.F.R. from human material cannot be very different from that used for bovine fluid.

Sintered glass filters of smaller pore size were unobtainable. Now η greatly varies with shear rate for these solutions and it was possible to decrease the shear rate occurring during filtration, by applying a smaller net filtration pressure than that of about one atmosphere normally used.

A trial was made with bovine synovial fluid (B.8.2.3.) and a sintered glass filter of number 4 grade:5-10 μ pore diameter. Various filtration pressures were applied and the filtrate checked for polysaccharide by adding acetic acid (protein being present). Mucin clots, indicating polysaccharide, in the filtrate, were obtained when the filtration pressure was greater than 2 - 3 $\frac{1}{2}$ cm. of mercury, but not at these low pressures, which only allowed a very slow rate of flow. The results of this experiment thus support the previous theoretical treatment.

Similarly an ultrafiltrate residue was obtained by very slow filtration of umbilical cord extracts on a sintered glass No.5 filter (pore size ca 1 μ). The method was, however, not a practical one, because bacterial growth could not be controlled during the long time taken (several

weeks) for the requisite number of washes.

The theoretical treatment of ultrafiltration suggested several more ideas:

- (1) A way of investigating, in vitro, the action of hyaluronidase as used clinically; this is separately described in (B.8.).
- (2) A mechanical function of hyaluronic acid-water systems in connective tissue and particularly in umbilical cord; to be described later (B.12).
- (3) A possible form of interaction of hyaluronic acid and other large molecules, in particular proteins; to be described now.

The results of the present work (Tab.3) are in accord with the conclusions of Ogston & Stanier⁹¹ that the hyaluronic acids are greatly hydrated. These polymers could themselves then be treated as porous plugs, and there might be similar limiting conditions at which they in turn act as ultrafilters for smaller molecules, in particular for protein. Qualitatively, carboxyhaemoglobin, applied to a pad of U.F.R. formed in a sintered glass filter, is held back to a considerable extent. The experiment was not elaborated because it would be difficult to ensure that such a pad of U.F.R. had, or developed no holes or channels.

B.10

DISCUSSION

At the end of each of the foregoing chapters there is a detailed discussion of the experimental techniques used, the results that were obtained by means of them and of the conclusions drawn.

It remains to give a general discussion. First are given some general considerations of the nature of inter-cellular space and of the materials found in it. This leads to some mechanical considerations of connective tissue. A hypothesis is then formulated for the part which hyaluronic acids can take in such a mechanical function of connective tissue, and this is applied in particular to Wharton's jelly and the vitreous humor of the eye. Finally there is a conclusion.

**B.11. SOME GENERAL CONSIDERATIONS OF MACROMOLECULAR SUBSTANCES
IN INTERSTITIAL SPACES**

At present only few functions can be deduced for the macromolecules of interstitial space, and of these mechanical and defensive functions seem to be the principal ones. To take a broader view two methods of approach will be used:

(1) to consider the local interaction of these interstitial substances with their neighbouring cells and (2) to regard the interstitial substances as part of the integrated physiology of the whole organism.

B.11.1. Local relationships

Following Claude Bernard we ask how far interstitial macromolecules could affect and control the immediate environment of cells. They can do so by chemical and by physical interaction. Of chemical interactions a metabolic role is unlikely, as substances such as epidermin, elastin, collagen etc. are as far as is known relatively inert. There may be other macromolecules, such as plasma proteins which have metabolic function. On the whole one would expect those macromolecules of extracellular space which have a pronounced function in the transport of metabolites, such as haemoglobin, to be primarily present in the circulatory system and not in the interstitial part of the extracellular space.

Kremer has suggested that the polysaccharides of interstitial space could act as glucose stores in the embryo, but in view of the high hydration of these macromolecules this would be uneconomical, and in an evolutionary sense unlikely.

Macromolecules could exert chemical effects on the immediate environment of cells by keeping certain chemical groups in this vicinity. They could modify the ionic environment (e.g. Ca^{++} , K^+ , Na^+ , Cl^-) by possessing anchored, charged groups, such as the $-\text{SO}_3\text{H}$ groups of the sulphated polysaccharides, and would of course at the same time affect the distribution and free energy of the vital medium, water, around the cells. Lastly, chemically, they could also protect a cell against untoward influences, either in a manner similar to the suggestion of Rogers that polyanions might act as general enzyme inhibitors (e.g. heparin and chondroitin sulphates) or by interfering with the diffusion of other, macromolecular matter. An example of this latter case is probably the ground substance in which the ripe ovum is often enclosed, and which can be removed by the haluronidase of the sperm.

Of physical interactions of macromolecules and cells, only a mechanical one appear significant. Such interaction of confinement, cohesion and support can either be direct or also involve water; this will be dealt with later.

It is interesting that some single celled organisms, such as pneumococci have a local macromolecular environment, a polysaccharide capsule, that some streptococci surround themselves with a hyaluronic acid similar to that found in the mammalian connective tissue, and that generally loose associations of cells are often found in a polysaccharide slime, as in the seaweed fucus.

B.11.2. General relationships

What are the physiological functions of connective tissue? There come to mind: (1) storage (2) an environment for undifferentiated cells (3) protective functions (4) mechanical functions.

(1) By previous argument connective tissue is unlikely to act as a store for metabolites, though it may act as a temporary water reservoir, as instanced in the various states of the body from oedema to severe dehydration, and may passively act as a store by occupying a certain space. Mineral metabolism, particularly that of calcium and of chloride, is linked with connective tissue and here macromolecular polyions will play some part.

(2) Undifferentiated or primitive cells are often found in a matrix of loose connective tissue. It is difficult to see what functions the macromolecules of such interstitial tissue could have towards these cells, apart

from those previously mentioned. Such macromolecules could exercise some general isolation and temporary support during the shaping of new cell masses, as might be instanced in embryonic development or in the partial isolation of a healing wound. Nature cells may of course be similarly supported.

(3) The discovery of spreading factors, and of inhibitors to them, showed that interstitial substances do exert a defensive function against foreign organisms and substances.

B.11.2.4 (4) Mechanical functions

These can be divided into two groups: (a) where interstitial substances act in an accessory fashion, and (b) where they occupy a primary role.

B.11.2.4a Accessory functions are lubrication and the holding together of small aggregates of cells.

Synovial fluid is a good lubricant and owes this property to the hyaluronic acid which it contains. The hyaluronic acid found in fascia may similarly allow tendons, muscles and other organs such as the lungs to glide smoothly over adjoining structures.

The holding together of small aggregates of cells is achieved both by interstitial cementing substances, as

in the walls of blood capillaries, and by a pervading system of fibres, as in liver. In order to transmit tension both effects must occur; the fibres of a tendon must be cemented together and linked to muscle cells.

B.11.2.4b Mechanical systems entirely composed of interstitial substances

All the mechanical forces occurring within a body can be resolved into a set of tensile and compressive forces. This includes such compound functions as bending and twisting. (Unless stated to the contrary the term compressive force is the vector quantity of mechanics and should not be confused with the non-directional pressure or compression of a hydraulic system). The mechanical components of the body may be separated into three categories:- (1) fibres, which only resist tension, (2) water which is an incompressible fluid, (3) skeletal matrix which can withstand both tensile and compressive forces.

The skeleton (particularly the endo-skeleton) alone is insufficient to maintain the structure of the body. The way in which tensile elements act in fascia and tendon is clear enough, but there must also be structures other than bone and cartilage which can resist compressive forces. The problem which will now be dealt with is how a combination

of fibres and water can provide such a structure.

First consider the matrix of bone: a fibrous network resistant to tension is embedded in a mass of inorganic crystallites resisting compression. To provide a similar system from water and fibres, the water must be prevented from moving, from being "squeezed out" when a pressure is applied. This could be done by enclosing the water in small bags, which are held together by cohesion of their walls or by fibres, and a qualitative proof of this is given in a subsequent section. It will also be shown that the smaller the bag, the better can the system function.

In most parts of the body cells conveniently act as such bags, the bulk flow of water across their membranes being greatly retarded. Indeed some cells may specialise in this mechanical function, in particular the fat cells which occur as pads in the hands, and feet and at other locations where considerable pressure is applied to a structure which must be flexible.

What system is there, however, to resist compressive forces where there is no skeleton and there are only few cells, that is in such regions as areolar tissue of skin, umbilical cord and vitreous humor of the eye? It is suggested that coils of certain interstitial molecules entrap water and so take the place of the cellular bags. First, however, this

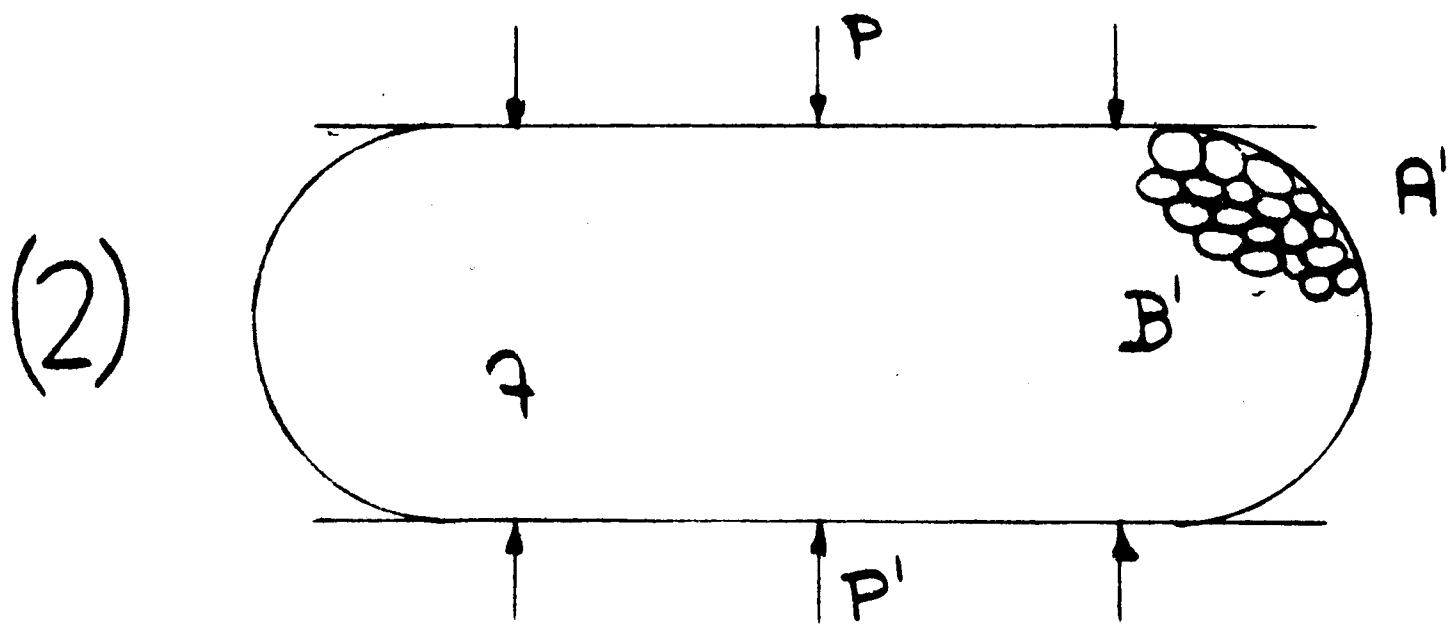
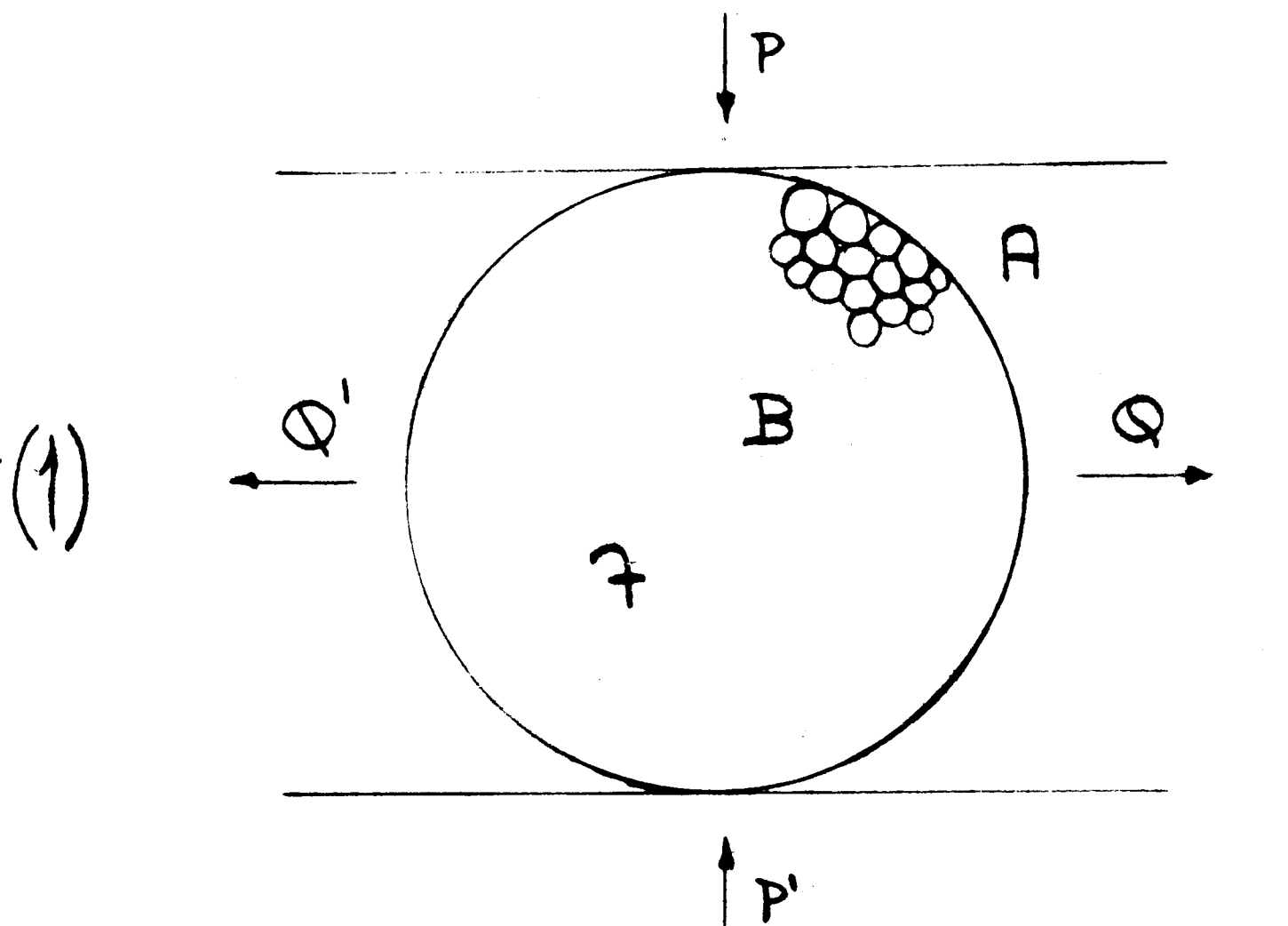
postulate of a system of bags will be more closely considered.

B.11.3. To show that a collection of bags, each holding an incompressible fluid and themselves held together, forms a structure which resists deformation

That a space filled with fluid under pressure forms a unit which can resist compressive forces, is familiar enough from the physiological mechanism of erectile tissue. It is not obvious, however, that if such a space is subdivided into separate compartments the total resistance to compressive forces is increased.

The basic concept is that the shape which has the smallest surface in proportion to its volume is a sphere, thus a balloon or bladder with elastic walls takes up the form of a sphere when filled with a (weightless) incompressible fluid. Any deformation of such a bladder, by squashing, must increase the surface area and thus work is done against the elastic forces of the walls of the bladder. Qualitatively it can be seen that as the work is done by stretching the containing wall, if the total area of wall which has to be stretched for a given deformation can be increased, the total work which has to be done is increased and therefore the resistance to deformation is increased. Subdividing the bladder into small compartments can cause such an increase

Fig. 11.



in wall area, until ultimately the resistance to compression simply depends upon the density, or fraction of total cross-sectional area, of the elastic material constituting the compartment walls. It is easier to visualise such systems in two dimensions, and a more formal derivation of the same system may clarify some points.

Consider (see (Fig.11)) the enclosure A to be made of elastic material and to contain an incompressible, two dimensional fluid F, which has been introduced under some pressure. The enclosure A will take up the form of a circle as in (Fig.11(1)).

If two linear enclosures are now brought up and a compressive force is applied along the line PP^1 , then deformation results along Q_1Q^1 with the conditions that the area enclosed by A remains constant (incompressible fluid). As A is no longer a circle its length (perimeter) must have been increased as in (Fig.11(2)), say to A^1 from its previous state and work must have been done on it.

Consider the original state of A and subdivide it into the set of enclosures B, small with respect to A, and made of the same material as A. Again fill the system with the two dimensional incompressible fluid so that A again

becomes a circle, of the same size as before and the units B will also be circular (really polygonal).

Apply compression along $P.P^1$ so that A again assumes the shape (and size) A^1 . In this process deformation of the system B to B^1 must have occurred, if no slip is allowed between adjoining enclosures of B. The total work done is now the sum of the work done in elongating A to A^1 , plus the work done in changing the system B to B^1 . This is greater than the work done in changing the system A alone to A^1 .

Now A and B can be made any desired size.

Therefore a system of small units (B) filled with an incompressible fluid will form a structure opposing deformation.

B.12 THE PART PLAYED BY HYALURONIC ACID IN THE MECHANICAL
FUNCTIONS OF CONNECTIVE TISSUE

B.12.1 In the previous section a model structure was proposed which would resist compression. In this section the following proposals are put forward and examined: that polymers of the type to which hyaluronic acid belongs can, together with water and fibres, form an analogous structure to the previous model, and that this does occur in regions of the body where there are few or no cellular 'bags'.

These proposals are conveniently separated and examined under the headings:-

- (1) That there are regions of the metazoan body which for reasons of development or of mechanics are poor in cells and which are exposed to compressive forces.
- (2) Such regions contain hyaluronic acids or related compounds and tensile elements (collagen and elastic fibres, sometimes strands of smooth muscle; any other type of fibre which might be discovered).
- (3) Hyaluronic acid offers a high resistance to both
 - (a) water being moved through it and
 - (b) it, the hyaluronic acid, moving with respect to the fibre system.

B.12.2. Discussion of the previous proposals

Proposal (1):- Regions of the metazoan body which are poor

in cells and are exposed to compressive forces include:-

(1) embryological structures in which rapid alterations of cells have to occur. (2) umbilical cord (3) the vitreous humor (4) some parts of the placenta (5) areolar tissue - as in the skin (6) to a small extent in lung and spleen (7) joint fluid (8) some of the near environment of nerves and blood vessels.¹⁴³

Proposal (2):- the substances mentioned occur in all the regions enumerated under postulate (1).

Proposal (3):- a high resistance of hyaluronic acid to being moved through tissues and to water moving through it is indicated by (1) the resistance offered to subdermal infusions, (2) the resistance to spreading of small quantities of solutions containing dye-stuffs⁷⁶ (3) the high frictional coefficient of hyaluronic acid-water systems as indicated by a low diffusion coefficient in vitro⁸⁹, (4) the known high viscosity of hyaluronic acid solutions, (5) experiments with hyaluronidase such as: (a) facilitating subdermal infusions and spreading experiments, (b) increasing the permeability of fascia²⁵, (c) enabling the hyaluronic acid containing material of the swollen sex skin of the Rhesus monkey to be displaced to other regions by applying mild pressure after hyaluronidase had been injected¹¹¹

Given that the previous three postulates are valid, how can a system of hyaluronic acid, water and fibres, oppose compression in line with the theoretical scheme previously given?

Ogston⁹⁵ has compressed two surfaces between which there was a free film of synovial fluid and showed that neither did the film break down, nor were the surfaces approximated as closely as in a control system without hyaluronic acid. He concluded that synovial fluid has a special capacity for forming a layer which has some degree of elasticity. A separation of 300-500A between the surfaces was calculated for the loads and concentrations used. This distance is of the order of magnitude of the diameters calculated for hydrated hyaluronic acid particles.

In the previous theoretical treatment the bags B need neither be within a common enclosure, nor need their surfaces be joined together rigidly, provided that there is sufficient interaction between adjoining bags. Thus it is possible to stand on top of a pile of filled hot water bottles.

Suppose that the individual hyaluronic acid particles correspond to the bags B (B.11.3). From sedimentation and diffusion studies we know that the hyaluronic acid polymer

offers considerable resistance to water flowing through it, and from Ogston's experiment we infer that the hydrated polymer particle resists compression. (A particle composed of a random coil resists deformation - which would make it less random).

Provided, then, that the particle-particle interaction of a hyaluronic acid system can be sufficiently increased, the system ought to withstand mechanical compression as previously outlined.

The high viscosity and visco-elastic properties of a solution of hyaluronic acid indicates considerable particle-particle interaction. Regard the whole solution of hyaluronic acid as a solvent and introduce long particles into it. The viscosity of the whole system will be greatly increased, as follows from such theories as Simha's¹²⁵ for the viscosity of solutions. In other words, the relative movement of different particles of hyaluronic acid is still further decreased by introducing fibres, and at a sufficient concentration of fibres the whole system may be able to resist mechanical compression. On compression such a system will of course flow, because it is only visco-elastic. The rate of yield being dependent on conditions; this rate of flow may be very low. In the animal body, the spaces which such a system fills are more in the nature of channels and thin

beds, than bulk cavities (except in the vitreous humor, where there is a definite enclosure corresponding to the enclosure A of the theoretical treatment (B.11.3.)), so that the system: hyaluronic acid-water-fibres has partial confining walls with which there is viscous interaction and thereby flow along the channel or bed will be further retarded. Traversing strands of fibres, vessels, cells and other inclusions will provide further resistance.

It is suggested that such a system holds for umbilical cord, which contains much hyaluronic acid and is pervaded by numerous fibres of varying size and aggregation.

Such a scheme may very well hold for vitreous humor which is composed of a dilute hyaluronic acid and protein gel traversed by thin (order of 500A) fibres of vitrosin⁴⁴, a protein of the collagen class. When removed from the eye the humor is a visco elastic solid which slowly flows. To quote Pirie¹⁰⁶,

"Although hyaluronic acid and the insoluble collagen-like protein of the vitreous humor do not seem to have any chemical relation in the humor, yet they do influence each other. Aggregated hyaluronic acid has a very large particle size, and the hyaluronic acid-containing jelly of the vitreous humor may be considered as an unstable structure that interacts with the structure of the insoluble protein. Lique-

fraction of the insoluble protein is slowed down by the presence of aggregated hyaluronic acid, and disaggregation of hyaluronic acid is slowed down by the presence of insoluble protein. Each structure tends to preserve the other, and it seems that pathological changes in one might also lead to change in the other."

A cock's comb is another example of a location where hyaluronic acid may function in this way. The comb is a fairly dense, cellular and fibrous tissue from which hyaluronic acid can be extracted¹². There exists a rudimentary vascular erectile tissue, of twisted and dilated vessels, but there are no cavernous spaces¹⁴¹. Both parts of the extracellular system, circulatory and interstitial, would here appear to combine with cells to form an organ of moderate rigidity.

As was pointed out earlier, the total strength of a system of 'bags' will increase with increasing density of tensile elements, until ultimately there would be little else but fibrous tissue. A convenient example of this is the baby hippopotamus, whose hide is a regular, three-dimensional weave of fibre bundles, separated by but little interstitial gel¹⁴².

B.13

CONCLUSIONS ON HYALURONIC ACIDS, IN PARTICULAR IN UMBILICAL CORD

In what has gone before it has been shown that extracts from human umbilical cord have physical properties closely akin to those of human synovial fluid. Further, that these properties can be expressed in terms of a large, hydrated particle (of molecular weight of about 4×10^6 and hydration of the order of 300 ml./g.) as a model for the polymer arranged in a partially rigid coil. It was shown that the viscous solutions of such a polymer will, in confined spaces, encounter a resistance to flow of the order of its resistance to water flowing through it, and the approximate diameter of such an equivalent capillary was calculated.

Histologically it was shown that Wharton's jelly is traversed by numerous fine fibres, of which at least many are collagen fibres. By a qualitative theoretical treatment it was indicated that a suitable system of hyaluronic acid, water, and randomly orientated fibres could offer a considerable resistance to mechanical compression. This is in accordance with the practical handling of pieces of dissected Wharton's jelly, especially if the cord or dissected piece has previously been swollen by immersion in water.

That the vital blood vessels of the umbilical cord remain patent through the whole period of gestation is remarkable. The pressure of blood in the umbilical arteries

is only some 30 mm of mercury, and in the vein it will be considerably less, perhaps 5-10 mm of mercury. The system will be nearly independent of intrauterine pressure, as this is transmitted equally, through the amniotic fluid, to foetus, umbilical cord and placenta. Although Wharton's jelly is a degenerative remnant of embryonic tissue, it should help to prevent kinking of the umbilical vessels, somewhat akin to rubber pressure tubing being less readily kinked than corresponding thin walled tubing. Wharton's jelly may also prevent premature occlusion of the umbilical vessels during birth.

A study of the action of hyaluronidase led to the conclusion that the enzyme, as it is used in clinical practice, probably causes such a change in the hyaluronic acid of ground substance, that the ground substance is pushed with the infusion liquid through the infiltrated tissues.

In order to make model experiments of hyaluronic acid-water-fibre systems which would be reasonably representative of conditions in vivo, the corresponding concentrations would have to be known. To determine these would however have required a large number of analyses of doubtful validity. Umbilical cords are inherently rather varied, and the treatment they receive after birth is also not uniform.

I was not in a position to obtain a number of cords that would have been neither hydrated nor desiccated by the treatment received at the hands of the nursing staff.

Estimation of the total concentration of hyaluronic acids in cord was therefore not attempted (there would also have been the difficulty of making exhaustive, representative extracts), and the estimation of fibres would have posed similar problems.

IT C

MCQUEEN COLLEGE

C.1.

GENERAL INTRODUCTION - COLLAGEN AND THE SOLUBLE COLLAGEN

Collagen is the name of a group of structural proteins occurring, with variations, in many parts of the animal kingdom^{5, 66}. The chief sources are tendon, bone and hide of mammals, and various parts, such as the swim bladder of fish. The group has a characteristic amino acid composition^{5, 66, 132}, in particular: (percentages for bovine collagen): a high concentration of proline (12%) and varying amounts of hydroxyproline⁴⁵ (10%), a large proportion of glycine (35%), a large number of total amino acids with non-polar side chains (51%), and an appreciable amount of hydroxylysine (0.7%). The content of aromatic and sulphur containing amino acids is very low, and there are not many polar side chains.

Most collagens are fibrous, or are related to fibrous material. Under the electron microscope most fibres show a characteristic pattern of cross banding^{5, 138}, in particular after staining with phosphotungstate or after metal shadowing. A major repeat distance of about 640A is normally found in mature vertebrate collagen, but a subspacing of 210-220A may be very prominent, especially in embryonic material¹⁴⁰. Quite a number of further subspacings all perpendicular to the fibre axis may be observed, and in the native material they form a pattern which is orientated

with respect to the direction along the fibre ("polarized"). Width and length of fibres vary over a wide range.

A third characteristic of the collagens is the type of X-ray diffraction pattern which they give⁵. This consists of two parts (1) wide angle and (2) small angle patterns.

(1) A prominent equatorial distance of 11-17Å is indicated, according to the state of hydration, but there is no real evidence for longer repeat units in this direction. Of the wide angle meridional spacings that of 2.86Å is the most characteristic, particularly distinguishing these proteins from the k-m-s-f group of fibrous proteins⁶⁹.

(2) Meridional small angle reflections are found, corresponding to the repeat distances of hundreds of angstroms seen with the electron microscope.

The representative members of mammalian collagen, such as the principal component of bovine Achilles tendon, are characteristically inert towards enzymes and chemical reagents, and are insoluble. On hydrolytic treatment of varying severity, in particular on boiling with acids or alkalis, such collagen fibres swell and then pass into solution as gelatin.

Gelatins are degraded collagens, having lost all long range order as indicated by X-ray or electron microscope

investigation³⁰. According to temperature the gelatin can exist in two forms: a "cold" form below 20-30°C and a "hot" form at higher temperatures. The two states will be discussed in detail later. From¹¹² infra red, polarimetric and Xray studies it has been concluded that material in the "cold" form resembles intact collagen, but not so in the "hot" form. For example, the wide-angle Xray diffraction pattern of gelatin resembles that of collagen only if the gelatin is in the "cold" form. An initial cleavage product of collagens has been postulated and called "parent gelatin".³⁸

In 1900 Zacharides¹⁴⁰ showed that very dilute acetic acid would dissolve some collagen from tendon. Hageotte^{86, 87} then found in 1927 that dialysis of such a solution against water or suitable saline, gave a precipitate, consisting of needle-like crystals as observed under the microscope. Like collagen, the precipitate stained with van Gieson.

When the electron microscope became available this needle-like precipitate proved to consist of fibres with 640A spacings, just like preparations made by ultrasonic disintegration from tissues. It is thus possible to re-constitute material with long range order from solutions of collagen, and in this respect soluble collagen differs sharply from gelatin.

The soluble collagens, then, with the exception of some invertebrate collagens which will not be discussed, are soluble substances which by amino acid composition are collagens, and directly or indirectly can be demonstrated to possess long range order.

A group of Russian workers under Orekhovitch^{16, 20, 96, 97, 98, 133} extracted soluble collagens from the skin and organs of various animals with citrate buffer pH 3.5. They partially purified this material by dialysis against water, salt solutions or phosphate buffer and made tentative estimations of amino acid composition and molecular size. They found that this material occurred more abundantly in the skins of young animals than in those of old ones, and somewhat enthusiastically named their substance, or group of substances, procollagen.

Gross, Schmitt & Higherberger^{41, 42, 52, 119, 121, 122} working in the United States made similar extracts, and in particular have investigated a collagen readily extracted from carp swim bladder⁴¹ by dilute acetic acid or citrate solutions, and named ichthyocol. In numerous studies with the electron microscope it became clear that the same material can be precipitated in different fibrous forms, showing differently spaced cross banding. The controlling

factors seem to be the presence of other substances in solution⁴²; thus when considerable amounts of such polysaccharides as hyaluronic or chondroitin sulphuric acid are present structureless fibres result, while with smaller quantities fibres with long spacing (P.L.S.) of the order of 2000A are produced, and in complete absence of extraneous material the normal 640A spacing is formed. In the presence of A.T.P. long spacing fibres only one segment long are formed, and these segments aggregate laterally (S.L.S.). These forms could be converted into each other after resolution. From such studies a primary unit, called the tropo-collagen molecule⁴², was postulated to exist in solution.

Boedtker & Doty¹³ have observed the light scattering, osmotic pressure, flow birefringence, sedimentation and viscosity behaviour of acid solutions of ichthyocol. They obtained closely similar molecular weights of $300,000 \pm 50,000$ and concluded that the material was homogeneous. The model of the particle which best fitted their measurements was a rigid rod 2,500 - 3000A long, 14A diameter, with an assumed hydration of 34%.

Gallop³⁴ working on a similar, but more crude extract obtained a molecular weight of 1.7×10^6 and concluded that the material was in the form of a stiff rod consisting of not more than three peptide chains.

The Russian workers have adduced various physical parameters to their extracts. Bresler¹⁶ gave a molecular weight of 70,000 and ignoring hydration made unwarranted calculations for an ellipsoidal model. On repeating the sedimentation and diffusion measurements, Orekhovitch now estimates a particle of weight 700,000, length 6,700A and width 13A.

Cohen²² found the specific optical rotation of solutions of ichthyocol in acid to be -350° and polarimetrically followed the conversion of this substance into gelatin.

A group of workers⁴⁹ at the National Institute for Medical Research, Mill Hill, injected young rabbits with ^{14}C labelled glycine and followed the incorporation of ^{14}C labelled glycine into various collagen fractions obtained from skin. The skins were successively and exhaustively extracted with phosphate buffer pH 8-9, and then with citrate buffer pH 3.5, and the insoluble collagen in the residue obtained as gelatin after autoclaving. On dialysing the phosphate extracts against water a precipitate resulted, which was shown by partial amino acid analysis to be a collagen, and was named "alkali soluble collagen". After

the water dialysis it could, however, not be dissolved in phosphate buffer, though it did readily dissolve in citrate buffer pH 3.5. From the extracts of skin made with citrate buffer, a collagen was precipitated by dialysis against water or 5% NaCl solution, and this material was called "acid soluble collagen". The specific radioactivities of glycine isolated from the various collagen fractions was measured. From the rates of incorporation of C^{14} it was concluded that alkali soluble collagen was rapidly formed and then converted to insoluble collagen, with acid collagen probably forming an intermediate stage.

Jackson⁵⁵ modified the extraction of alkali soluble collagen, using phosphate buffer pH 7.0. The material was salted out at half saturation with NaCl, and could again be dissolved in phosphate buffer at pH 7.0. The procedure was repeated several times. At this stage, Professor A. Neuberger and Dr. Jackson invited me to make physical measurements on this substance, and a number of batches of material were made available to me. The investigations which are to be described were principally made on this substance, which I also isolated myself according to a slightly modified procedure. The substance has been named "neutral salt soluble collagen" and must be very closely related to, if not identical

with the previous "alkali soluble collagen". I have found it soluble also in acid. Collagen extracted with citrate buffer pH 3.5, after removal of neutral salt soluble collagen from the skins, will be referred to as acid soluble collagen.

Crude extracts, equivalent to solutions of neutral salt soluble collagens, have been prepared by the American workers⁴³, who have also used physiological saline and serum ultra filtrate as extraction media. They independently found the "heat precipitation" which was found in this investigation, and their paper was published while this work was in progress. Orekhovitch⁹⁹ has compared materials isolated by the methods of the Hill Hill workers and by his own, and his findings will be mentioned later. He also showed that the incorporation of C¹⁴ into soluble collagens is practically abolished in scorbutic guinea pigs.

Jackson⁵⁶ has recently studied the rate of formation of the various collagens in granulomas which were induced in guinea pigs by subdermal injections of the sulphated polysaccharide carageenin. His findings are in accord with previous studies.

C.2. STORY OF THE INVESTIGATION OF NEUTRAL SALT SOLUBLE COLLAGEN

(The subject is not presented in the chronological order of the investigation)

The material was supplied to me so that I might make an estimate of homogeneity and molecular dimensions on the basis of some physical measurements. This was done, and among the conclusions were that (1) the material was not homogeneous (2) most of it ought to be visible under the electron microscope. Electron microscopic studies confirmed the general molecular dimensions deduced from the measurements made in solution. Evidence that the material consisted of three main fractions was obtained from a set of experiments based upon the following property of the material.

When solutions of neutral salt soluble collagen are warmed to 15-37°C a precipitate forms, which has been called "heat precipitate". This precipitate has two characteristics: (1) it is fibrous and (2) part of it will dissolve again on standing in the cold. Evidence for heterogeneity of the original solution was obtained in experiments in which this variation of state with temperature was used.

The fibrous nature of heat precipitate was further investigated with the electron microscope, which showed the fibres to be closely akin to those of mature collagen.

The nature of the change from solution to fibrous state was therefore thought to be interesting. From various kinetic studies it was concluded that the change has some of the characteristics of protein denaturation.

When the material was first supplied to me it was only known that it readily changed to an insoluble form, which was suspected to be acid collagen. Some of the studies on heat precipitation were indeed made in order to devise conditions under which the solutions would be stable during physical measurements, and such conditions were found.

It was further found that the material remains in solution, under certain conditions, at acid pH. This was made the basis for a partial separation of the components of the material. Polarimetric studies on solutions of whole neutral salt soluble collagen at different temperatures showed it to be closely similar to acid soluble collagen in specific rotation and in its conversion to gelatin.

When the material had been shown to be heterogeneous, it became necessary to investigate the method of isolation. An investigation was made of how often the procedures devised by Jackson had to be repeated in order to obtain material of constant composition, as judged by certain criteria. Coupled with this investigation was an estimation

of the concentration of phosphorus and of material absorbing ultraviolet light. The phosphorus estimation was made because of the possible occurrence of phosphorhydroxylysine in neutral salt soluble collagen; the measurements showed this to be unlikely. The measurements of material absorbing in the ultra violet range were made to estimate tyrosine, and possible purine and pyrimidine contaminants.

Description of the work is arranged as follows:

- a) Preparation of the soluble collagens.
- b) Factors affecting solubility and precipitation of neutral salt soluble collagen.
- c) Studies on the partial re-solution of the "heat precipitate" of neutral salt soluble collagen.
- d) Studies on the isolation of neutral salt soluble collagen and the phosphorus content of the material, also the rate of incorporation of ^{32}P into it.
- e) Investigations with the optical and electron microscopes.
- f) Behaviour of neutral salt soluble collagen at acid pH.
- g) Polarimetric studies.
- h) Physical measurements.
- i) Conclusion

N.B. "Neutral salt soluble collagen" is used both as a generic term and with reference to particular samples. The source of particular samples is to be understood to be rabbit skin, unless another source is named.

C.3. PREPARATION OF NEUTRAL SALT SOLUBLE COLLAGEN⁵⁵

C.3.1. Material: Rabbits: of Himalayan strain 4-8 weeks old,
killed by air embolism.

Rats: Oxford laboratory strain, 80-100 g.
decapitated.

C.3.2. Extraction and purification

The animals were skinned immediately after death; the skins, cleaned of any adhering tissues and washed with demineralised water, were made into small rolls, frozen in solid CO₂ and then finely sliced. The material was extracted with neutral phosphate or versene buffer overnight, using about 300 ml. of buffer per rabbit or 75-100 ml. per rat. The mince was strained off with muslin and two further extracts were prepared in the same way. Each extract was filtered through sgar paper (Green's 904¹), and in the rat preparations were clarified in the preparative ultracentrifuge (Rotor 20, 19,000 r.p.m., 1 hour). Solid NaCl was added with gentle stirring to 16-17% w/v (18 for versene extracts) and the precipitate was allowed to settle overnight. After siphoning off some of the supernatant, the precipitate was collected on the centrifuge (for versene extracts the ultracentrifuge was used as before). The

material was dissolved in fresh buffer, of about $1/20$ th the original volume, and the solution clarified; in the last rabbit preparation and in all the rat preparations the ultra-centrifuge was used for this. 30% NaCl solution, or solid salt, was added until a permanent precipitate just formed, which was left to stand overnight, and the various procedures were then repeated. The material was precipitated 4-5 times in all by the addition of NaCl, and the final precipitate was stored at -30°C .

It was most important not to allow the material to warm up to room temperature at any stage, as this would cause it to become insoluble at neutral pH.

C.3.3. Preparation of acid soluble collagen

After the minced skins had been extracted three times at neutral pH, they were similarly extracted with citrate buffer at pH 3.7. The material separates from the extracts at 5% NaCl concentration and was purified by repeated precipitation, either with salt, or on dialysis against tap water, followed by dialysis against demineralised water.

C.3.4. Preparation of solutions of soluble collagens

An appropriate amount of material was quickly weighed out from stock and then the appropriate volume of buffer

was added in small portions, gently stirring and "mashing" all the time. The suspension was dialyzed against three changes of buffer, each at least 50 x the volume of the suspension and for at least 12 hours. The suspension was clarified on the preparative ultracentrifuge (Rotor 44 39,000 r.p.m. usually for 1 hour). Supernatants were crystal clear or had a slight opalescence when viewed against the light. For some of the earlier experiments the preparative ultracentrifuge was not available. Material was kept at below 10°C throughout.

**C.4. FACTORS AFFECTING THE SOLUBILITY AND PRECIPITATION OF
NEUTRAL SALT SOLUBLE COLLAGEN**

These will be considered under the headings:

- a) Method used for determining "precipitation time".
- b) General
- c) The history of the material
- d) Temperature
- e) Nature of buffer
- f) pH
- g) Other substances present
- h) Concentration
- i) Various studies connected with "precipitation time".
- j) Conclusions

The time taken for a definite precipitate just to appear under given conditions, is called the "precipitation time". It was determined in the following way:

C.4.1. Method used for determining "precipitation time"

Small test tubes (5 cm x 0.5 cm) were labelled in their uppermost portion with wax pencil. The tubes were stoppered and kept in ice as a set. The necessary solutions were made up, cooled in ice, and then quickly added to the tubes, to a total volume of 0.05-0.5 ml.

Labels were drawn out of a hat and the tubes arranged in a corresponding order in a linear rack, so that

the markings could not be seen. Each tube was provided with a small glass stirring rod and the contents were quickly and carefully stirred, the rod being left in the tube.

A linear rack had been placed in an illuminated and thermostated waterbath fitted with a glass front. The tubes were now picked at random, quickly blotted with filter paper and without looking at the marking suspended in serial order from the rack in the water bath. A stop watch was started when the first tube was placed in the water bath and the time was noted (some 30 seconds later) when the last tube was introduced. The positions of water bath, rack and level of water had been so arranged that, the markings on the tubes could not be seen.

The tubes were now regarded in linear sequence in the rack and they were stirred in turn. In due course a faint haze would appear in each tube, which could usually quickly be coalesced to a precipitate and then to a clot adhering to the small stirring rod.

When I decided that a definite precipitate had just formed in a given tube, the time was noted. The interval between this time and the time of immersion of the tube in the thermostat is called the "precipitation time".

C.4.2. General

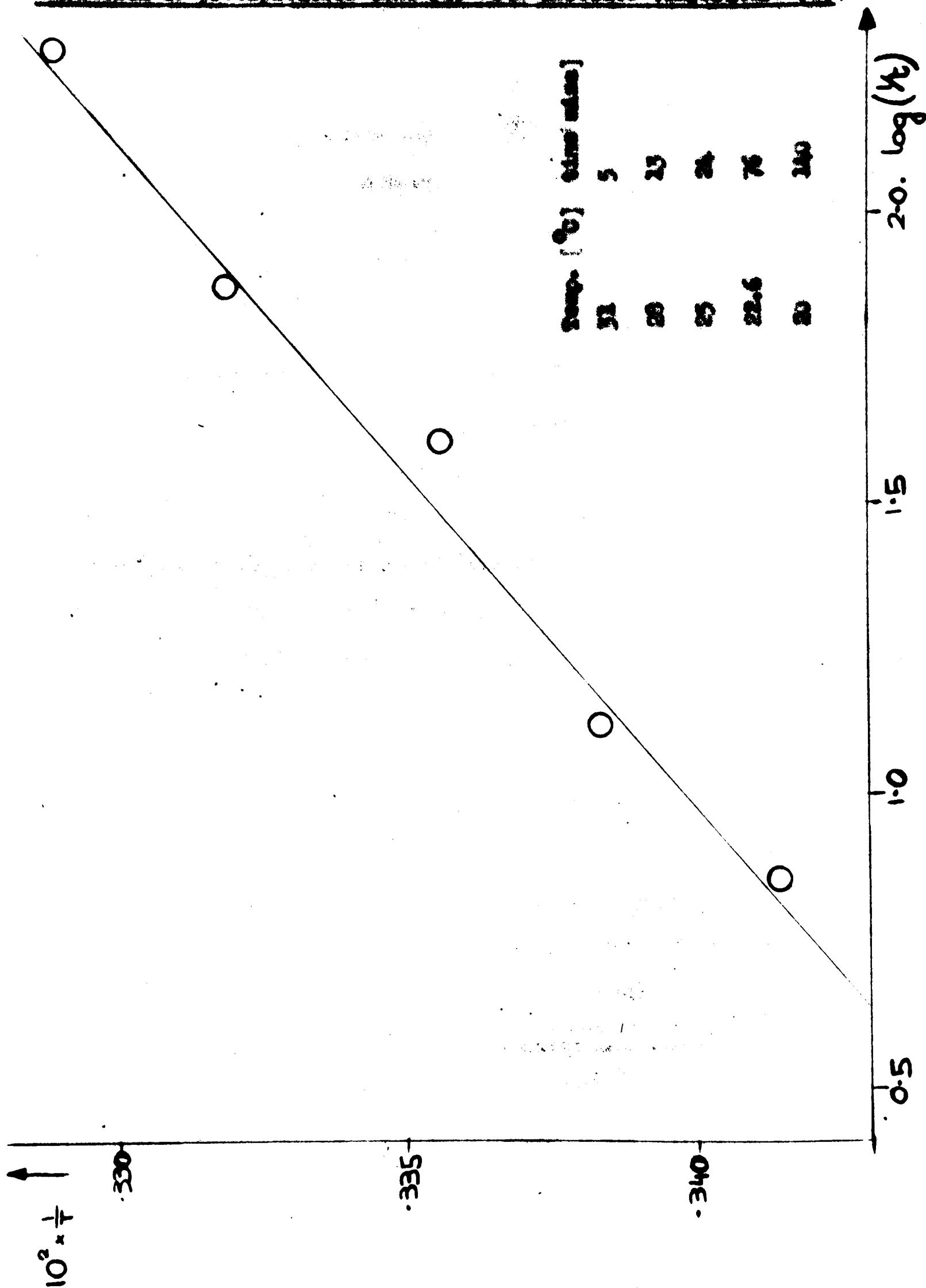
Precipitation time is dependent upon many factors, including the state of the material and of the observer. In (Fig.14) all observations made in one set of tubes (C.4.1.) are denoted by the same symbol, and it is clear that there is greater consistency within a set of observations than between them. Accordingly "precipitation times" have been used only as relative measures within a set of observations, and unknown proportionality constants would have to be applied to interrelate different sets of observations. For example: in (Fig.13) two different samples of collagen both showed a linear relationship between "precipitation time" and hydrogen ion concentration, but the slopes of the two lines are different.

C.4.3. History of the material

If the preparation had not been carried out speedily in the cold, or if the solid had been kept for any length of time above -20°C , or if an attempt had been made to remove water from the material, an increasing proportion of the material became insoluble at neutral pH. Some insoluble material always appeared. A representative sample was sample IV and an analysis of it, made by weighing and refractometry after suitable dialysis and centrifuging, was:

Fig. 12.

Variation of precipitation time (θ) with absolute temperature (T).



total non-dialysable solids = 5% w w.

of <u>these</u> :	soluble in phosphate pH 6.8	55%
	soluble in citrate pH 3.7	11%
	insoluble	33%

C.4.4. Temperature

Below 10°C a solution at pH 7.4 had not formed a precipitate after several months, a solution at pH 6.8 would give one after several weeks, and one of pH 6.4-6.6 within a few days. A solution at pH 6.8 would form a precipitate in several hours at room temperature and the variation of "precipitation time" from 20-31°C is given in (Fig.12). At 37°C most solutions give a precipitate within a few minutes.

Calculation of energy of activation E:

By the Arrhenius equation:

$$\ln k_v = \text{const} - \frac{E}{RT} \quad (29)$$

where k_v is the rate constant of the process, T the absolute temperature and R the gas constant

$$\text{Now: } k_v = q \left(\frac{1}{t} \right) \quad (30)$$

where q = a proportionality constant

t = "precipitation time"

Substituting (30) in (29) and differentiating with respect to $\left(\frac{1}{t} \right)$:

TABLE 5Some "precipitation times"

0.05 ml. of solution P8 of sample IV in phosphate pH 6.8, plus
 0.02 ml. of the following ($\frac{M}{10}$) solutions adjusted to the same
 pH (glass electrode). Temp. 29.4°C.

<u>Substances added</u>	<u>"Precipitation time"</u>
{ α -picoline	2' 40"
{ glyoxaline	3' 05"
{ collidene	3' 20"
ascorbic acid	4' 10"-5' 50"
{ phosphate buffer	5' 40"
{ phosphate buffer washing	
{ P12 of a heat ppt.	6' 10"

. Similar experiment with 0.05 ml. of solution P7 of sample IV
 and 0.02 ml. TRIS buffer pH 7.3, ionic strength 0.1:

<u>Substances added</u>	<u>"Precipitation time"</u>
{ TRIS	2' 00"
{ Supernatant from:	
{ TRIS + some of sample IV	2' 05"
phosphate	6' +

$$E = -R \frac{d \ln \left(\frac{1}{\tau} \right)}{d \left(\frac{1}{T} \right)} \quad (31)$$

Using values of (Fig.12) in equation (31):

$$E = 79 \text{ k cal/mole/degree} \quad (32)$$

Such a value for the energy of activation is of course only approximate, but it is of the order met with in protein denaturation and would indicate that the rate limiting step in the formation of a heat precipitate is of this nature.

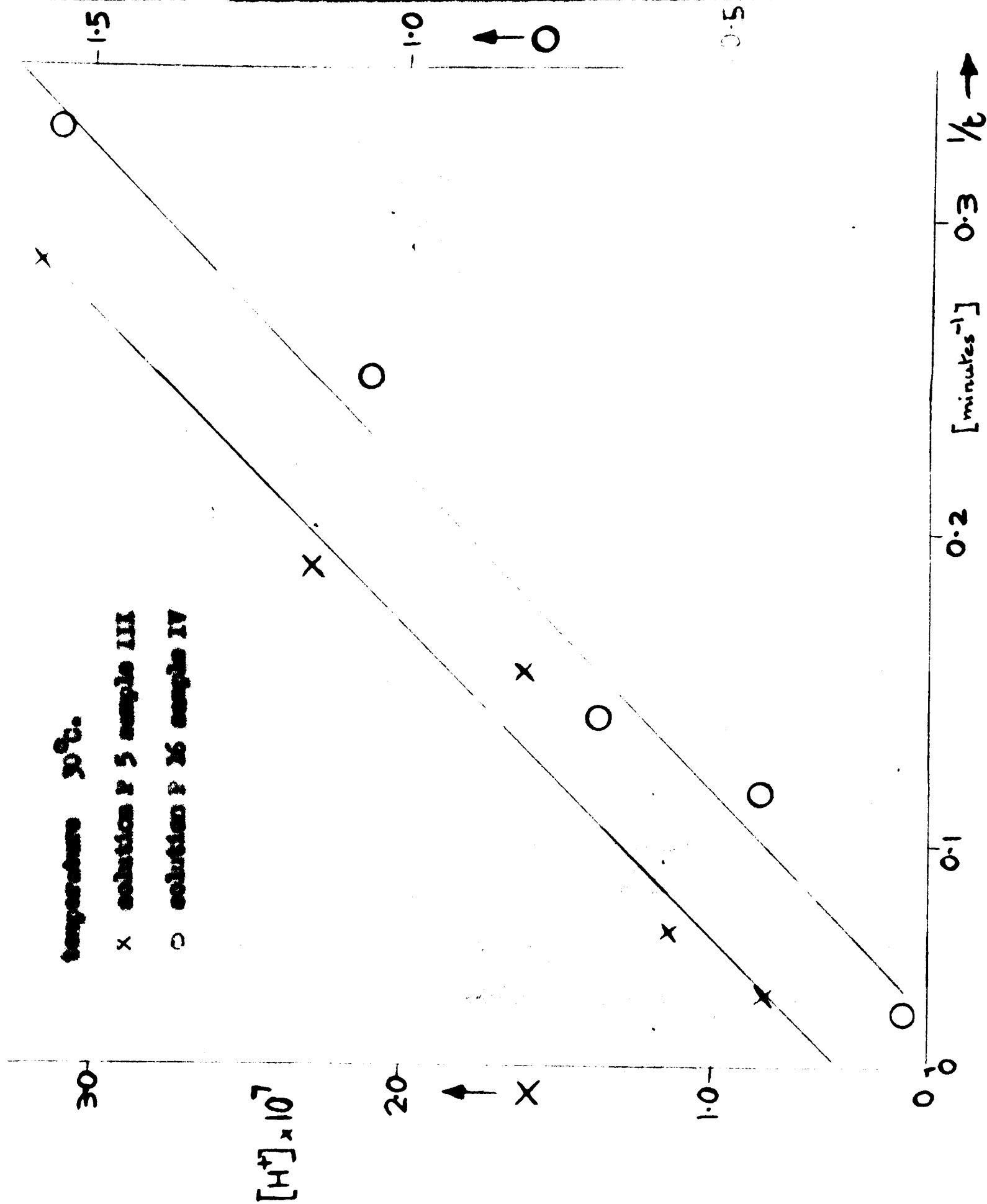
C.4.5. Nature of buffer

It was suggested that phosphate might cause aggregation and therefore other buffers were tried. Glyoxaline, γ -picoline and collidene all accelerated precipitation (Tab.5) and such action had previously been shown for TRIS buffer (Tab.5) $[\text{tri}-(\text{hydroxymethyl})\text{amino methane}]$ to indicate that the buffer would eventually completely precipitate the material in the cold. Indeed this was an irreversible process as only very little of the precipitate formed by TRIS buffer pH 7.3 in the cold could afterwards be taken up in phosphate pH 6.8, using exhaustive dialysis.

Versene, in contrast, retarded precipitation, 5 mg. ml. preventing a precipitate from forming in 17 hours at room

Fig. 13.

Variation of precipitation time (t) with hydrogen ion concentration.



temperature, where a control gave one in 4 hours. This effect was not due to the removal of heavy metal ions, which were separately shown to reduce precipitation time only very slightly (Co^{++} , Fe^{+++} and Al^{++} were tried. Hg^{++} gave an immediate precipitate). Versene is a poor buffer above pH 6.8, but it could be used to buffer NaCl solutions to this or slightly higher pH (A.4.4.)

C.4.6. pH

In the range pH 6.5 - 7.4 the "precipitation time" varies inversely as the hydrogen ion concentration (Fig.13) Following on this finding physical measurements were mostly made on solutions of pH 7.4. The "precipitation time" becomes very short below pH 6.5, around pH 5.0 a precipitate forms at 2°C, and as will be discussed later no "heat precipitate" is formed below pH 5.0-4.5

The linear dependance of the rate of this process on hydrogen ion concentration suggests that either (1) hydrogen ions act catalytically, or, more likely (2) the particles must be in a particular state of ionisation for the change to occur, or (1) and (2).

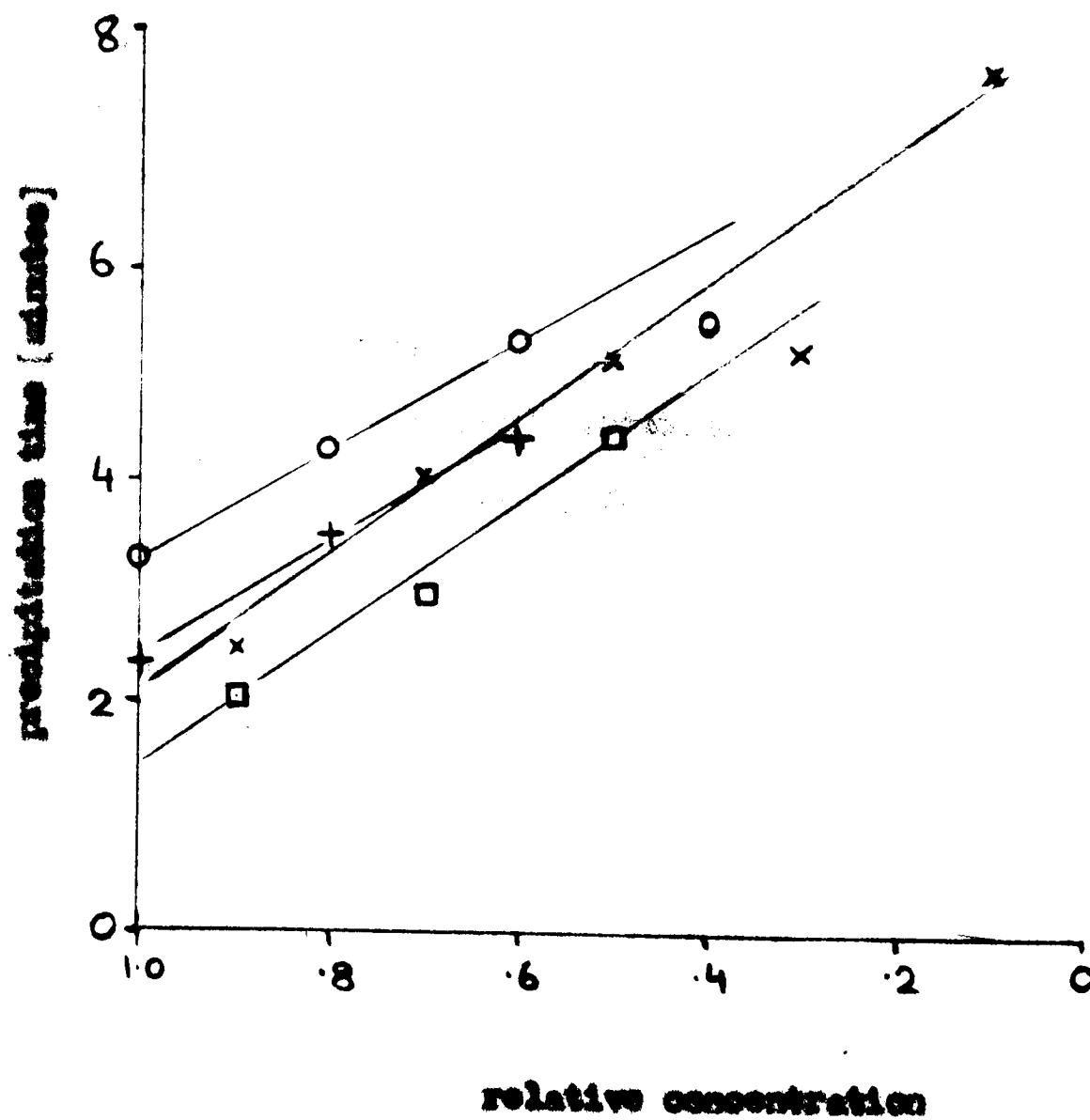
C.4.7. Presence of other substances

In general it would seem that positively charged basic N ions speed up the precipitation process, e.g. previously mentioned buffers, while negatively charged ions retard it. These effects may be masked by the effect of ionic strength, precipitation occurring more rapidly as the NaCl concentration is increased. Chondroitin sulphate, cyanide and sulphite particularly retard precipitation, to as much as 20 minutes at 37°C , but eventually precipitation still occurs.

The extracted neutral salt soluble collagen must have existed in some form in vivo at 37°C , but it is quite unknown whether this state was solid or solution. If it is present in solution in vivo, it could be that during isolation it had been separated from components essential to keep it in solution. With this in view a sample of the total protein¹⁰² which remain in the extract after the first salt precipitation was tested, but it had no effect on the precipitation time. Ascorbic acid (Tab.5) also does not appear to affect it, chondroitin sulphate has been previously mentioned, and hyaluronic acid (synovial fluid U.F.E., buffered) forms an immediate slight cloudiness with the soluble collagen, which does not coalesce upon heating unless hyaluronidase is added.

Fig. 14c

The variation of precipitation time with concentration.



The same material was used for all experiments; all results obtained in a given experiment are denoted by the same symbol.

C.4.8. Concentration

Precipitation time increases approximately linearly with dilution (Fig.14). Precipitation times could not be measured with sufficient accuracy to distinguish between different orders of reaction.

C.4.9. Various studies connected with precipitation time

(1) Artificial, partial alignment of the particles does not cause a precipitate to form; thus shearing at 180 sec^{-1} for 6 hours at 4°C did not cause precipitation.

(2) Cyanide or iodoacetamide retard the appearance of a precipitate. Coupled with the effect of sulphite this might imply that SH groups could be involved. No nitroprusside reaction was obtained, and the inhibitory action of chondroitin sulphate makes a specific function of sulphydryl reagents unlikely. British Anti Lewisite (B.A.L.) quickly formed a precipitate with the material.

(3) In pilot experiments no catalytic action was found when "heat precipitate", supernatant, or washings of a "heat precipitate", were added to another solution which was then worked.

C.4.10. Conclusions

The formation of a "heat precipitate" appears to be

a form of protein denaturation, as judged by the activation energy; the dependence on hydrogen ion concentration is consistent with this view. Ions with multiple negative charge slow down the process, basic nitrogenous ions accelerate it.

**C.5. STUDIES ON THE PARTIAL RE-SOLUTICE OF THE HEAT PRECIPITATE
OF NEUTRAL SALT SOLUBLE COLLAGEN**

These will be described under the headings:

- a) Introduction
- b) Method of preparing gels by warming and subsequent analysis of these gels
- c) Gel ratio, the variation of gel ratio on incubating for increasing lengths of time
- d) Recovery, and gel ratio of material after standing in refrigerator.
- e) Factors affecting recovery:
 - (1) pH
 - (2) concentration
- f) Repeated gelling and recovery of material
- g) Conclusions

C.5.1. Introduction

When it was first shown that material precipitated on warming a solution of neutral salt soluble collagen, it was thought that this could be due simply to a reduction of solubility with temperature. Accordingly some precipitate was shaken for several hours with fresh phosphate buffer

pH 6.8 in the cold, but only less than one per cent dissolved. Studies on "precipitation time" were then made. It was found that if a solution was not disturbed while it was warmed, only an opalescence appeared. The introduction of a stirring rod made it clear that a gel had formed, whose rigidity would be greater, the greater the concentration of the original solution. The gel would quickly synerese about the rod. If a gel was not disturbed, but placed in the refrigerator and prodded after several days, it was apparent that its rigidity had greatly decreased (as compared with a control which was prodded immediately after warming), and if the gel had not been disturbed much, its rigidity could again be increased by warming. This suggested that some process was reversible with respect to temperature, but that once a gel had syneresed to a small clot, only a small part of the solid was exposed to solvent, and as the appropriate diffusion coefficients are small, re-solution and equilibrium at lower temperatures would only be reached very slowly.

A method was devised for preparing gels by warming, keeping the gels in the refrigerator and analysing the product after various time intervals.

C.5.2. Method of preparing gels by warming and the subsequent analysis of these gels (see also (Fig.20) C.5.4.)

C.5.2.1. Preparation of gel by heating:

0.6 ml. or more of the solution (called "parent solution") was placed in a clean "lustroid" centrifuge tube (as for Spinco ultracentrifuge, rotor 40) and the lightly stoppered tube placed in a waterbath at 37°C . The tubes were firmly stoppered after a few minutes. At the end of the appropriate incubation time, usually 1 hour, the tubes were dried and placed in the refrigerator without disturbing the contents.

C.5.2.2. Determination of of gel ratio of parent solution

Often one tube was removed from the waterbath before the others, for determination of the "gel ratio". Its contents were quickly fragmented and stirred with a small glass rod, and the tube then topped up with medicinal paraffin previously warmed to 35°C , and balanced against a balance tube. The tubes were quickly introduced into a Spinco preparative rotor 40, which had been equilibrated to 35°C , and the material was centrifuged, for 20 minutes at 39,000 r.p.m. The aqueous supernatant was withdrawn through a Pasteur pipette and then stored. The rotor temperature did not fall below 27°C during these operations. The refractive increment of this supernatant was measured (A.3.5.) as was that of the original solution.

Gel ratio is defined as:

$$= 100 \times \frac{\left[\begin{array}{l} \text{refractive increment of supernatant,} \\ \text{refractive increment of solution from} \\ \text{which the supernatant was made} \end{array} \right]}{\text{refractive increment of solution from which the supernatant was made}} \times 100$$

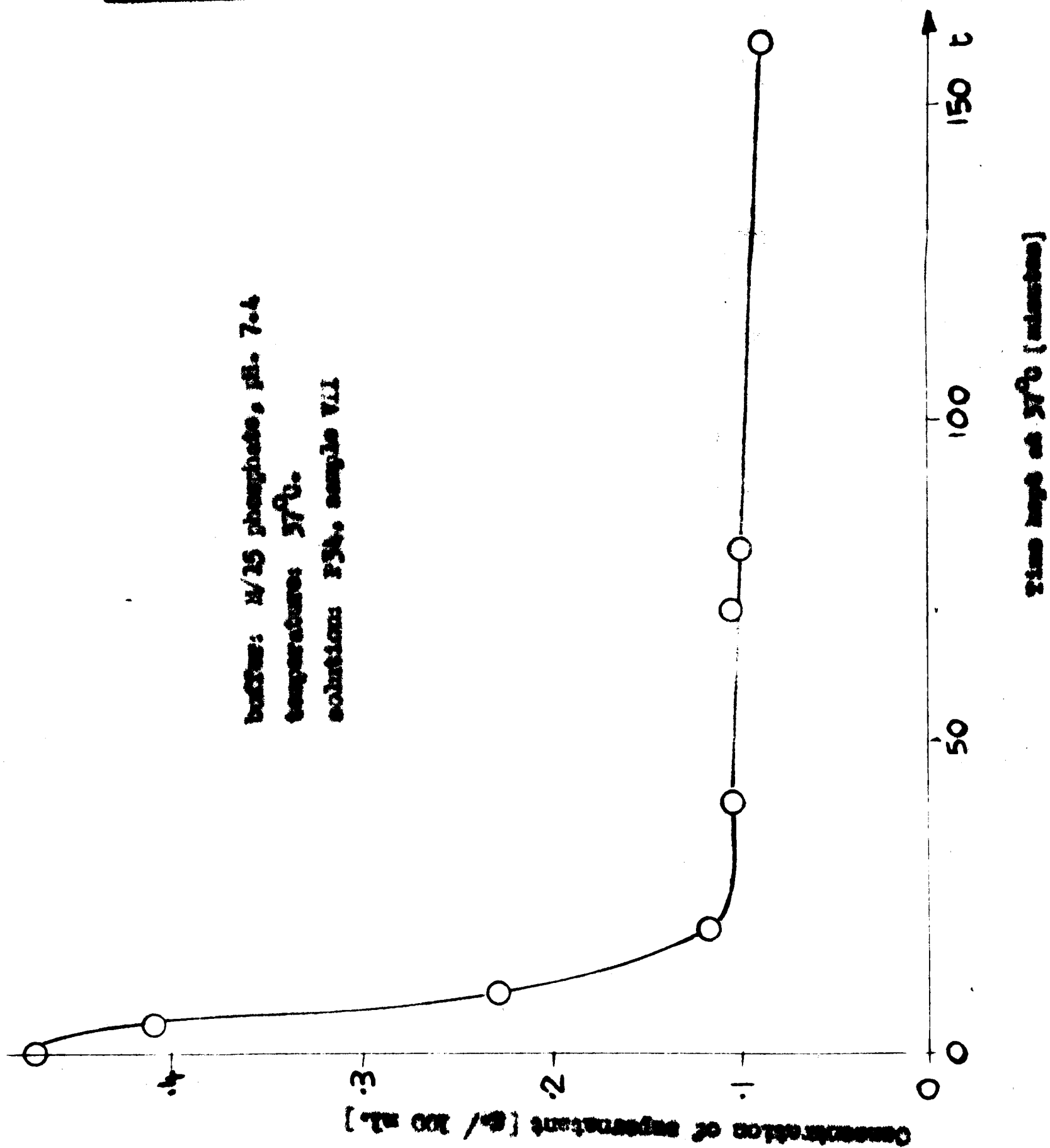
C.5.2.3. Collection of material which had re-dissolved in the cold

After varying intervals of several days the Spinco rotor and medicinal paraffin was equilibrated in the refrigerator, any residual gel quickly fragmented, and, proceeding as in the previous section but at about 4°C, the material was centrifuged and the supernatant called "off-spring solution" collected.

This supernatant was used for further experiments, or all, or some part of it, was used for the determination of its "gel ratio". To do this the refractive increment was first measured and the solution was then incubated for 1 hour at 37°C. Procedure was as in the previous description for determining gel ratio, except that a small angle centrifuge (9,000 r.p.m.) and its glass tubes (7.5 x 1.2 cm) were used in place of the Spinco ultracentrifuge. The rotor of the small centrifuge did not cool below 22°C during such an experiment.

Fig. 13.

Rabbit neutral salt soluble collagen: variation of beef
precipitation with time kept at 37°C.



C.5.3. Gel ratio, the variation of gel ratio on incubating for given lengths of time

It was first necessary to establish standard conditions for preparing the gel by warming, and to ensure that as much as possible of the material would gel.

37°C was chosen as a temperature at which gel formation should proceed rapidly, probably to a maximum extent, and yet at which it was hoped that no cleavage to gelatin might occur (as it is known to at 40-50°C and above).

Solutions at pH 7.4 were incubated for 5 - 160 minutes at 37°C, and following the method of (C.5.2.) the refractive increments of the supernatants were obtained immediately after gelling (Fig.15). It was concluded that:

- (1) little further change occurred beyond 40 minutes' incubation, when a gel ratio (C.5.2.2.) of 83% is obtained.
- (2) formation of gelatin is unlikely, as what little change there is after 40 minutes corresponds to more, not less material being removed from solution.
- (3) No simple mathematical relationship was found between gel ratio and time.

Fig. 16.

Rabbit neutral salt soluble collagen; variation of heat
precipitation with time kept at 22°C; initial stage only.

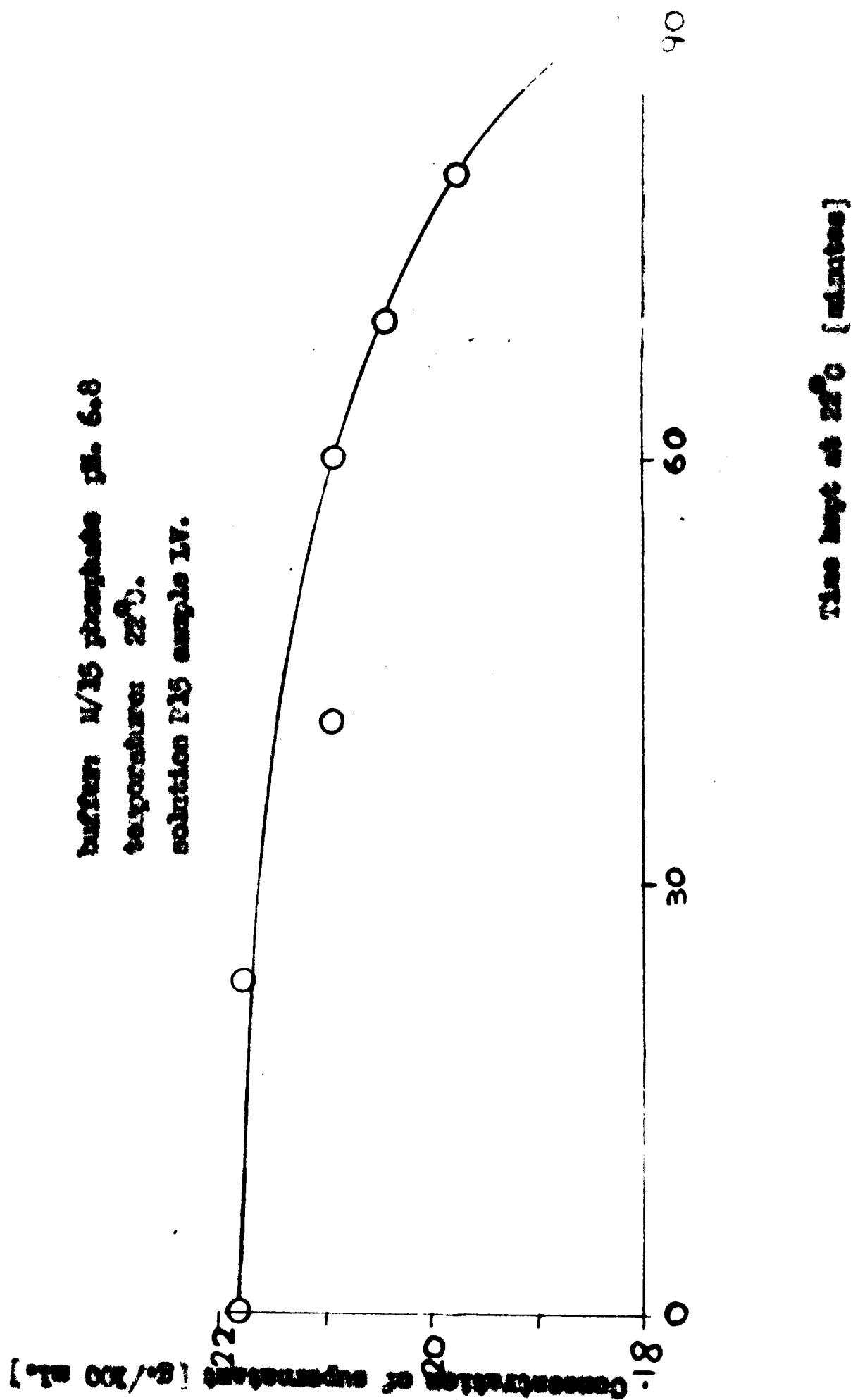


Fig. 17.

rat neutral salt soluble collagen: the variation of heat
precipitation with time kept at 37°C.

[buffer: versene - NaCl (A.4.4.), pH. 6.7]

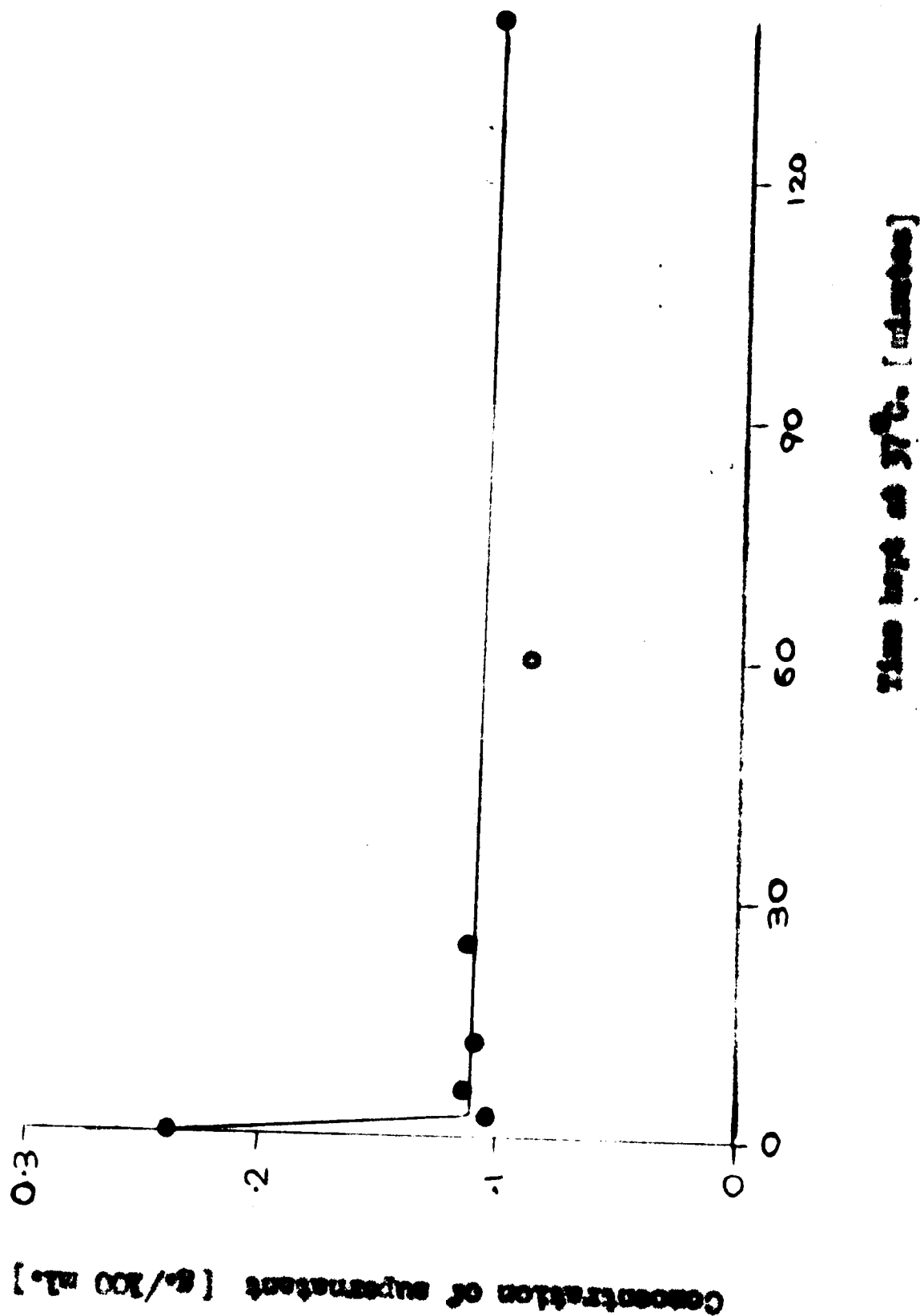
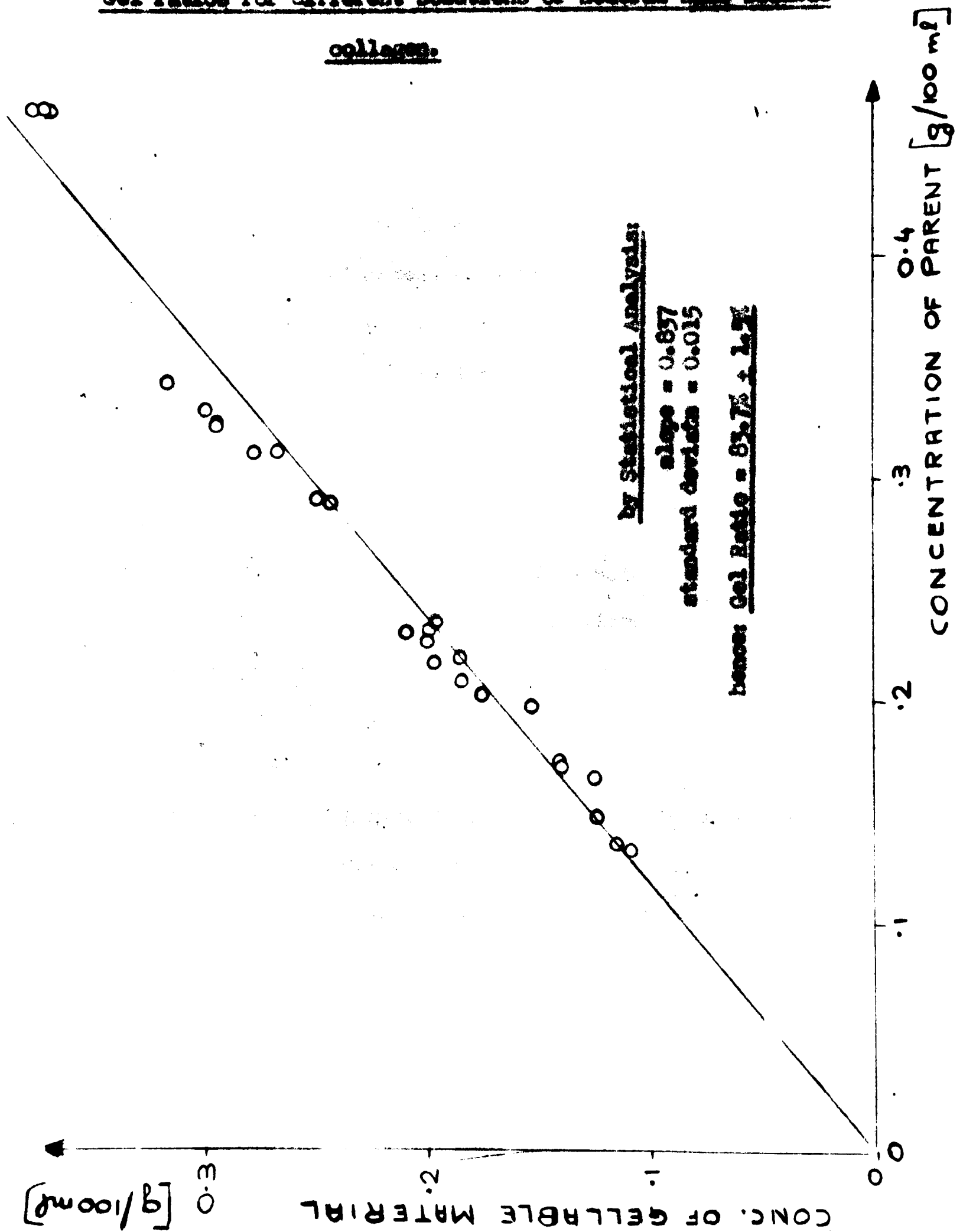


Fig. 18.

Gel ratios for different solutions of neutral salt soluble
collagen.



The initial stages of precipitation had previously been investigated under different conditions (in an attempt at some kinetic work) and are shown in (Fig.16), indicating that the total precipitation-time curve is sigmoidal. The results of a similar experiment with partially purified material from rats, in versene-NaCl buffer pH 6.7, is shown in (Fig.17).

$$\text{Gel ratio} = 100 - \left[\frac{\text{refractive increment of supernatant}}{\text{refractive increment of solution from which supernatant was prepared}} \right] \times 100$$

Gel ratios of 80-90% were regularly obtained for purified material. All gel ratios obtained with purified material are given in (Fig.18), which shows a linear relationship between concentration of the parent solution and concentration of gellable material. Statistical fitting of a straight line to these results gave a mean gel ratio of $(83.7 \pm 1.5)\%$. The line passes through the origin (i.e. + 0.0007g./100 ml. when gellable = 0). This independence of the gel ratios on total concentration makes it very unlikely that there is an equilibrium relationship between gel and its supernatant.

Gel ratios will be further discussed in the section on purification (C.6.3.) Similar values were also obtained under different conditions, e.g. solution 18 Sample IV gave

Fig. 30.

Re-solution in the refrigerator of head gals.

Concentration of gillable material in solution
 at the start of the experiment is 100%.

- Curve [1]: Concentration of total material in solution after standing in refrigerator
- × Curve [2]: Concentration of material left in solution after head galling and centrifuging corresponding solutions denoted in curve [1]
- △ Curve [3]: Net concentration of gillable material which has re-dissolved on standing in the refrigerator, calculated by difference of corresponding points of curves [1] and [2].

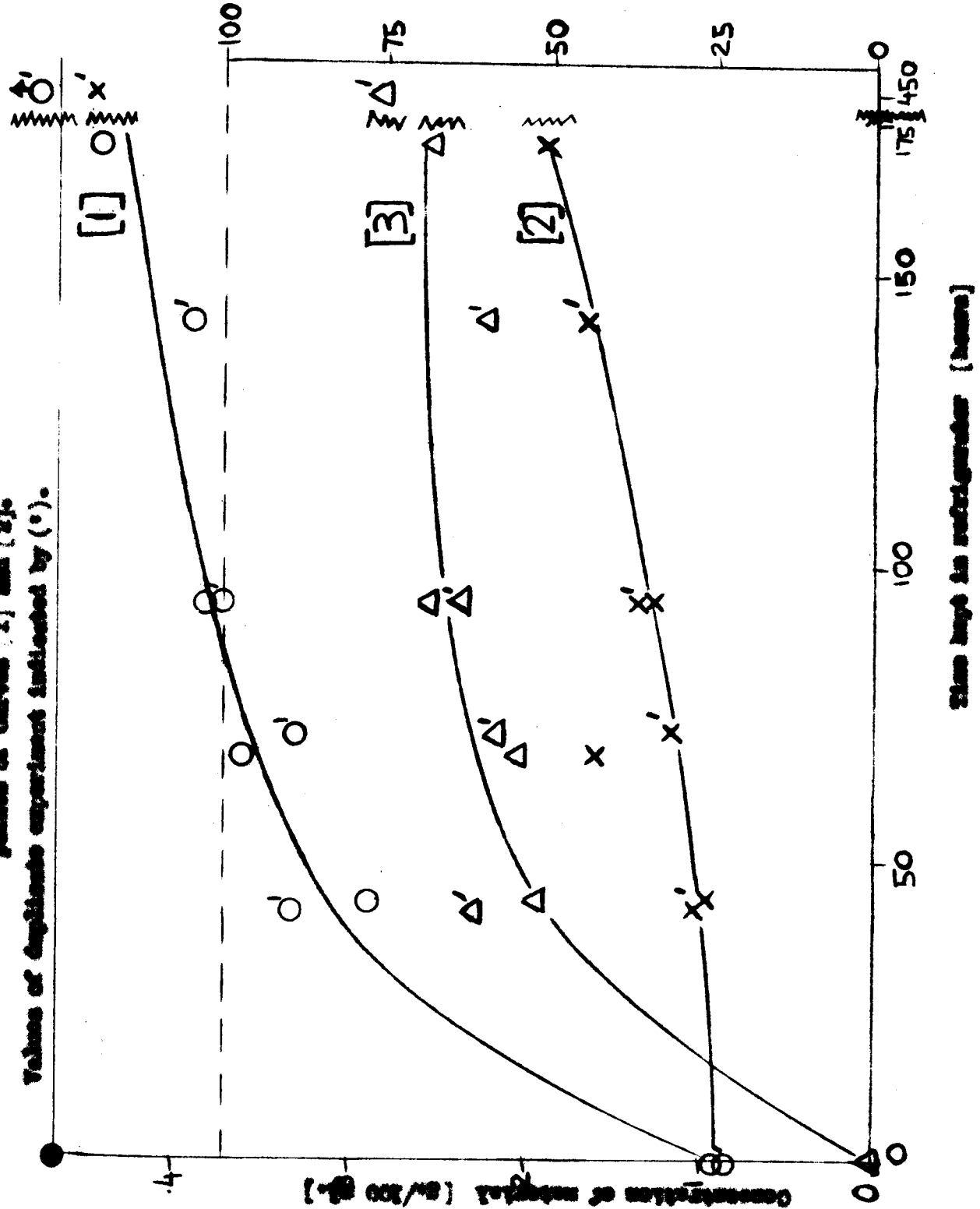
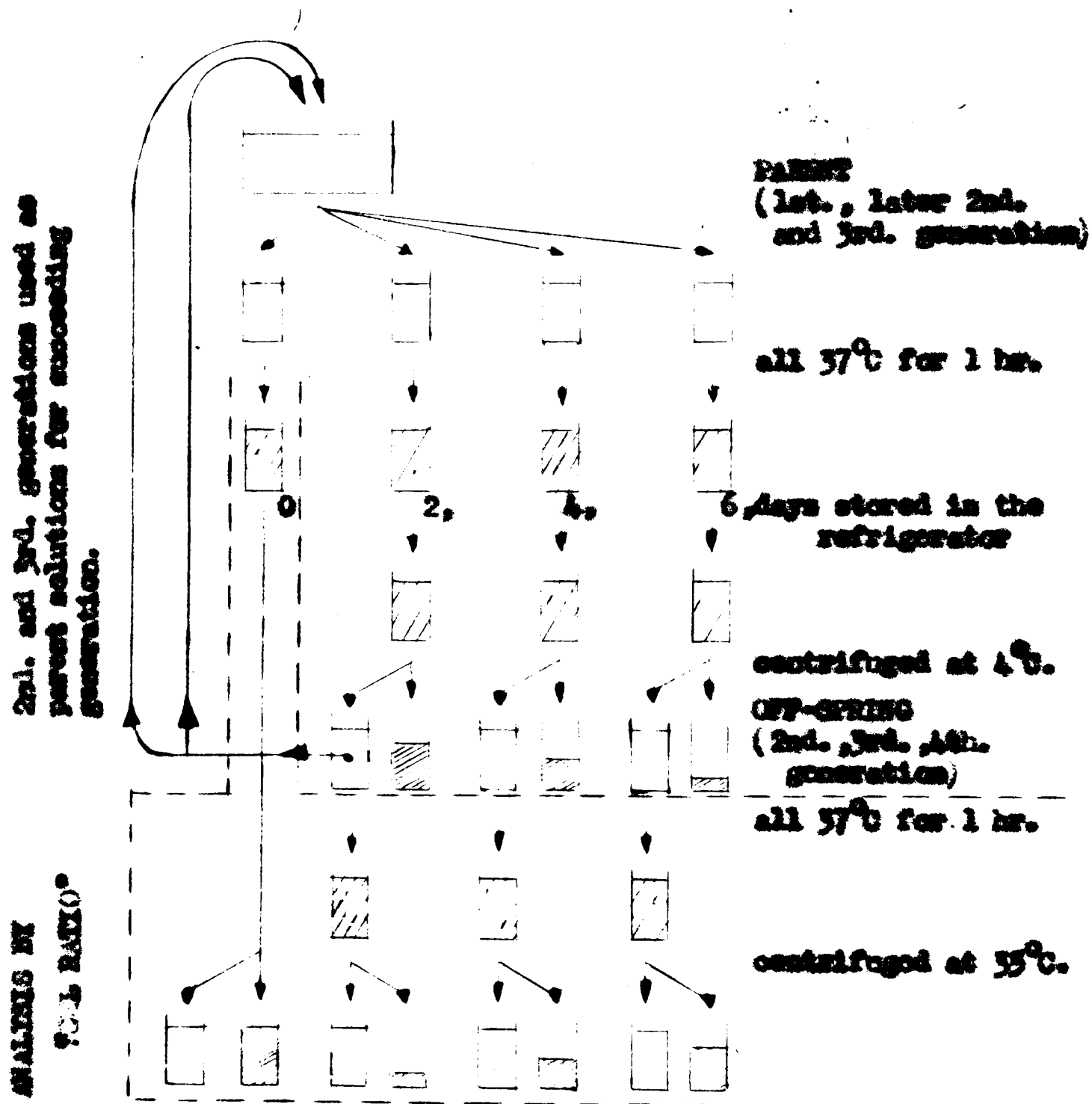


Fig. 20.

**Outline of method used for investigating the recovery of gels
made by warming solutions of neutral salt soluble collagen.**



This represents the method of section (C.5.2.), used for experiments (C.5.4 and 5); for (C.5.6.) it was continued to 3rd. and 4th. generation. Shaded areas represent gel or precipitate; clear areas indicate solutions whose refractive increments were measured so as to obtain their concentrations.

a gel ratio of 80% after 30 minutes at 50°C and pH 6.8. Again, as will be shown later (C.5.6.) gel ratios were reproducible to within a few per cent on repeated warming of solutions.

Summary:- standard conditions for making gels were decided, namely: 37°C , 60 - 70 minutes, when 83.7 of the total refracting material precipitates, independently of concentration.

C.5.4. Recovery and gel ratio of material after standing in refrigerator

+ illustrated in Fig 20

Following the method described in (C.5.2.) a set of gels were prepared and then centrifuged after standing 2 - 19 days in the refrigerator. The experiment was duplicated after some weeks and the second set of points are marked with a dash in (Fig.19). Curve (1) of (Fig.19) shows how with time the refractive increment of the supernatant approaches that of the original solution and even exceeds it. Such refractive increment represents total material in solution. To obtain values for the material which has dissolved and can again be heat precipitated, the supernatants were in turn incubated at 37°C and the precipitates formed at once spun off (C.5.2.3.). The refractive increments of the final supernatants so formed

are shown in curve (2) (Fig.19). Subtraction of curve (2) from curve (1) gave curve (3) of (Fig.19). Curve (3) therefore represents the refractive increment of the "heat precipitate" obtained from material in solution at the end of a given period in the refrigerator; in other words it is material which was heat gelled, dissolved in the cold, and heat gelled again. For convenience a percentage scale is also given, the total material which can be precipitated at the first heating being taken as 100%; or in other words $100\% = (\text{refractive increment of original solution}) \times \text{gel ratio}$.

From curve (3) it is apparent that not more than about 70% of the originally gellable material can be recovered. In agreement with this a gel was always found on taking tubes out of the refrigerator, even though mechanically the gels obtained were progressively weaker with increasing time of standing in the refrigerator. Bacterial action and/or evaporation through the collodion tubes must, however, be occurring as otherwise curve (1) (Fig.19) could at no point exceed the value of the initial refractive increment, and presumably the increase in curve (2) (Fig.19) is due to similar causes; this is particularly shown by the values obtained after 19 days' standing in the refrigerator. In view of the sensitivity which this substance shows to practically all materials that are added to its solution (see C.4.7.), and the difficulties

which had been encountered in the search for an antiseptic for hyaluronic acid solutions (B.5.7.), no attempt at bacteriostasis was made. There are indications, to be mentioned later, (C.5.6.) that bacterial action alone does not account for the 30% of originally gellable material which cannot be recovered. In subsequent experiments of varying duration not more than 70% of such material was recovered, and the values ranged from 55-70% (Fig.24)

Most of the scatter of the points in (Fig.19) is probably due to centrifuging. The problem, which was not investigated in detail is the following. Pilot experiments showed that the fibres, of which such a gel consists (C.8.1.) sediment only slowly in the viscous collagen solutions under the centrifugal acceleration applied by an ordinary, 9000 r.p.m. angle centrifuge. Should the supernatant be contaminated with submicroscopic fibres, their refractive increment will, in an unknown fashion, be added to that of the supernatant, in the measured refractive increment values. To avoid this, it was necessary to employ an ultracentrifuge. Under the conditions used some 5% of the macromolecules in true solution could readily be sedimented out. The precipitate obtained after ultracentrifuging never had quite sharp boundaries, and these would further be stirred up by the currents induced during the careful pipetting out of

the small volume of viscous supernatant through a small orifice; finally the descending paraffin boundary would induce surface tension effects and thermal convection in the tube was also not excluded.

When the supernatant did not contain macromolecules which would make it viscous, syneresis of the precipitate followed by centrifuging at 9000 r.p.m. was adequate, and this method (C.5.2.5.) was used when possible.

Conclusion:- some 70% of material originally gellable dissolved upon standing in the cold and could be recovered at 37°C. The remaining 30% might not have been recovered because: (1) bacterial action had occurred (2) a fundamental change had taken place in the material (3) it represented a different material which did not re-dissolve, or only did so very slowly (4) the original solutions might have been supersaturated and the concentration of the recovered gellable material, about 0.25 g./100 ml., might have represented the true limit of solubility.

C.5.5. Factors affecting recovery

(1) pH

(2) concentration

Fig. 22.

Fig. 21.

Re-solution in the refrigerator of heat ppt made from parent solutions of different pH. values; expressed as percentage of soluble material of parent solution.

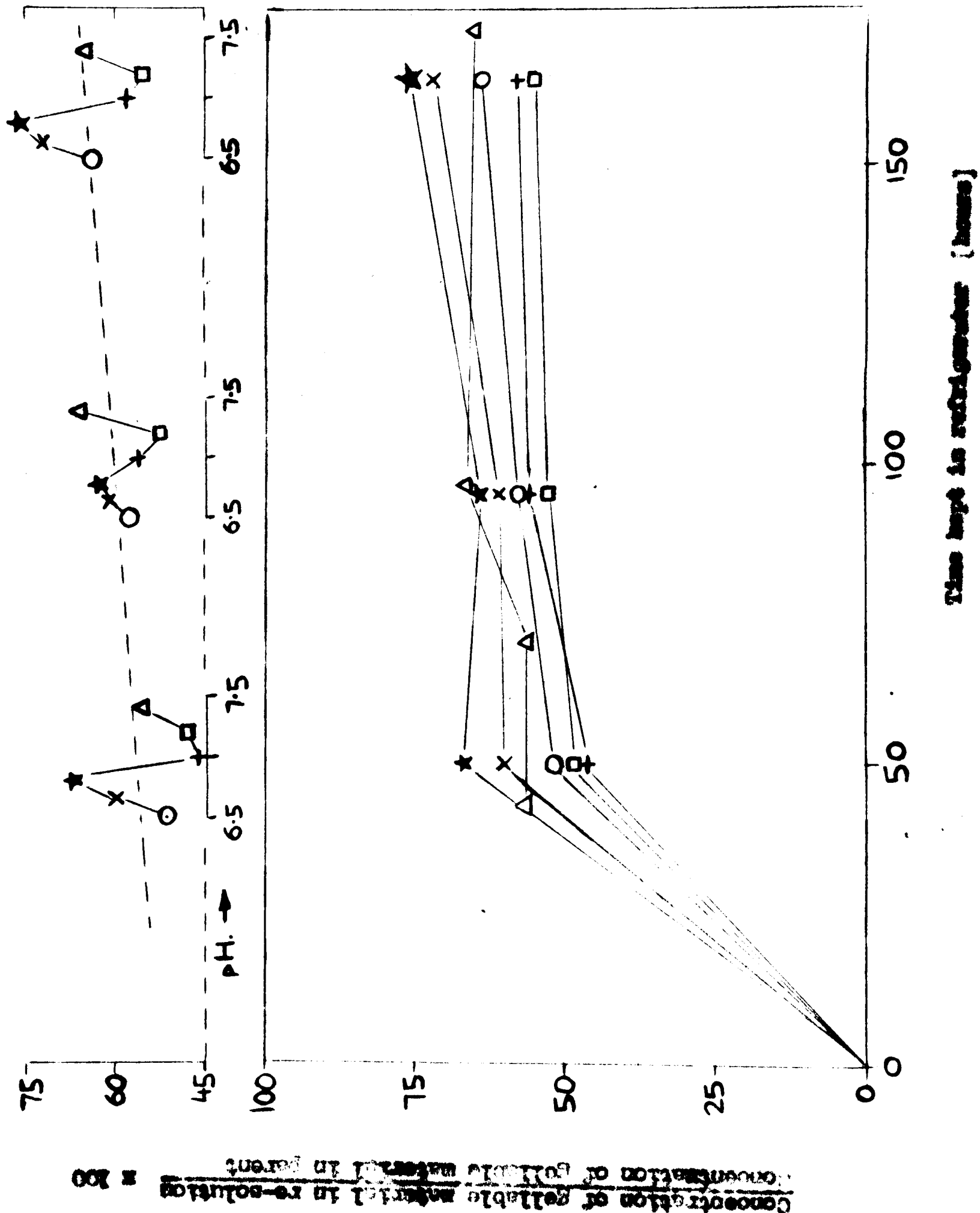
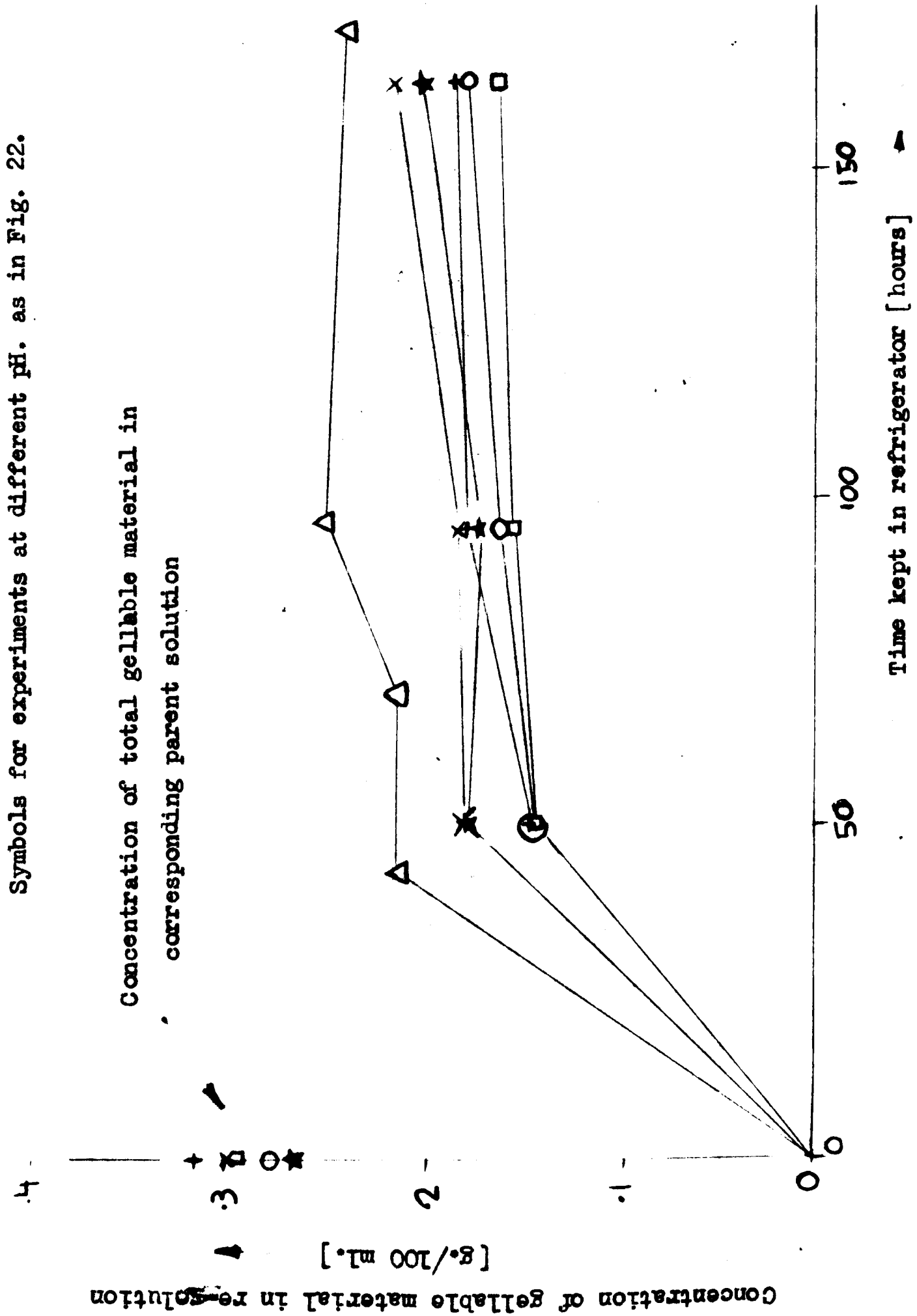


Fig. 23.

Re-resolution in the refrigerator of heat gel made from parent solutions of different pH.; expressed as concentration of gellable material in solution.



C.5.5.1. pH

The experiment described in the previous section (C.5.4.) was repeated at pH 6.5, 6.65, 6.8, 7.0, 7.2. Graphs of the percentage recovery of originally gellable material (corresponding to curve (3) (Fig.19) expressed as percentage) are shown in (Fig.21), and curve (3) of (Fig.19) is also plotted. In (Fig.22) the percentage recovery is plotted against pH for different times of standing in the refrigerator. In (Fig.23) the equivalent refractive increment of recovered gellable material is plotted against time, and curve (3) of (Fig.19) is again included. The gel ratios varied from 85 to 92.

Conclusions:- (1) variation of pH from 6.5. - 7.4 did not affect the gel ratio or the percentage of gellable material recovered in any obvious, clear out fashion
(2) the results fitted in well with those of (Fig.19) when expressed on a percentage basis, but not when expressed in terms of concentrations; this makes it unlikely that the original solutions were supersaturated (Conclusions, C.5.4.)
(3) the mean value of the percentage of gellable material recovered slowly increased with time of standing in the refrigerator.

C.5.5.2. Concentration

The previous type of experiment (C.5.4. and C.5.5.1.)

Fig. 24.

Re-solution in the refrigerator of heat gel made from parent solutions of different concentrations; expressed as a percentage of gellable material in the parent solution.

Symbols as in Fig. 25.
Shaded area: limiting values of Fig. 21. (this area includes curve (3) of Fig. 19.)

Concentration of gellable material in re-solution $\times 100$

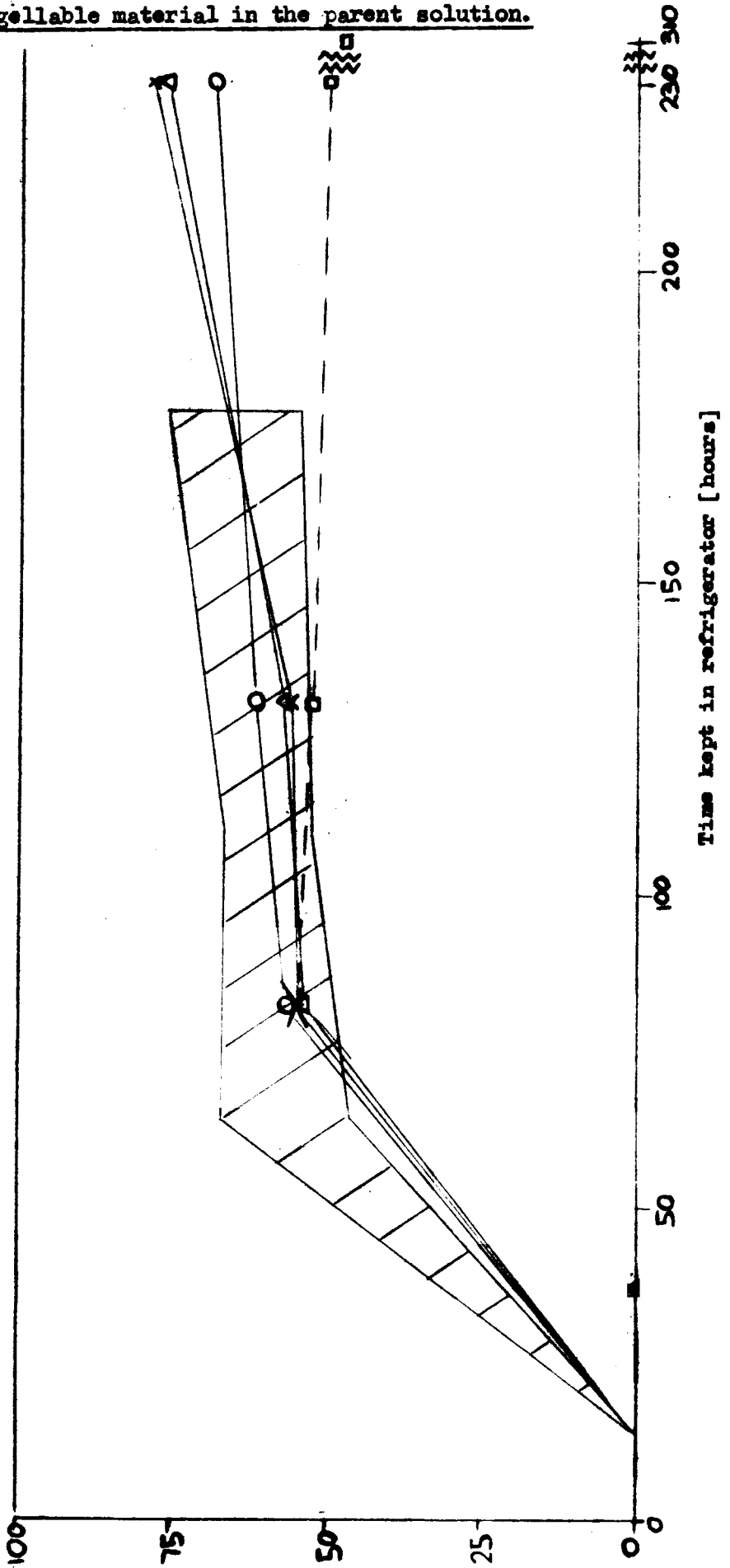
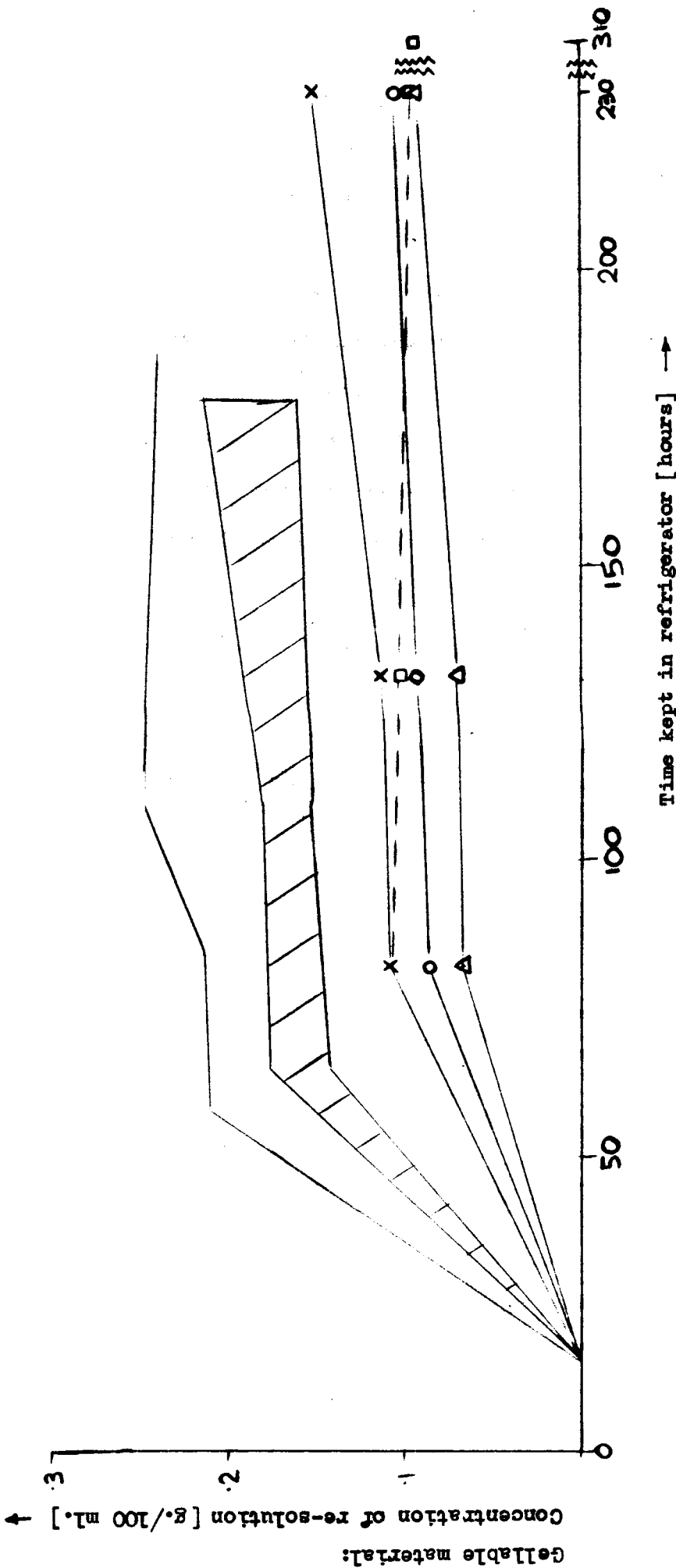


Fig. 25.

Re-solution in the refrigerator of heat gel made
from parent solutions of different concentrations.

Concentration of parent solution: x 0.238 g./100 ml.
 o 0.198 g./100 ml.
 Δ 0.163 g./100 ml.

Line without marks: curve (3) of Fig. 19.
Shaded area : limiting values of Fig. 23.



was repeated, but keeping the pH constant at 7.0 and varying the concentration of starting material: 0.163, 0.199, 0.238 g./100 ml. The results are shown in (Fig.24), plotted as percentage of originally gellable material recovered against time, and in (Fig.25) as refractive increment of recovered gellable material against time (For the present ignore the symbols $\square$ and the dotted line joining them). The shaded areas in the two graphs are bounded by the limiting values of (Fig.21) and (Fig.23) respectively, and the line not marked with any symbols in (Fig.25) is a transposition of (Fig.19 curve (3)). The precise position of the "knee" in these graphs has no significance, as it depends solely on the first time interval and this was chosen arbitrarily for different experiments.

Inspection of (Fig.24) and (Fig.25), with or without considering the transpositions of the other results, does indicate that agreement between different series of gels is much closer when expressed on a percentage than on a concentration basis. While (Fig.25) does not indicate any limiting concentration, such as would be expected from a saturated solution, the values of (Fig.24) do fall around a mean of about 60%.

Conclusion:- that material which gels on warming is composed of two parts: one, some 60%, which within a few

days re-dissolves on standing in the refrigerator, the other, some 40%, which does not.

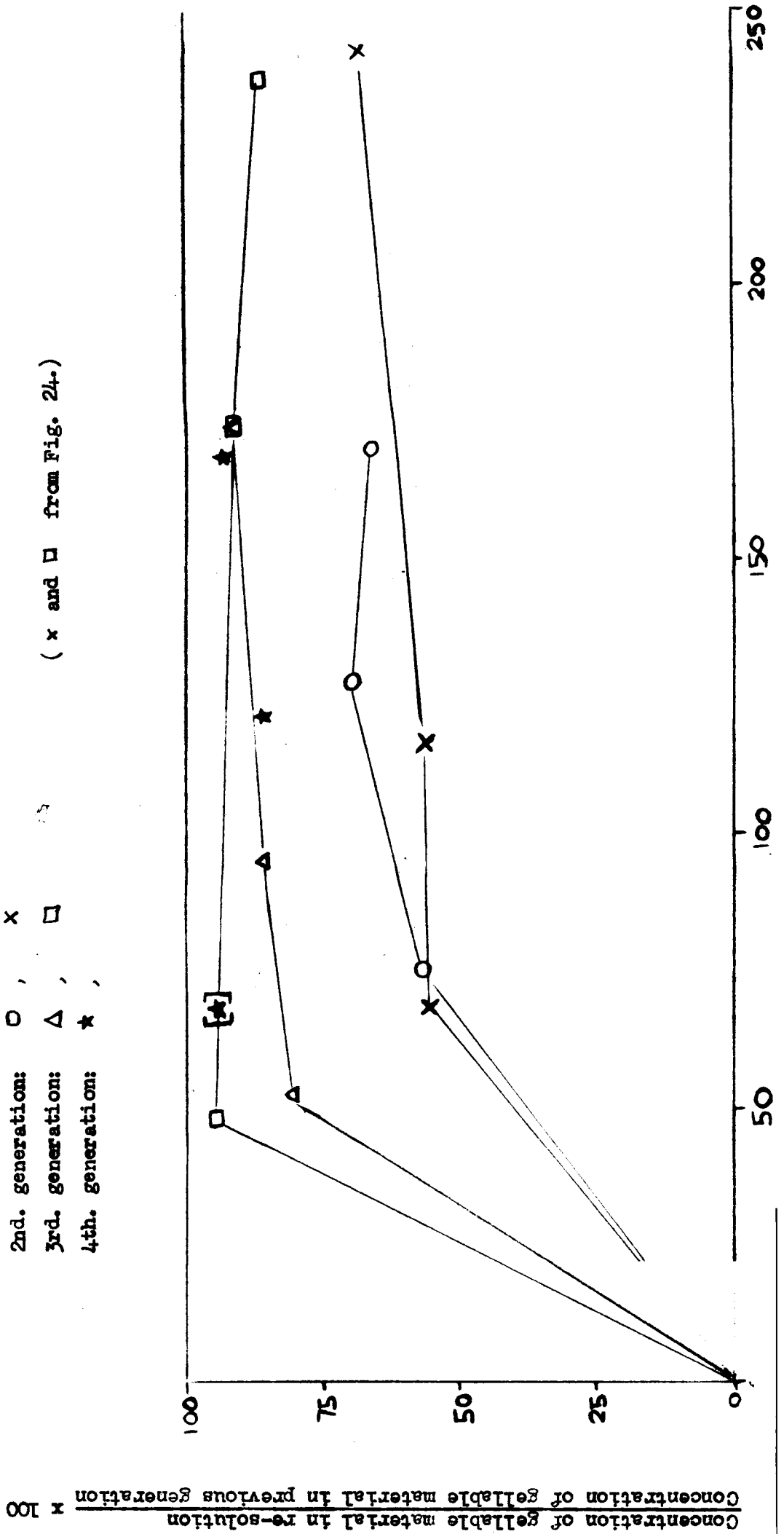
C.5.6. Repeated gelling and recovery of material (see Fig.20)

The experiments to be described now provide further evidence for the hypothesis that there are two gellable fractions, one of which re-dissolves rapidly and the other not. The experimental method used so far was to warm samples of an original solution, called the first generation, then to allow the gel partly to redissolve on standing in the refrigerator, and the resulting solutions, after centrifuging off remaining gel, are called the second generation. The amount of gellable material in the second generation was determined by a separate analytical technique, that of determining "gel ratio", which itself happened to involve warming to 37°C and centrifuging off the heat precipitate immediately.

By hypothesis all the gellable material present in solution in the second generation should be of the kind which, if gelled, rapidly re-dissolves again on standing in the refrigerator. A third generation of solution was made in precisely this fashion and itself analysed by the technique of gel ratio. After this had been established in a preliminary experiment, to be described soon, another

Fig. 26.

Repeated re-solution in the refrigerator of heat gel.



experiment was made which was similarly taken to four generations of solutions. The behaviour, in terms of resolution of gel in going from the second to the third generation, was closely followed in going from the third to the fourth generation, a near 100% recovery of the particular gels being obtained.

In the experiment described in the previous section (C.5.5.2.) a subsidiary experiment was made in which the solution of second generation marked x in (Fig.24) and (Fig.25) was made the parent of a third solution marked $\square$ in (Fig.24) and (Fig.25). It is apparent from both (Fig.24) and (Fig.25) that the amount of gellable material recovered in the third generation, is only little less than that present in its parent. This is expressed in another way in (Fig.26). For the present only regard the points marked x and $\square$ which represent the same solutions as in (Fig.24) and (Fig.25). In (Fig.26) the abscissa denotes time of standing in the refrigerator as before, the ordinates marks, the amount of gellable material recovered from an off-spring solution expressed as a percentage of the gellable material present in its immediate parent. Thus while in the second generation (x) some 60% was recovered, as previously shown, in the third generation ($\square$) the first recovery was 95% and then fell off with time to 87%. The gellable material present in the second generation,

in contrast, increased with time. Qualitatively the materials were also different: the tubes from which the solutions of second generation were made always contained some gel, however weak, when taken out of the refrigerator; in contrast the tubes from which the solutions of the third generation were made contained no gel on taking out of the refrigerator, and there was practically no residue after ultracentrifuging. The decrease of gellable material recovered in the third generation with time, is probably due to bacterial action. If this is representative of bacterial action, then it is insufficient to account for the 40% or so of original gel which cannot be recovered.

After this promising experiment another one was made like it, but taken to four generations. The results are shown in (Fig.26): second generation is represented by $\odot$, third by Δ and fourth by $\star$.

The spinco tube holding the first sample of the fourth generation fractured during centrifuging and only a small volume could be recovered for measurement. The value calculated from this is unreliable and the point is bracketed in (Fig.26).

While the scatter of points is greater than could be desired the points of (Fig.26) do fall into two groups: those representing second generation and those representing third and fourth generations.

It is concluded that the 60% or so of originally gellable material which dissolved rapidly (within 3 days) on standing in the refrigerator, did so again, not only once but also twice.

0.5.7. Conclusions

Warming solutions of neutral salt soluble collagen to 37°C indicated the presence of three components:

- (1) material which did not gel - called fraction B
- (2) material which gelled and the gel dissolved rapidly in the cold - called fraction A
- (3) material which gelled and the gel did not dissolve rapidly in the cold - called fraction C

The proportions of these fractions did not vary markedly with concentration or pH. From this it is concluded that they are unlikely to represent different parts of a system in equilibrium; for example if B represented a saturated solution of neutral salt soluble collagen, then the proportion of B should vary with concentration of starting material, but it did not (Fig.18).

The fractions A, B and C may not have existed as such in the original solution, and might have been produced on warming to 37°C. Whether the initial warming

produced a change or not, subsequent repeated heating did not cause a further change (e.g. Fig.26).

Evidence from electron microscopy (C.8.2.1.) and flow birefringence (C.11.4.3.) does indicate that the original material was heterogeneous. Especially the various lengths of particles seen under the electron microscope, would suggest groups of substances, rather than three precise species corresponding to the fractions A, B and C. Indeed the evidence obtained from the previous solubility studies points to fractions of a continuous series, rather than to three species.

Fraction B could be one or more of the following:

- (1) non-collagenous impurity
- (2) collagenous material which does not "heat precipitate"
- (3) material split off from neutral salt soluble collagen when it forms a heat precipitate.

Fraction B is macromolecular, at least on exhaustive dialysis of a heat supernatant against phosphate buffer pH 7.4, the refractive increment did not decrease (within an experimental error estimated at 5%).

C.6 STUDIES ON THE ISOLATION OF NEUTRAL SALT SOLUBLE COLLAGEN
AND THE PHOSPHORUS CONTENT OF, AND INCORPORATION INTO, THE
MATERIAL

Two experiments were made and after an introduction will be described and discussed separately.

C.6.1. Introduction

The first experiment was arranged to give information on several, different points of interest, and also to act as a preliminary for the second experiment.

The points of interest were:

- (1) The state of purity of the preparations of neutral salt soluble collagen.
- (2) The nature of fraction B, that fraction of neutral salt soluble collagen which does not gel on warming a solution to 37°C
- (3) The nature of the process of heat gelation, in particular whether a substance containing tyrosine and/or phosphorus is split off.

The quantities measured were:

- (1) refractive increment
- (2) absorption in the range 250-285mμ.
- (3) counts of radioactive disintegration of ^{32}P
- (4) phosphorus content (only in the second experiment)

and these were made on samples which had been taken to various stages of purification, and each sample was further separated into fractions (A & C) and (B) (see C.5.7.) by heat gelling. Now to describe the points of interest separately and to show why the particular measurements were made:

(1) The state of purity of the preparations of neutral salt soluble collagen:- Evidence for heterogeneity of the material had been provided by (a) flow birefringence measurements (C.11.4.3.), (b) electron microscopy (C.8.2.1.) and (c) the experiments involving repeated warming and cooling (C.5.7.). Heterogeneity in this sense can either be due to the presence of different members of the same family of substances (such as polymers of different particle size) or due to contamination with unrelated substances which will be called impurities. It was not intended to search for a new method of preparation, but to find how far the existing method of Jackson⁵⁵ had to be taken to obtain material of constant composition.

(2) The nature of the fraction B, that fraction of neutral salt soluble collagen which does not gel on warming a solution to 37°C:- It was pointed out in (C.5.7.) that this could be (a) non-collagenous impurity, (b) collagenous

material which does not heat precipitate, (c) material split off from neutral salt soluble collagen when it forms a heat precipitate.

(3) The nature of the process of heat gelation, in particular whether a substance containing tyrosine and/or phosphorus is split off:- The full nature of this process is unknown. Now neutral salt soluble collagen and acid soluble collagen must be closely related, and a partial conversion of the former to the latter could occur during heat gelling. This would be connected with a change in tyrosine content, and might be connected with a change in phosphorus content (if these substances contain any phosphorus at all) as follows:

Ultraviolet absorption spectra:-were chiefly of interest as measures of aromatic amino acids. Although the tyrosine content of mature collagen is small¹³², that of acid soluble collagen is even less¹⁵ 0.25% (N of total N), so that Bowes, Kenton & Moss¹⁵ postulated that "procollagen" (i.e. acid soluble collagen) combines with a "macropolysaccharide" rich in tyrosine and low in hydroxyproline, alanine and serine, to form mature ox hide collagen. Now in preliminary ultraviolet absorption studies I had found indication of an appreciable amount of tyrosine. Either then this was present as an impurity, or it would be split off when acid

soluble collagen was formed from neutral salt soluble collagen.

Phosphorus content:-was of more remote interest. Phosphorhydroxylysine had been isolated from calf embryo³⁹, while, to my knowledge hydroxylysine has so far only definitely been found in collagen^{26, 29, 135}. It was possible that the phosphorylation of hydroxylysine had occurred during isolation. It was also possible that: the apparently ready conversion of neutral salt soluble collagen to acid soluble collagen consisted of the hydrolytic removal of a few phosphorus atoms attached to hydroxylysine residues. Indeed similar ideas recently caused van Slyke and co-workers¹²⁶ to investigate, and show that P^{32} is not incorporated into nature collagen. Schmitt et al¹²² claimed a special action of A.T.P. in promoting a particular formation of collagen ("S.L.S."). Following this, in brief qualitative experiment, I did find that A.T.P. added to a solution of acid soluble collagen made it behave like neutral salt soluble collagen, in that it was soluble at neutral pH and could be reversible precipitated on warming. Lastly, my most readily available source of neutral salt soluble collagen was the skin of young rats which had been given P^{32} (intraperitoneally) by Dr. L. A. Stocken. The likely phosphorus content of

neutral salt soluble collagen was so small, that it would be reasonable to estimate it only after thorough purification, which was going to be studied in any case and for which radioactive counting techniques were convenient.

The actual estimation of phosphorus content was done after the method of purification had been worked out, and this forms the second experiment together with a study of the rate of incorporation of ^{32}P into the material.

Refractive increment was used as a convenient measure of concentration. Estimations of hydroxyproline might have been useful, but, together with any radical changes in the method of purification, this belonged to Dr. D.S. Jackson's domain, according to the way we shared out the work between us. He was going to make these measurements, but so far has not done so.

C.6.2. Experimental

Eight rats were given P^{32} phosphate (50 $\mu\text{C}/100 \text{ g.}$) 2 hours before sacrifice and the skins were treated as described previously (C.5.), avoiding extraneous phosphate and using veronal-NaCl buffer (A.4.4.) and applying four precipitations with NaCl. After each such precipitation a

sample was removed and exhaustively dialysed against three changes of buffer. The refractive increment and ultraviolet absorption spectra of the sample were measured, and radioactive counts were taken on the sample and its last dialysis buffer. A known volume of sample was then kept at 37°C for 1 hour and the gel rapidly fragmented and removed on the centrifuge. Refractive increment and ultraviolet absorption spectra were taken on the supernatant, and radioactive counts measured from the supernatant and from the heat precipitate dissolved in 10% acetic acid. To some samples a known volume of phosphate buffer was added just before "heat gelling" (this will be mentioned in greater detail later). The concentrations of samples was 0.16 - 0.3 g./100 ml.

C.6.2.1. Estimation of radioactivity

A Vell liquid counter¹³⁶ of 10 ml. capacity was used in conjunction with standard scaling equipment. About 1000 counts were recorded for each sample so that the standard deviation in counts was about 3% $\left[= \frac{(1000)^{1/2}}{1000} \times 100 \right]$. To observe this number of counts usually took about 1 hour. Background counts were taken with water at the beginning and end of each day's experiment, and were 9-12 counts per minute.

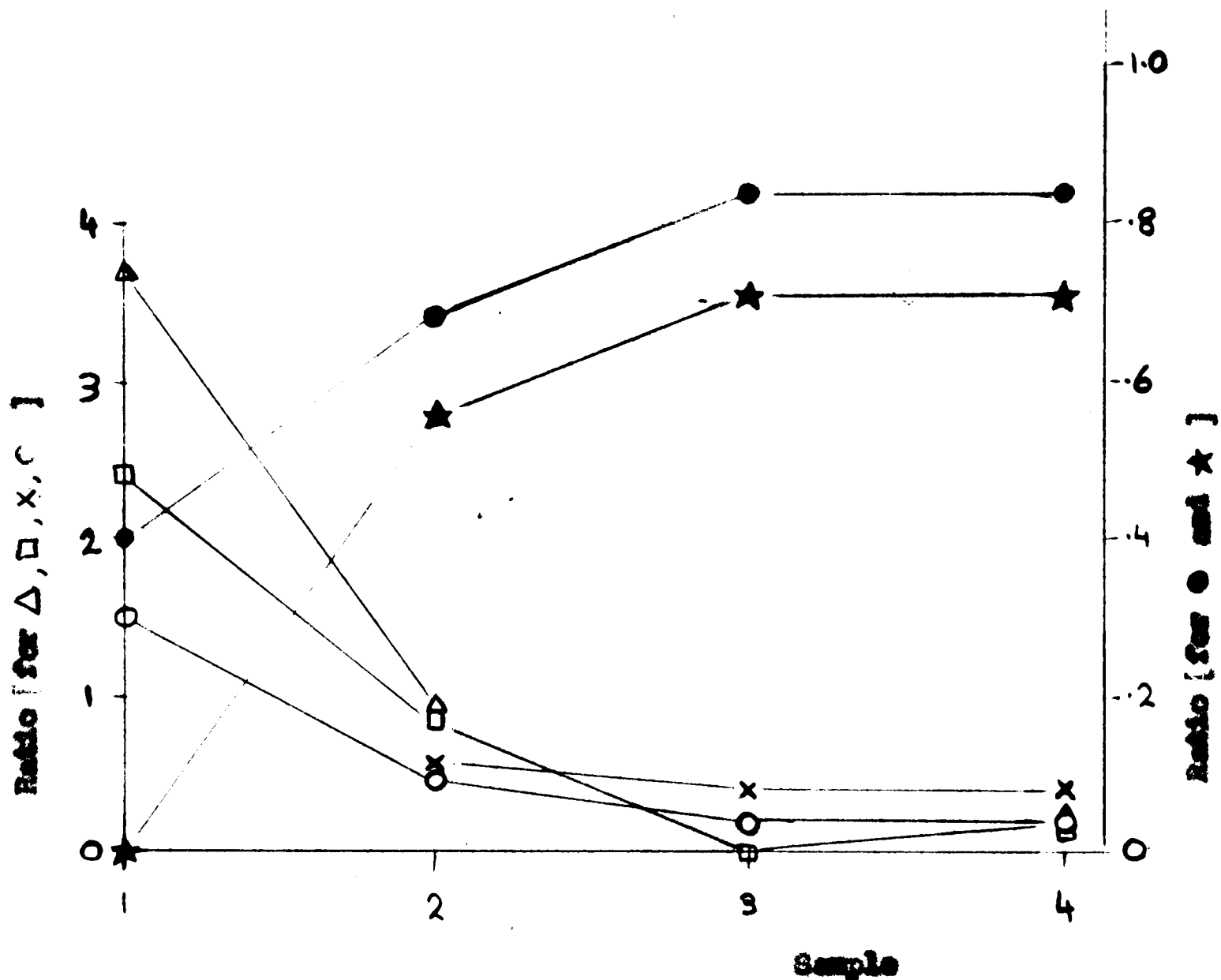
G.6.3. Results and discussion

The results are given in (Fig.27-32); in all but (Fig.32) the abscissa is not a scale, and the successive stages of purification are marked off as 1 - 4 at convenient intervals. Radioactivity counts were usually taken for 1 hour (G.6.2.1.), when 800 - 1,400 counts were scored. Backgrounds were measured for corresponding times and also the counts for the corresponding dialysis buffers, of which only those for samples 1 and 2 had a measurable radioactivity. "Counts" (i.e. number of observed disintegrations per hour) are given as corrected for background, buffer and decay from time of injection, and standard deviations shown are those calculated for counts. In the final samples counts were of the order of 250 counts per hour per 10 mg. protein (see Fig.28), which is very low. The animals had, however, been given the P^{32} only two hours before death, and even if this is incorporated into neutral salt soluble collagen, the uptake is likely to be small as it is so for C^{14} glycine even after 8 hours⁴⁹. The net zero count calculated for supernatant from heat precipitating sample 3 is probably fortuitous.

Measurements on intact samples, i.e. before warming, and called parent solutions, had to be done fairly rapidly to avoid gelling at room temperature. Supernatants were

Fig. 27.

Purification of neutral salt soluble collagen: ratios of single variables for supernatant and gel of different samples.



● refractive increment of gel
refractive increment of parent

C refractive increment of supernat
refractive increment of gel

★ absorption at 275 mμ of gel
absorption at 275 mμ of parent

X absorption at 275mμ of supernat
absorption at 275mμ of gel

□ counts per hour from supernatant
counts per hour from gel

Δ counts per hour from supernatant
counts per hour from gel

} phosphate added before heating

rather dilute (order of 0.05 g./100 ml.) and measurements correspondingly inaccurate.

Ultraviolet absorption is given at 275 mμ, unless otherwise stated.

In (Fig.27) each of the variables : refractive increment, counts, ultraviolet absorption, is treated separately, the relationship between parent solution, supernatant and gel being recorded for each sample. Inspection shows that there is little change between samples 3 and 4. Thus as judged by successive "gel ratio" expressed in terms of any of the variables (refractometric concentration, or counts, or ultraviolet absorption), nothing is gained in purity by carrying out more than four NaCl precipitations.

Compare the final ratios obtained for different variables. Those for refractive increment and for counts agree i.e. $\frac{\text{supernatant}}{\text{gel}} = 0.2$ for both

$$\text{or: gel ratio} = \frac{\text{gel}}{\text{parent}} = \frac{\text{gel}}{\text{gel \& supernatant}} = \frac{1}{0.2 + 1} = 0.8$$

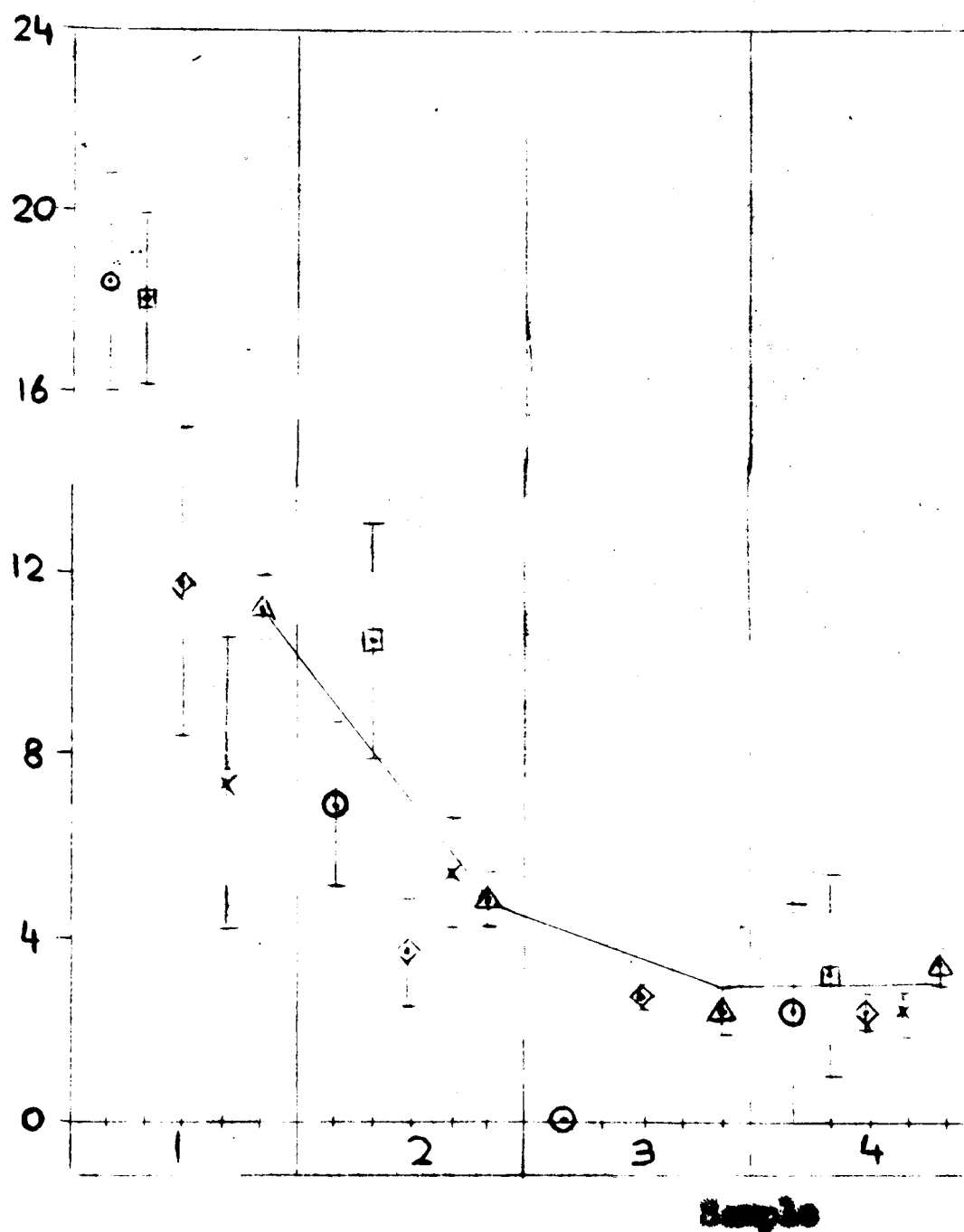
Thus counts also show an 80% gel ratio. Ultraviolet absorption in contrast gives: $\frac{\text{supernatant}}{\text{gel}} = 0.4$

Thus there is relatively more ultraviolet absorbing material in the supernatant than in the gel. The composition of fraction B therefore differs from that of (A and C), and

Fig. 22

Relation of radio activity with purification, and t
relation of the parts into which a sample can be separated
expressed as radioactivity per unit (refractometric
concentration.

**$10^{-2} \times$ number of radio active disintegrations per hour
per 10 mg. of protein.**



		SUPERNAT.	PPT.
HEATING ALONE		○	◇
HEATING WITH PHOSPHATE		□	×
PARENT	△	—	—

it cannot solely be a saturated solution of (A and C₂).

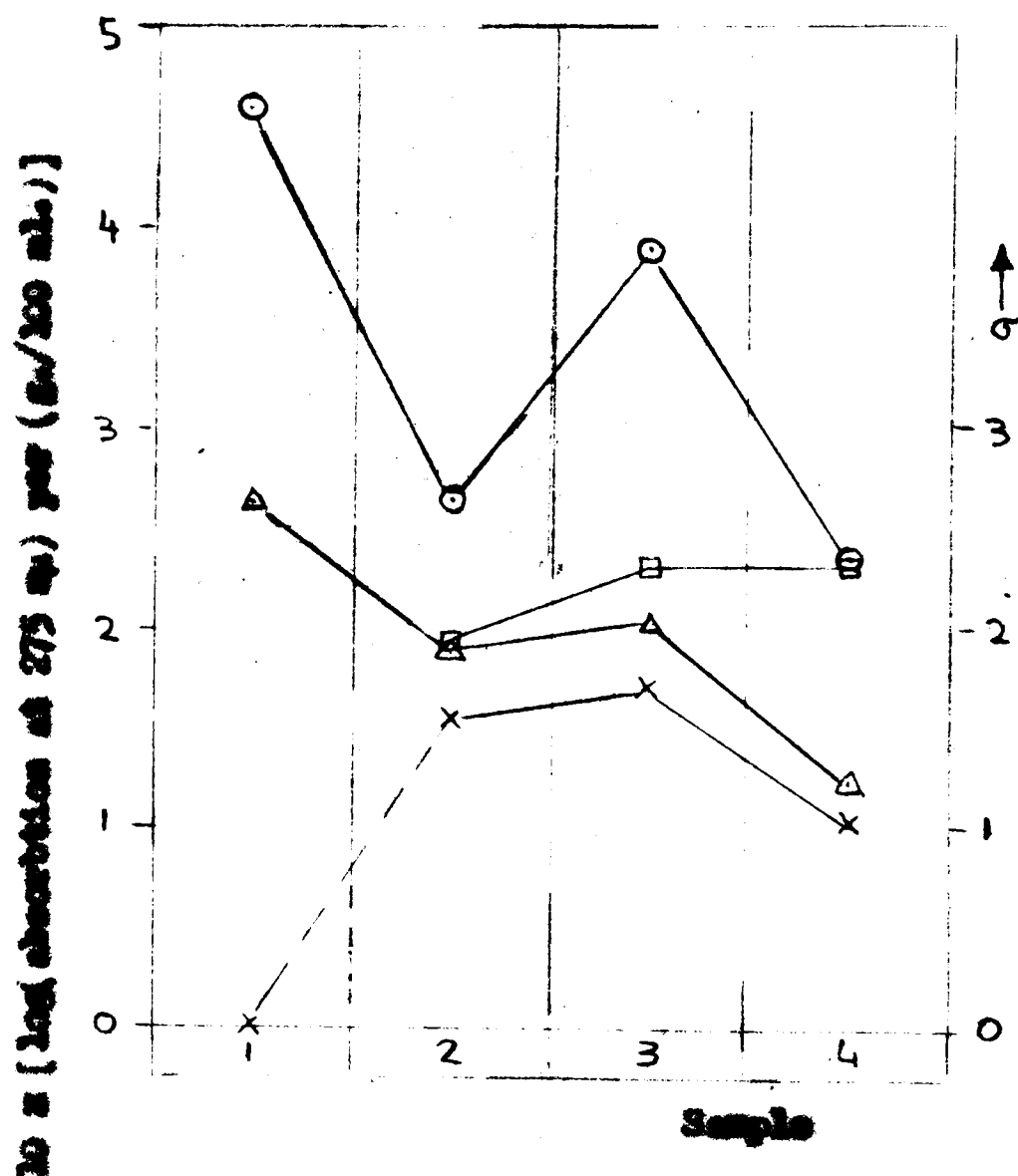
In (Fig.28, 29, 30) the three variables are combined in the three possible pairs.

(Fig.28) shows the counts per unit refractive increment. Again it is seen how after the first two samples the activity levels out, and that there is no difference in activity between supernatant and gel. This means that phosphate is neither split off nor taken up on forming the heat gel. Adsorption of radioactive phosphate, either initially on the molecules, or finally on the gel, could obscure the deductions. To investigate this samples 1, 2 and 4 were each divided into two parts which were heat gelled separately. Phosphate buffer of the same pH, 6.7, was added to one set to a final concentration of $(N/200)$ some 5-20 minutes before warming. A rough estimation of phosphorus was made on one supernatant and it was evident that no appreciable fraction of the added phosphate had been removed by the gel. The results are shown in (Fig.27) and (Fig.28). A significant effect was obtained only with the crudest sample 1, and perhaps there is just an effect with sample 2.

It is concluded that in the purified samples the distribution of radioactive phosphate with respect to concentration of material is not affected by warming, and

Fig. 2.

Absorbtion at 275 m. per unit concentration (refractometry)
for samples at different stages of purification, and for the parts
into which each sample can be separated by heat rolling, in
particular the relationship of these values for supernatant
and gel.

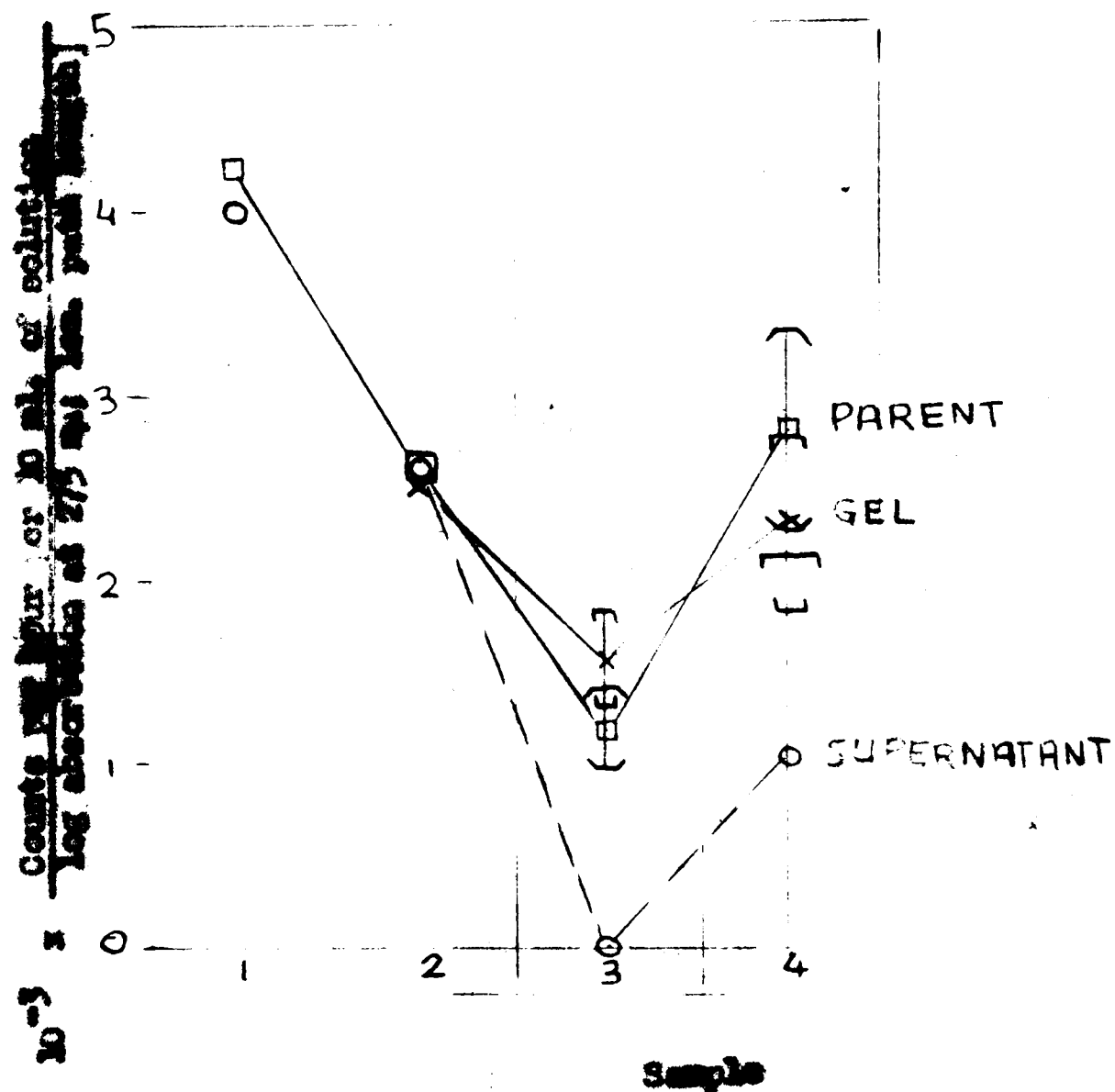


$$\sigma = \frac{[\log \text{absorbtion at } 275 \text{ m. per (g./100 ml.)}] \text{ of supernat.}}{[\log \text{absorbtion at } 275 \text{ m. per (g./100 ml.)}] \text{ of gel}}$$

- Δ percent
- supernatant
- × gel
- values of σ

Fig. D.

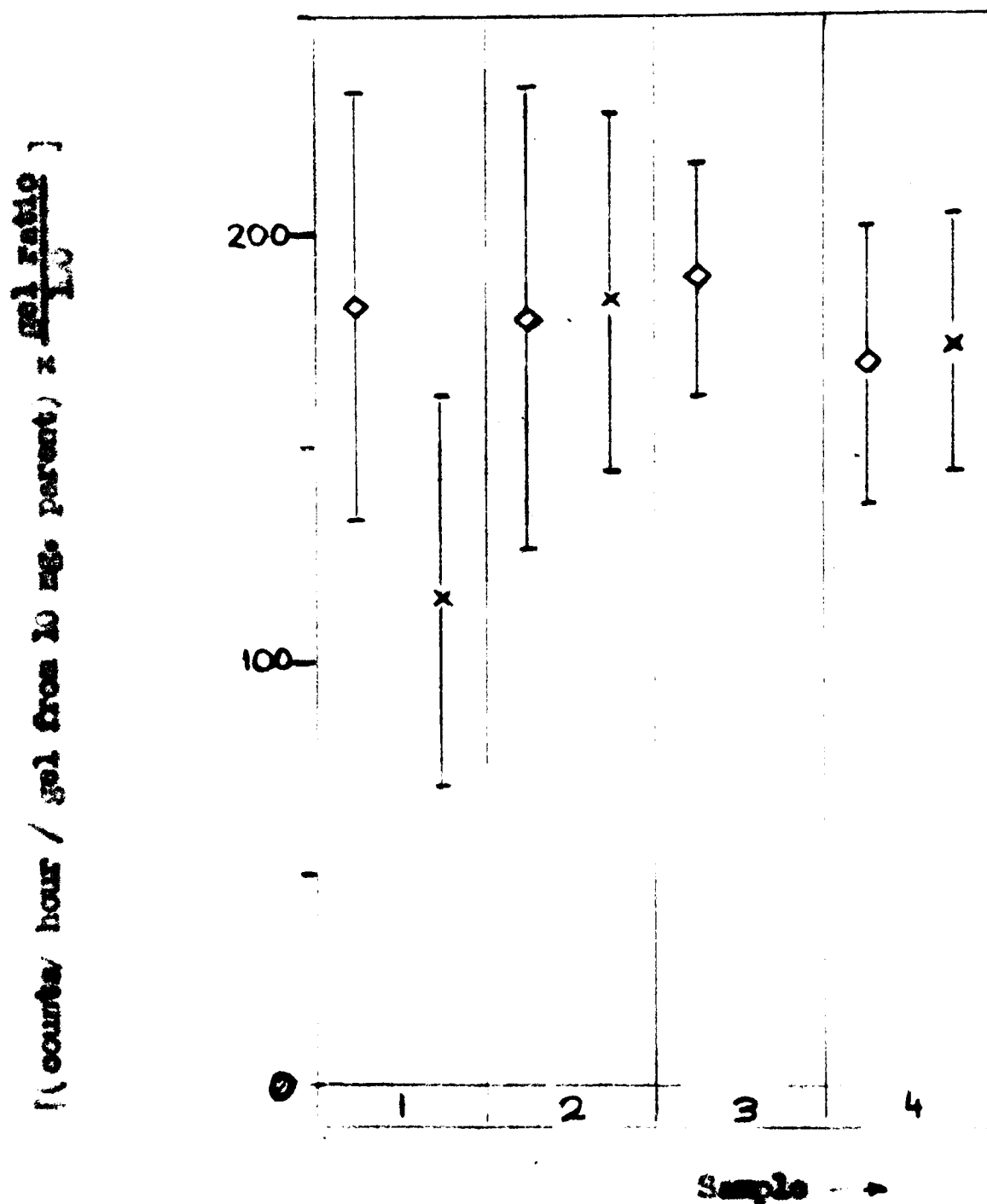
Radio activity per unit of absorption at 275 mμ for samples at
different stages of purification, and for the parts into which
each sample can be separated by heat gelling.



[Standard deviations, calculated only on the basis
of the number of counts taken, are indicated]

Fig. 31.

(Gel ratio x ratio active disintegrations per hour given off by gel made from 10 mg. parent), for samples at different stages of purification.



x heating after the addition of phosphate buffer

◇ heating without adding phosphate buffer

that radioactive phosphate is not adsorbed on these samples.

(Fig.29) shows the ultraviolet absorption per unit refractive increment, and inset is the ratio of these ratios for supernatant/gel, which steadies at 2.3, i.e. supernatant shows 2.3 times as much ultraviolet absorption per unit (refractive) concentration, as gel does.

The proportion of material absorbing in the ultraviolet seems to decrease with purification for parent, gel and supernatant, though perhaps not significantly so for the last. Thus more than four NaCl precipitations might yield slightly purer material.

The results given in (Fig.30) of counts/ultraviolet absorption show considerable scatter. This ratio appears to remain constant or to increase slightly in passing from samples 3 to 4; an increase will occur if material absorbing in the ultraviolet continues to be removed while ^{32}P material is not.

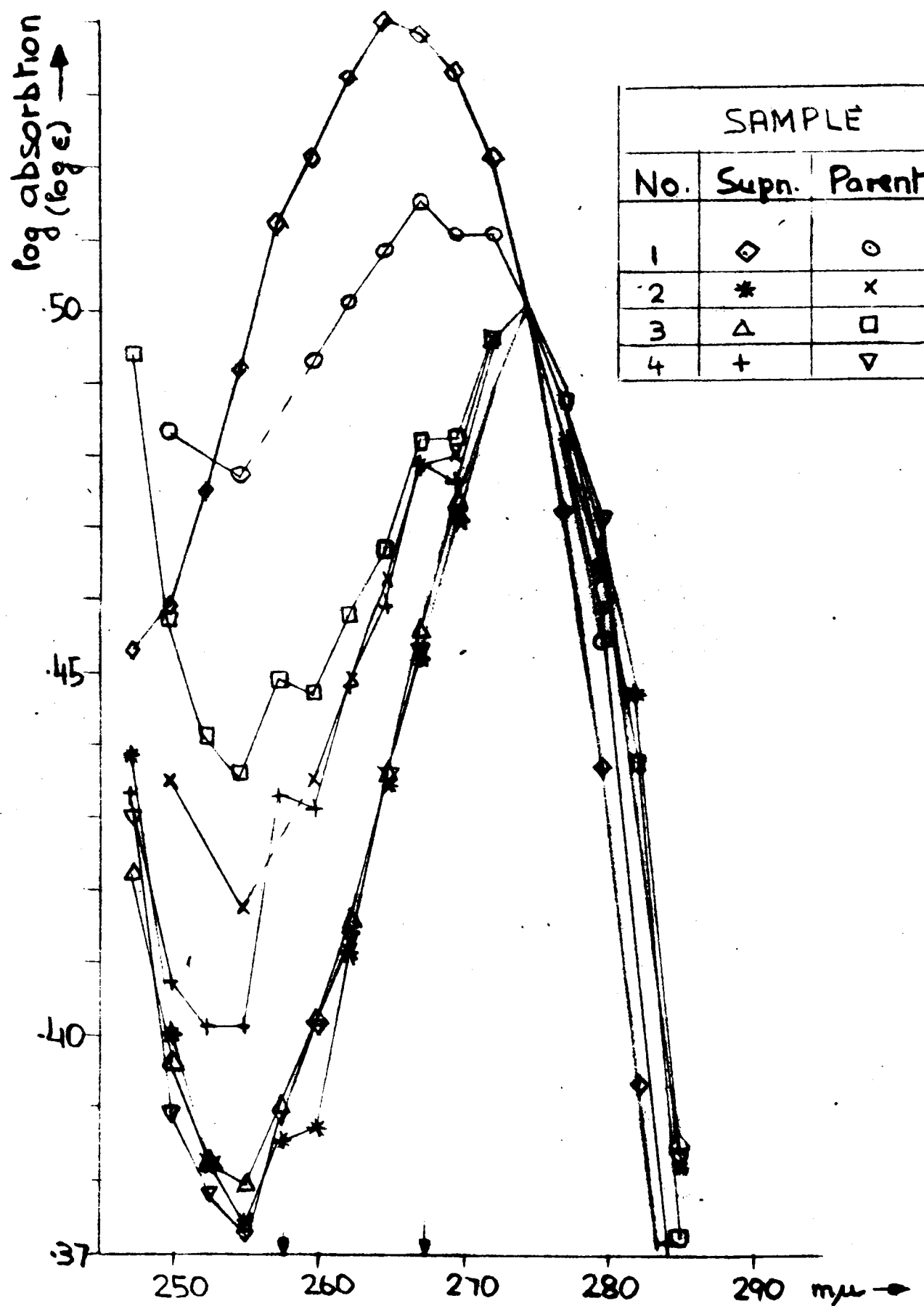
In (Fig.31) is plotted the function:

$$[H \times (\text{refractive}) \text{ gel ratio}]$$

where H = counts per hour emitted from a gel made from unit quantity (10 mg.) of parent.

Fig. 12.

Ultra violet absorption spectra of samples and of their constituent parts, at different stages of purification corrected to a log absorption = 0.500 at 275 m μ and 1 cm. path length.



From (Fig.31) it appears that the product of these two quantities is constant, so that we can write:

$$H \times \text{gel ratio} = k^1 \quad (33)$$

where k^1 is a constant.

It is difficult to interpret this constancy, which may be fortuitous. One way is the following. Rewriting equation (33) we have :

$$H = k^1 / \text{gel ratio} \quad (34)$$

Whatever the gel ratio of pure neutral salt soluble collagen might be (if it gels at all): $k^1 / \text{gel ratio}$ will be finite, and therefore H will be finite, which means that a definite amount of radioactive phosphorus will be included in the neutral salt soluble collagen.

In (Fig.32) the ultraviolet absorption spectra of parent and supernatant solutions are plotted between 247.5 and 285 mμ, the absorption scale of each curve being corrected to give a log extinction of 0.500 at 275 mμ. Shoulders at 257.5 and 267.5 mμ are apparent (absorption was only measured at 2.5 mμ intervals) and it is clear that the material which absorbs over this range of wavelengths is decreased with purification relative to the material absorbing at 275 mμ. This is interpreted as progressive removal of compounds containing purine and pyrimidine, such as nucleic acids and their fragments.

The peak at 275 mμ is presumably due to tyrosine, though, with the shoulders, it could also be adenosine.

C.6.4. Summary and conclusions

- (1) Little was gained by carrying this method of purification through more than three or four NaCl precipitations; only the content of ultraviolet absorbing material was thereby slightly decreased.
- (2) The composition of supernatant and precipitate, formed by warming, differed in that the supernatant contained some 2.3 times more absorbing material per unit refractometric concentration than did the precipitate. (A corresponding ratio of 1.7 had previously been obtained in an experiment on less pure rabbit neutral salt soluble collagen, sample IV.)
- (3) The relative compositions of supernatant and "heat precipitate" were constant, as judged by all criteria used, after 3 - 4 NaCl precipitations.
- (4) The purified material appeared to have a definite phosphorus content. The distribution of phosphorus with respect to material was not affected by warming, i.e. phosphorus was neither split off nor taken up on "heat precipitation". Radioactive phosphorus was not adsorbed, as phosphate, to any extent on the parent material or "heat gel".

(5) Traces of purine and pyrimidine compounds could have been present, as judged by absorption at 257.5 and 267.5 mμ, though it would have been difficult to distinguish these from small quantities of phenylalanine. The proportion of material absorbing between about 250 mμ and 280 mμ was decreased with respect to absorption at 275 mμ, during purification.

(6) The phosphorus content of purified material, as indicated by radioactivity, could easily have been due to traces of nucleic acids, their fragments, or related compounds.

There were two sets of evidence against such a source of the phosphorus. (a) It was found that radioactivity and refractometric concentration varied in parallel, both with respect to successive stages of purification and to distribution between supernatant and heat precipitate, within any sample, (b) Qualitatively, the decreasing proportion, within the ultraviolet absorbing material, of material most likely to be associated with phosphorus, was not matched by a decreasing phosphorus count during the last stages of purification.

(7) The supernatant formed on heating was not entirely composed of the same material as the precipitate, though it may have contained the same material admixed with one or more materials possessing higher specific ultraviolet

absorption.

(8) If the absorption at 275 mμ was identified with tyrosine, some 5-15 tyrosine residues were calculated per molecule of weight 3.4×10^5 .

C.6.5. Related work

(1) Measurements of ultraviolet absorption made on various solutions during investigations on neutral salt soluble collagen showed various small peaks or shoulders in the range 255 - 270 mμ. Also material which was likely to be more pure, whether due to having undergone more NaCl precipitations, or having been clarified with the ultracentrifuge at some stages, showed a deeper minimum around 250 mμ than less pure solutions in which that region would only be a shoulder. This supports the previous finding, that with increasing purification, ultraviolet absorbing material is removed, particularly that of the region 255 - 270 mμ, a range in which nucleic acid constituents absorb.

(2) A dialysed solution of neutral salt soluble collagen was dialysed against TRIS buffer of same pH and ionic strength, when a precipitate formed. The supernatant was collected and its ultraviolet absorption spectrum and refractive increment were measured. These were qualitatively similar to those of a corresponding supernatant obtained by

heating the solution of neutral salt soluble collagen. The precipitate obtained with TRIS buffer was only sparingly soluble on subsequent exhaustive dialyses against phosphate buffer. These findings suggest that there is a similarity between "heat precipitation" and the precipitation obtained by adding TRIS buffer (Compare G.4.7.)

C.7.

ESTIMATION OF PHOSPHORUS CONTENT OF NEUTRAL SALT SOLUBLE,
AND OF THE RATE OF INCORPORATION OF PHOSPHORUS

A second experiment with P^{32} showed that:

- (1) There was only one g. atom of P per 340,000 g. of neutral salt soluble collagen.
- (2) Maximum incorporation of P^{32} occurred after more than 18 hours, and before 36 hours, following intraperitoneal administration.

C.7.1. Experimental

Rats were given P^{32} in batches of four, and killed 2, 10, 18, 36 and 94 hours after administration. There were two batches for the 2 hour value, one of which was irradiated (1000 roentgens each for Dr. L. A. Stocken's experiment) and denoted as 2R, the others were controls, denoted as 2N. Neutral salt soluble collagen was isolated as in (C.6.2.) using versene-NaCl buffer pH 6.9 and four NaCl precipitations. The final material was taken up in versene buffer and after exhaustive dialysis clarified on the ultracentrifuge, but it was then dialysed against three changes of acetate buffer pH 3.6. This ensured that no "heat precipitation" would occur during prolonged measurements at room temperature.

Refractive increment, ultraviolet absorption, radioactivity and phosphorus content were measured on each

sample. Radioactivity and phosphorus content were also measured on corresponding samples of the supernatant, after the first NaCl precipitation, of the skin extracts and of samples of plasma from blood collected at death (exsanguinated blood; cells removed at once on the centrifuge; proteins precipitated by trichloroacetic acid of final concentration 6%; filtered).

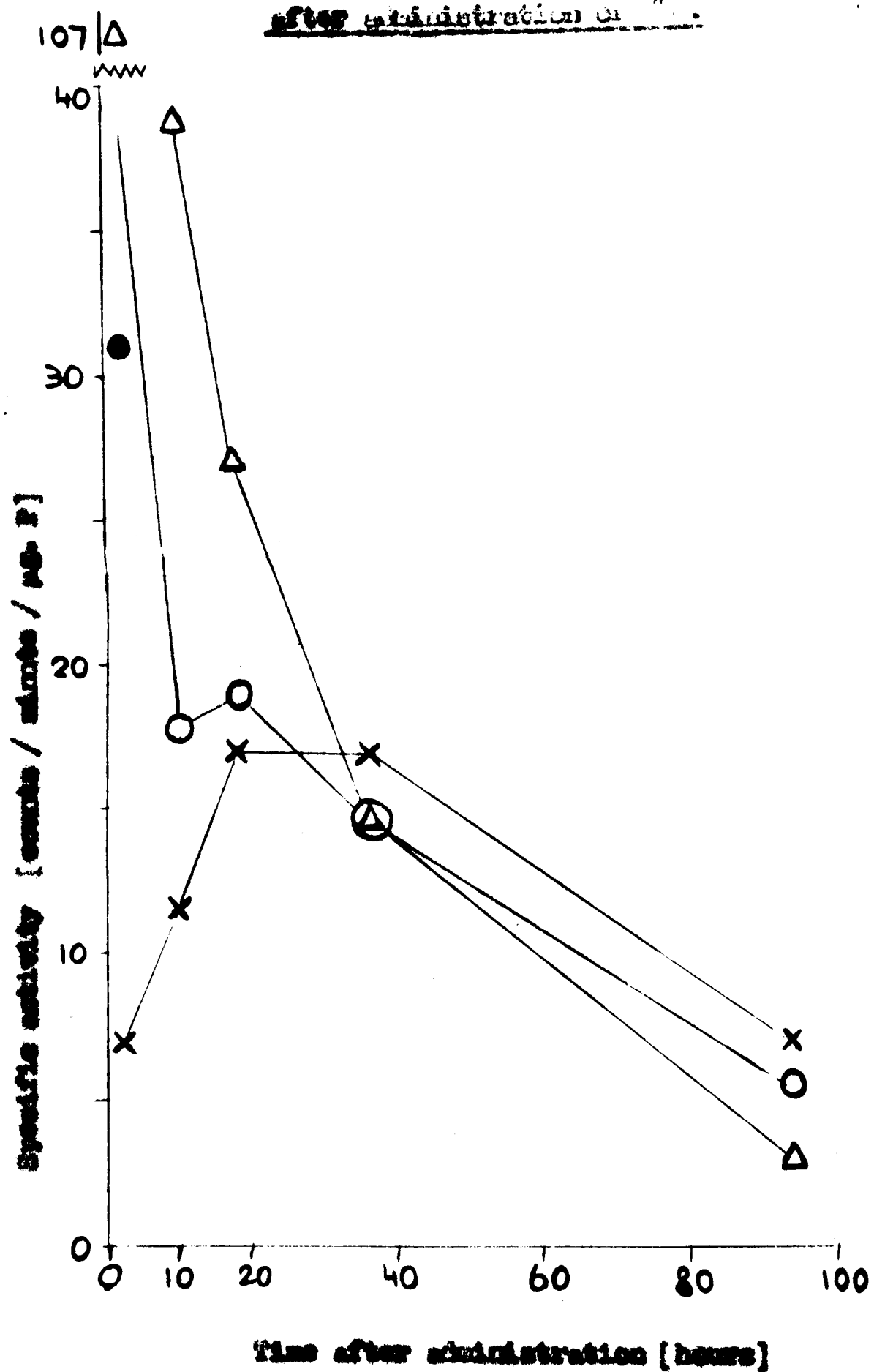
C.7.1.1. Phosphorus estimation

The method of Beerenblum & Chain⁷ was used.

About 5 ml. of sample were ashed with 2 ml. concentrated nitric and 0.5 ml. of concentrated sulphuric acids for 4-5 hours. After cooling the residue was hydrolysed with about 5 ml. of water by heating to boiling point for 30 minutes.

The extract was quantitatively transferred to a separating funnel, the total volume of water being 10 ml. There were added: 10 ml. isobutanol, 2.5 ml. ammonium molybdate (5%), 0.5 ml. 1N H_2SO_4 and the mixture was shaken for 20 secs. The aqueous phase was run off and two washes each with 10 ml. 1N H_2SO_4 given with 15 sec. shaking. The isobutanol layer was quantitatively transferred to another separating funnel, using not more than 2 ml. of isobutanol for rinsing. Reduction was then performed with 5 ml. of $SnCl_2$ solution (1 g. $SnCl_2$ in 2.5 ml. conc. HCl,

Specific radio activity of ^{32}P in samples taken at various times after administration of ...



- x neutral salt soluble collagen
- o residues of skin extracts after removal of neutral salt soluble collagen. [o animals irradiated]
- Δ plasma

TABLE 6

Phosphorus and tyrosine content of neutral salt soluble collagen,
sample XII, prepared from rat skin (C.7.)

<u>Sample</u>	<u>µg.P per 100 mg. protein</u>	<u>Tyrosine %N of total N</u>
10	8.62	0.63
18	9.62	0.89
36	9.3	0.84
94	8.92	0.72
mean	9.16	0.77

diluted 1 in 200 with H_2SO_4 , shaking for 30 secs.

After running off the aqueous phase, the isobutanol layer was transferred to a measuring cylinder, cleared with a few drops of ethanol and made up to 10 ml. with isobutanol. The solution was filtered and read in the Hilger Uvispek spectrophotometer, using light of 680 mμ wave length and cells of 1 or 4 cm. path lengths.

Suitable controls, standards and duplicates were run.

C.7.2. Results

The respective specific activities (corrected counts per minute, per μg P) are given in (Fig.53); the phosphorus content, and the tyrosine content calculated from ultraviolet absorption at 275 mμ, are given in (Tab.6). Little reliance can be placed on values for neutral salt soluble collagen for samples 2N and 2R. Very concentrated solutions (order of 0.4%) were used. While this ensured reasonable counts of 1600 or more, counted in under 1 hour, the solutions were not perfectly clear for light absorption measurements; an approximate correction for scatter was made from readings at 550 mμ and allowing for a fourth power wavelength relation. The samples used in phosphorus estimation contained 2-4 μg.P.

C.7.3. Discussion and conclusions

The mean phosphorus content of samples 10, 18, 36, 94 was 9.16 $\mu\text{g P}$ per 100 μg protein (Tab.6), from which the widest deviation was by 6%. This is equivalent to 1 g. atom of P in 340,000 g. of neutral salt soluble collagen.

According to (Fig.33) the maximum incorporation of P^{32} occurred about 24 hours after administration. About this value is also found for the incorporation of C^{14}

glycine into neutral salt soluble collagen^{49, 56, 99}. By comparison with these experiments, the appreciable incorporation of P after 2 hours appears suspicious, while the decay within 94 hours is in agreement with them.

The question again arises as to whether the radioactive phosphorus determined in the final material was due to some impurity which had been carried over. Determinations were made of the specific activity of phosphorus in the residues left after the removal of neutral salt soluble collagen from the skin extracts, in order to give some information on this point. If the specific activities of the residues and of the purified material had been found to be comparable in the various batches of animals, then this would have been evidence for the phosphorus in the neutral salt soluble collagen being an impurity. Inspection of (Fig.33) shows that these two sets of specific activities

were found to be quite different, in particular that the specific activities of neutral salt soluble collagen were greater (and outside experimental error) than those of the residues after 36 and 94 hours. The only conclusion which can be drawn is: that there is no evidence to show that the phosphorus found in the neutral salt soluble collagen is an impurity.

This phosphorus could still be an impurity, provided the following had occurred. The residues may have contained several phosphorus compounds, of different specific activities, and it could have been that only some of these, of relatively high specific activity were carried over with the neutral salt soluble collagen during isolation.

The ultraviolet absorption spectra did show small shoulders at 258 mμ, and perhaps at 266 mμ. It is not possible to measure the content of purine and pyrimidine material from this, but qualitatively, there could be just about enough nucleic acid like material to account for the phosphorus.

The mean tyrosine content found is only approximate: calculated as 0.77%N of total N from absorption at 275 mμ; Eastoe²⁹ found: 0.45 (though Eastoe's analysis was done on rabbit material).

Whether the phosphorus found associated with the neutral salt soluble collagen was an impurity or not, 1 g. atom of phosphorus was found per 340,000 g. of neutral salt soluble collagen, and correspondingly only one mole of hydroxylysine residue could be phosphorylated in this weight of material. Now Eastoe's²⁹ analyses show that 15 mole residues of this amino acid are present in 340,000 g. of neutral salt soluble collagen. It is therefore unlikely that a dephosphorylation of hydroxylysine is connected with any of the changes undergone by neutral salt soluble collagen.

Lastly, whatever material the phosphorus may be bound to, it is optimally incorporated in about 24 hours.

C.8. INVESTIGATIONS MADE WITH THE MICROSCOPE ON NEUTRAL SALT
SOLUBLE COLLAGEN AND ON HEAT PRECIPITATE

C.8.1. Optical microscope:

Heat precipitate consisted of fibres, though due to synereisis of the precipitate, such fibres could often only be distinguished with difficulty. They could usually be seen at the borders of clumps of synereised precipitate. The best preparations were obtained under conditions which caused a slow formation of precipitate, in particular a pH of about 6.7 and a temperature of about 10°C; judged subjectively versene and particles which could act as nuclei also seemed to aid the production of large fibres. Unstained fibres could be seen readily at 80 fold magnification, but were not easily photographed because of poor contrast. Usually high power (x 320) or oil immersion (x 800) were required to see the fibres. They could also be fixed in formal and readily took up van Gieson stain. Material treated in this way is shown in (bot.12). The apparent thickness of the fibre is no measure of its true width, as an interference pattern is formed.

The nature of the fibres, as observed in the light microscope, was independent of the pH (6.5 - 7.4) of the

parent solution when the precipitate was formed fairly rapidly.

Phase contrast microscopy showed up some very fine fibres. It seemed that all precipitates were really fibrous, in particular that formed by the addition of TRIS to a solution was so, and at least some of the insoluble material from a store of neutral salt soluble collagen was fibrous.

Characteristically the fibres curved gently along bands of fairly large radii of curvature and unless apparently mechanically kinked did not show sharp angular deviations. After washing with water they were more curly, taking up bands of smaller radii of curvature.

C.8..2 Electron microscope

C.8.2.1. Material in solution:- the appearance of the material depended on preliminary treatment. If it was fixed with formaldehyde a precipitate formed which could be dispersed by ultrasonic vibrations. The resulting material, after staining with phosphotungstate, showed as fibres of various length, but of width of only about 100A (Phot.13). There was multiple cross-banding, with particularly pronounced double bars at about 5000A intervals. Half way between these major repeats were smaller, but pronounced, doublets.

Similar material was obtained when the solution was first placed on the electron microscope grid and then fixed in formal vapour. It seemed possible that formaldehyde caused end-to-end linking of rods 2,500 A or 5,000 A long.

At this point it should be explained that as no electron microscope is to be found in Oxford, all this work was done with Dr. Valentine at the National Institute for Medical Research, Mill Hill, and while I was most courteously received there is a limit to which a guest may tax his host's patience. I am convinced that both formaldehyde and phosphotungstate, the electron microscopist's household reagents, alter the nature of neutral salt soluble collagen, and there was therefore the problem of finding other conditions for fixing the material on the electron microscope grid.

Eventually the following method was evolved. Keeping everything at cold room temperature, a drop of solution was placed on the electron microscope grid and then most of it was removed with filter paper. The film was fixed in osmium tetroxide vapour, and after washing in water was shadowed with Pt-Pd. The supporting film was a layer of carbon. Rods of uniform width were observed, the ends of several often meeting in a star shaped cluster, but there were also isolated ones. The rods appeared to be simple multiples of some standard length of about 2,500 A and showed nodes at this interval if

they were of multiple length. This photographic plate was lost by Dr. Valentine's assistant, and the experiment was going to be repeated at a later date. It has been repeated, but not under the same conditions. In place of carbon supporting films only those made out of nitro-cellulose were available and these are weaker, have a non-uniform structure, and their wetting properties are different. Further, in place of Pt-Pd shadowing, staining with phosphotungstate was used. Using this method there were examined samples of solutions which were also investigated by the various physical methods (Section C.11.4). The solutions were: (1) neutral salt soluble collagen, sample F71. (2) solutions of the fractions A, B, and C into which F71 was separated, (3) sample P87 which had been prepared by heat gelling a solution of F71, dissolving the gel in 1% acetic acid and dialysing back into phosphate buffer. Details of the preparation are given in section C.11.3.

The appearance of the complete neutral salt soluble collagen did not lend itself to any particular interpretation. (phot. 18 and 19). The irregular criss-cross pattern of strands could simply be a form of drying down of the material, though it need not be so.

The separate fractions each gave a characteristic appearance, and a combination of all these characteristics

was shown by sample P87, but not by the parent solution P71.

Fraction A only showed circular areas of structureless matter containing some more densely stained specks (Phot.20). These "blobs" must be artefacts, as some 60 particles of molecular weight 5×10^5 and partial specific volume 0.71 would be required to agglomerate into a cylindrical volume of this cross sectional area and only 1A thick.

In fraction B a number of relatively large, striated fibres were present (Phot.21 and 22). With well defined major repeat distances of 550-650A and many unit distances long, they are typical collagen fibres and must be an impurity carried over from the heat gel of fraction A, from which fraction B was separated (Spinco rotor 40, 10 mins. at 35,000 r.p.m.). It is surprising that they should not have been removed during centrifugation, but presumably the frayed out ends of many of them could interfere with rapid sedimentation. The main constituent of fraction B appears as a criss-cross mat of short threads, with distances of the order of 500A between junctions. Closer inspections shows lack of any regularity of length or width over given lengths, yet the order of width is throughout the same as that of the finest frayed out or tapered ends of the contaminating fibres. Probably the whole structure is some artefact, though in contrast to fraction A, it has a pronounced linear character.

Electron micrographs of fraction C (Phot. 23 and 24) showed no background of finely dispersed material and only a number of tangled fibre networks, rather resembling spiders' cobwebs in appearance. The constituent fibres are thin, of the order of 100A, and their numerous junctions appear as anastomoses as well as crossings-over. I interpret these "cobwebs" as being remnants of disintegrated heat gel, and they could have been formed by an incomplete disintegration of thicker fibres (in acetic acid), with some of the points of lateral adherence remaining intact. They may be preparative artefacts, but it seems unlikely. That such structures should not have been removed by centrifuging at 35,000 r.p.m. for 1 hr. is surprising, and is further discussed in section C.11.6.

Sample P.87, formed from re-dissolved whole heat gel, gave an appearance which was simply a combination of those of fractions A, B and C. Only few "cobwebs" were present, and they were small.

Separate tests showed that on heat gelling the isolated fraction A, characteristic collagen fibres were formed, similar to those formed on heat gelling whole neutral salt soluble collagen.

C.8.2.2. Heat precipitate

This appeared as fibres of varying width: 100 - 1000A and more, and of considerable length. Staining with phosphotungstate and preferably shadowing with Pt-Pd, brought out pronounced cross banding of the kind found in mature collagen. Examples are shown in (Phot.15) which was made from the same material as (Phot.12). The fibres seemed to be readily distorted (see (Phot.15) at the crossing over of two thick fibres) and so the length of the major repeat varied; thus in 13 lengths of fibres it varied: 580 - 830A with a mean of 680A. (It should be noted that in mature collagen fibres the length of the major repeat distance varies, thus Schmitt¹²⁰ gives a distribution of lengths varying from 400A to 940A, with a peak at 640A, for fibres from rat tail tendon.) Various sub-bandings were found and, as in mature collagen, the arrangement of their pattern was orientated with respect to the direction of the fibre (polarized). Many fibres had tapering ends, a property associated by some¹³⁴ with collagen fibres obtained from such growing tissue as connective tissue of young animals and certain tumors.

C.8.3.

Conclusions

The transfer of neutral salt soluble collagen from solution to the stage of the electron microscope is too drastic for any definite conclusion to be reached on the state of neutral salt soluble collagen in solution. Rods some 50-100A thick and a few thousand Angstrom units long may exist; in any case there appears to be a tendency for the formation of elongated structures. Side to side aggregation is absent. An intricate fine banded structure was observed after fixation with formalin, but this is not related to the pattern of mature collagen; the overall appearance approached more that of F.L.S. - "long spacing fibrils" of the American workers¹²².

The process of heat gelling and re-dissolving either the whole material or the separated fractions via 1% acetic acid, does change the material; new structures, some of which may be artefacts but are nevertheless characteristic, became apparent. Fraction C appears as fragments of anastomosing networks of fine fibres; Fraction B gives a fine, linear deposit; fraction A gave circular aggregates and upon heating fraction A fine fibres of the characteristic appearance of mature collagen are formed, and some of these fine fibres were present as contaminants in fraction B.

Heat precipitate, whether formed from whole material or from isolated fraction A, consists of long fibres, often with tapering ends and of varying width, whose cross banding was very similar to that of mature collagen.

C.9. BEHAVIOUR OF NEUTRAL SALT SOLUBLE COLLAGEN AT ACID pH

C.9.1. Introduction

At the beginning of this work acid pH was avoided because (1) hydrolysis to gelatin was feared (2) it appeared that acid pH accelerated the formation of "heat precipitate" (C.4.6.), which at the time was suspected to be acid soluble collagen; indeed "heat precipitate" was shown to be soluble in acetic acid and at least to disperse in citrate of pH 3.7. When it became clear that at least some of the heat precipitate could again be dissolved at neutral pH (C.5.4.), the question arose whether neutral salt soluble collagen might be soluble at acid pH. This was of interest (1) because it might help with the separation of the fractions deduced to exist from the experiments made using successive warming and cooling, and (2) because the variation of the process of heat precipitation had been studied only from pH 7.4 down to pH 6.5 (C.4.6. and C.5.5.1.).

After some preliminary experiments it was shown that: (1) "heat precipitation" did not occur below pH 4.5 (2) neutral salt soluble collagen formed a true solution in acetate buffer pH 3.7, in which it could be kept at

35°C for 1 hour without the amount of gellable material being thereby reduced.

The latter property was used to make some polarimetric studies.

C.9.2. Variation of the nature of heat precipitate with pH

Samples of neutral salt soluble collagen in phosphate buffer were brought to pH: 6.4, 6.0, 5.5. and 4.5 in the cold and then warmed to 37°C for 1 hour. Gels were formed at pH 6.4, 6.0 and 5.5 with increasing rapidity, but the solution at pH 4.5 remained quite clear. Thus heat gelling does not seem to occur at a pH below 5.0 - 4.5.

The nature of the gels, as judged by their opacity and rigidity, changed progressively with pH. This had already been noted in the pH range 7.4 - 6.5 (C.4.6.) and the present series confirmed that with decreasing pH, mechanical rigidity and perhaps opacity of the gel decrease; it was especially noticeable in the range pH 7.0 - 5.5.

The sample at pH 4.5 was cooled and NaOH was added slowly to bring the pH to 7.2 (Johnson Indicator paper): a cloudiness occurred at pH 5 - 5.5 which subsequently cleared. The solution at pH 7.2 gave a gel in the usual way on incubating at 37°C, so that the previous treatment

TABLE 7

Exposure of neutral salt soluble collagen to acidpH in the cold

<u>Exposure of neutral salt soluble collagen to acid pH in the cold</u> [Solution P49 sample VII. Concentration of g/100 ml.]					<u>Time (hours)</u>	
	<u>P49/1</u>	<u>P49/2</u>	<u>P49/3</u>	<u>P49/4</u>	<u>Interval</u>	<u>Total</u>
<u>Dialysis buffer</u>						
1st lot	0.0% acetic	citrate pH 3.7	H ₂ O dist.	H ₂ O dist.	1% acetic	16
2nd lot	"	"	"	"	"	26
3rd lot	"	"	"	citrate pH 3.7	"	26
4th lot	phosph. pH 7.0	phosph.	phosph.	"	10% acetic	24
5th lot	"	"	"	phosph. pH 7.0	phosph.	6
6th lot	-	-	-	"	"	110
Ppt. on removing transient in 1st dialysis then clear	transient in 1st dialysis then clear	none	1st dialysis	1st dialysis then cleared in citrate	none	208
Ppt. on back to phosph.	much	trace	some	trace	none	
Nature of ppt. fine st. needles	no fibres	fibres and amorph.	fibres and amorph.	amorph.	-	
Conc. solution recovered	0.224	0.316	0.261	0.306	0.349	a
Conc. after Ht. pptn.	0.040	0.041	0.053	0.044	0.035	b
∴ Conc. of gellable	0.184	0.275	0.208	0.262	0.313	c-a-b
Gel ratio	82%	87%	80%	86%	90%	c/a
Vol. starting soln. ml.	0.58	0.58	0.77	0.77	0.77	

had not grossly affected it.

The next step was to find whether at acid pH the properties of neutral salt soluble collagen would be affected in an irreversible manner in the cold: gel ratio was a convenient index.

C.9.3. The effect on gel ratio of exposing neutral salt soluble collagen, in the cold, to acid pH

Small volumes of an 0.5% solution of neutral salt soluble collagen in phosphate buffer pH 7.4 were placed in dialysis sacs. They were then dialysed against various solutions as recorded in (Tab.7), including acetic acid, citrate pH 3.7 and distilled water. Their behaviour was observed and they were finally exhaustively dialysed back into phosphate pH 7.0. Any precipitate was removed on the centrifuge and examined. "Gel ratios" were determined on the supernatants and on a control (C.5.2.). The experiment was done in the cold room.

The following observations were made (the state of the materials while within the dialysis sacs could of course only be judged qualitatively, by appearance):

- (1) Neutral salt soluble collagen was soluble in: citrate 0.1M, pH 3.7, and in acetic acid 1% and 0.05%.

(2) A precipitate was formed on dialysis against water, (similar to that reported by Harkness et al⁴⁹) and a transient precipitate was also formed on dialysis against 0.05% acetic acid.

(3) The precipitate formed on dialysis against water was soluble in citrate pH 3.7 (as⁴⁹), but also the material remained in solution on subsequent dialysis into phosphate pH 7.0.

(4) A precipitate formed on dialysing the solution in 0.05% acetic acid against phosphate pH 7.0, and about 50% of the material was thereby removed. This precipitate was later dissolved in 1% acetic acid and on neutralising gave a clear solution which formed a precipitate in the usual way on warming to 37°C.

(5) The precipitate formed by dialysis against water was only with difficulty soluble in phosphate (similar to report in⁴³).

(6) The final solutions recovered all appeared to consist mostly of neutral salt soluble collagen, as far as could be judged by their "gel ratios" of 82-90%.

Discussion and conclusion of these results:

(1) The following conditions did not convert neutral salt soluble collagen into gelatin or acid soluble collagen in the cold:

(a) acetic acid 1% for 68 hours followed by acetic acid 10% for 24 hours.

(b) citrate 0.1M pH 3.7 for 68 hours.

(2) Imperfect recovery resulted afterdialysis against water or 0.05% acetic acid. It is unlikely that the precipitates formed represented some fundamental change for:

(a) it has been reported⁴³ that a similar precipitate formed with water can, with perseverance, be redissolved in phosphate, and (b) the precipitate formed with 0.05% acetic was eventually brought into solution as in (4) above.

It is more likely that under certain ionic conditions neutral salt soluble collagen precipitates in a form from which it can be readily redissolved only via an acid pH.

* 12 001

C.9.4. The recovery of total gellable material after heat gelling followed by solution in 1% acetic acid

The previous experiment (C.9.3.) indicated only that the material which was recovered was unchanged, as judged by gel ratio. In this experiment the quantitative recovery of gellable material was measured and a heat gelling stage introduced at the same time. .

Experiment:- 0.72 ml. of a solution of neutral salt soluble collagen (solution P40 sample VII) in phosphate pH 7.0 was placed in a dialysis sac, and this in a small volume of buffer in a test tube. The whole was warmed to 37°C for 75 minutes, and then cooled to 4°C, taking care throughout not to disturb the sac. This was now gently transferred to cold 1% acetic acid and after 2 hours' dialysis the contents were fluid and clear. After exhaustive dialysis against phosphate buffer pH 7.0 the contents of the sac were quantitatively collected and weighed. The clear solution was ultracentrifuged (Rotor 40, 39,000 r.p.m., 40 minutes) and its "gel ratio" determined in the usual way (C.5.2.) as was that of a control.

Results:

	Put in	Recovered
Volume of solution	0.72 ml.	0.704 g.
Concentration of gellable material (g./100 ml.)	0.194	0.197
Total gellable material	1.40 mg.	1.39 mg.

Discussion and conclusions

It was concluded that heat gelling followed by solution in 1% acetic acid had no appreciable effect on the recovery of gellable material.

The next point to be investigated was whether the material was in true solution at acid pH, or was only dispersed.

C.9.5. The state of neutral salt soluble collagen in buffers of acid pH

C.9.5.1. Introduction

Preliminary experiments had shown that:

- (1) observed under the microscope an apparent solution of neutral salt soluble collagen in citrate $M/15$ pH 3.7 contained numerous very small pieces of swollen precipitate at whose borders fibres were visible.
- (2) an appreciable amount of precipitate could be removed on the ultra-centrifuge from an apparent solution of neutral salt soluble collagen in citrate pH 3.7.
- (3) gellable material could be recovered from a solution which had been previously kept at 37°C at acid pH (compare C.9.2.)

The suitability of acetate and citrate buffers of pH 3.7 as solvents for neutral salt soluble collagen at 4° and at 37°C was therefore investigated.

0.9.5.2. Experiment:-

1 ml. samples of a solution of neutral salt soluble collagen in phosphate pH 7.4 were exhaustively dialysed against $N/5$ acetate and $N/15$ citrate buffers, both pH 3.65, in the cold. After refractometric measurements one sample in each buffer was kept at 35°C for 1 hour and then ultra-centrifuged at that temperature and the refractive increments of the supernatants taken. Corresponding operations were carried out on a pair of samples maintained at 4°C . All the supernatants were then dialysed back into phosphate pH 7.0 and their gel ratios determined.

Results:-

These were only semi-quantitative due to the unknown losses occurred while ultracentrifuging small samples.

(0.5.4.) However, percentages of material removed by ultracentrifuging were:

	at 4°C	at 35°C
acetate solution	8%	13%
citrate solution	34%	15%

Gel ratios of the solutions recovered after the indicated treatment were:

	4°C	33°C
acetate solution	sample lost	80%
citrate solution	75%	43%

C.9.5.3. Discussion and conclusions:-

- (1) Of the material which was removed on the ultracentrifuge 8 - 15% could be accounted for by the process of centrifuging together with an appreciable surface denaturation which must occur during repeated handling of small samples (an insoluble film was left behind on all glass ware which required 10% acetic acid to remove it). The 34% lost from the citrate "solution" was, however, appreciably greater than this.
- (2) The gel ratio of 80%, measured on acetate solutions which had been exposed to 33°C, made it unlikely that the value for the corresponding solution kept at 4°C would have been much less, especially in view of the good recovery obtained previously (C.9.4.) under analogous conditions.
- (3) The low gel ratio of 43% for citrate solution which had been kept at 33°C may in part be due to the microbiological action which so readily occurs in this buffer, apart from any adverse action which the buffer may have had. In view of the findings with cold citrate solution, the matter was not further investigated.

(4) In conclusion then:- acetate buffer pH 3.7 appeared to be a suitable solvent for neutral salt soluble collagen, both at 4° and at 37°C, while in contrast citrate buffer was not. (Indeed a further attempt at making a solution in citrate gave a direct precipitate in the cold room).

0.9.6. The solution of neutral salt soluble collagen in $\frac{M}{15}$ acetate pH 3.7 buffer.

This solution of some 11 ml. was made for polarimetry, but before that it was shown as follows, that no aggregate was formed by neutral salt soluble collagen in this buffer.

An 0.222% solution of neutral salt soluble collagen (sample VII) was exhaustively dialysed against $\frac{M}{15}$ acetate pH 3.7. Refractometrically the resulting solution had a concentration of 0.220 g./100 ml. A small sample was run in the analytical ultracentrifuge and only a single peak corresponding to the usual neutral salt soluble collagen component was observed. The remaining 11 ml. were ultracentrifuged at 4°C (Rotor 40, 39,000 r.p.m., 1 hour), when there was practically no sediment and the supernatant had a refractometric concentration of 0.219 g./100 ml., i.e. within the accuracy of the experimental methods no material had been removed.

C.10. POLARIMETRIC STUDIES

C.10.1. Introduction

These measurements were made in the hope of obtaining some insight into the nature of heat precipitation. This was not achieved but an indication of the close relationship between neutral salt soluble and acid soluble collagen was obtained from yet another viewpoint, and also an index found which could help in future assessments of neutral salt soluble collagens.

The argument was briefly this: It is known that gelatin radically and reversibly changes its properties on altering the temperature from about 10° to 40°C, and that a reasonable model for this transition is a change in the helical structure of the collagen peptide chain which presumably remains in some form in gelatin. (Some form of helical structure is now most probable for collagen⁵³.) Heat precipitation of neutral salt soluble collagen involving not only a change in solubility, but also, as displayed under the electron microscope a change in structure, naturally comes to mind as perhaps a close parallel. Infra red and X-ray studies indicate that only the cold form of gelatin resembles collagen⁶⁶.

The transition is accompanied by a change in optical rotation, the "cold" state having twice the specific optical rotation of the "hot" form¹¹². Cohen²² has polarimetrically followed the conversion of acid soluble collagen into gelatin and the subsequent behaviour on varying the temperature.

In the denaturation of proteins, denaturation and aggregation can be separate stages^{31, 108}. It was then possible that some fundamental rearrangement occurred in neutral salt soluble collagen on warming and that this was separately followed by aggregation to "heat precipitate". In acid solutions the first stage might still occur, but conditions could prevent subsequent aggregation. A reversible change in optical rotation might then be displayed by solutions of neutral salt soluble collagen at acid pH, on varying the temperature.

The following experiments were made:

- (1) The specific rotation of neutral salt soluble collagen was measured before and after heat precipitation.
- (2) The variation of specific rotation of neutral salt soluble collagen with temperature was measured at pH 7.0.
- (3) Repeat of (2) at pH 3.7.
- (4) Repeat of (3) with acid soluble collagen.

C.10.2. The specific rotation of neutral salt soluble collagen before and after heat precipitation

A solution of neutral salt soluble collagen was made in the usual way in phosphate pH 7.4 (solution P57, sample VII, 0.228 g./100 ml. gel ratio 87%). The specific rotation was determined. The solution was heat gelled, and dialysed against 1% acetic and a succession of phosphate buffers pH 7.4 as before (C.9.4.) and after ultracentrifuging (Rotor 40, 39,000 r.p.m. for 40 minutes) the specific rotation was again determined at 24°C.

Results:

Specific rotation of original material : -376°

" " " recovered " : -378°

Conclusions:- within experimental error the specific rotation of neutral salt soluble collagen was not affected by heat gelling and subsequent recovery via acetic acid.

C.10.3. The variation of specific rotation of neutral salt soluble collagen with temperature at pH 7.0

A solution of neutral salt soluble collagen was made in the usual way in phosphate pH 7.0 (solution P55, sample VII, 0.154 g./100 ml. gel ratio 80%). The specific rotation was measured starting at 0°C and at

Fig. 54

Variation of specific optical
relations of soluble collagens
with temperature.

[buffer: M/15 acetate pH. 5.7]

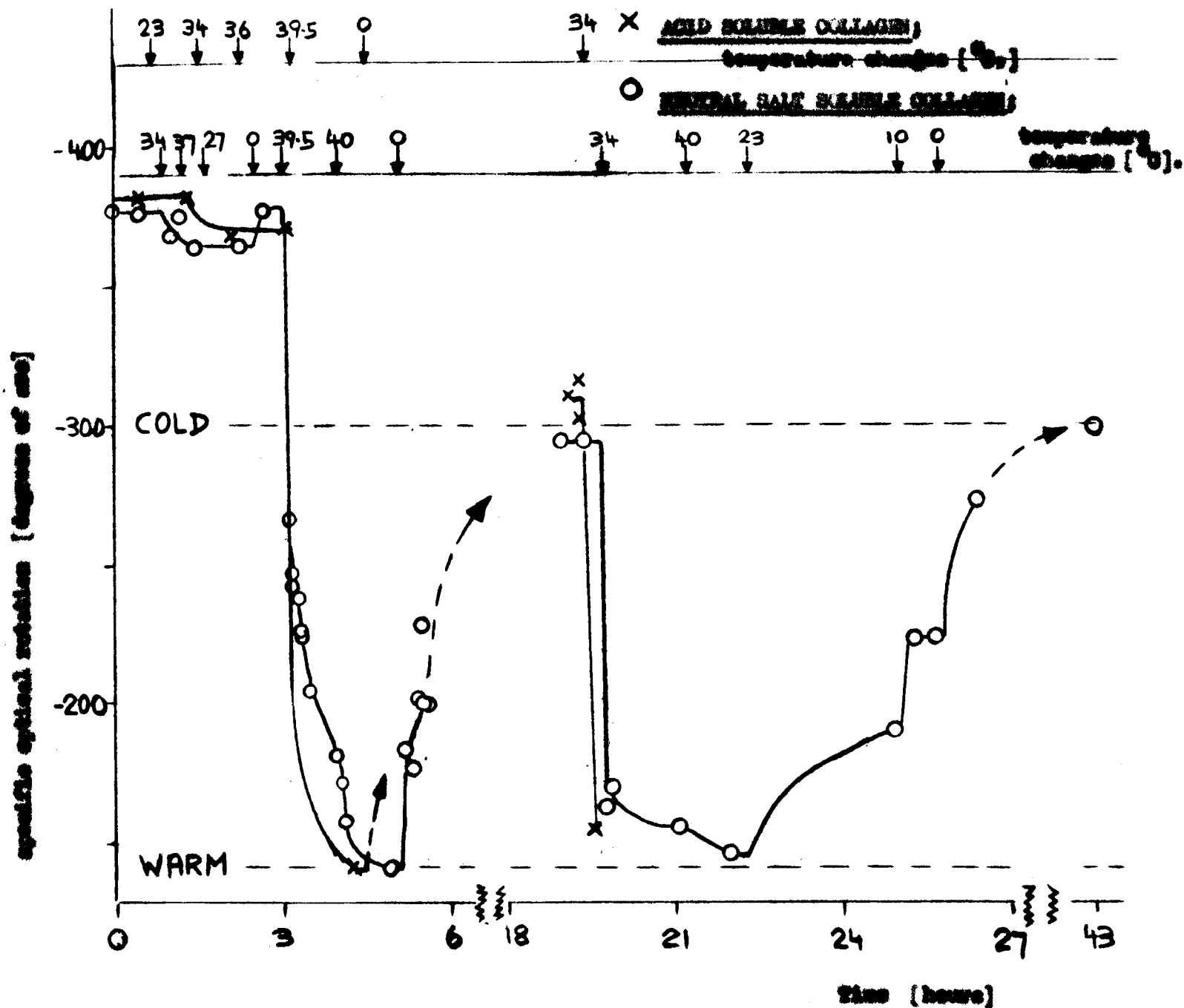
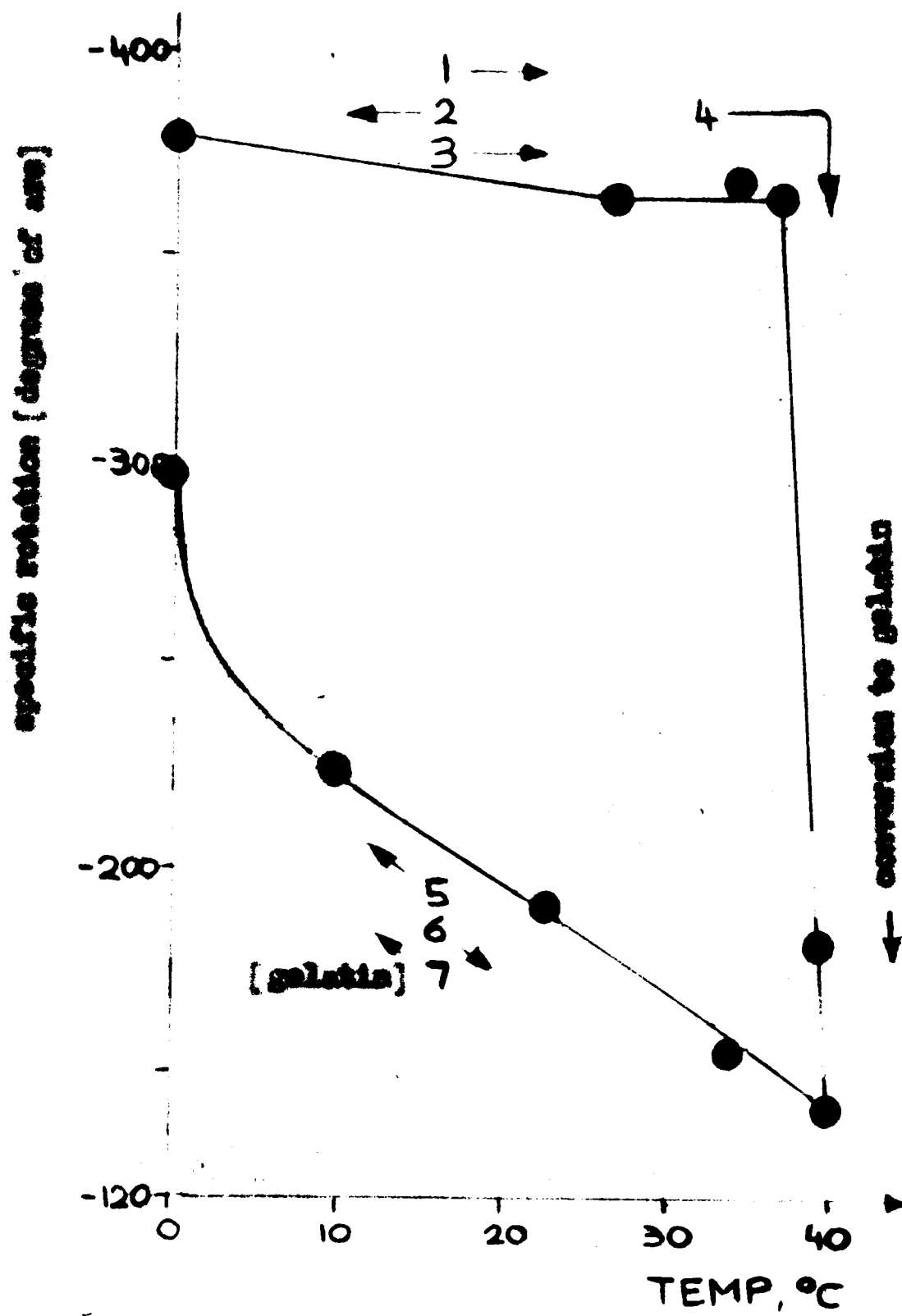


Fig. 3.

Variation of the specific rotation of neutral salt soluble
collagen with temperature.



intervals of 4 or 5°C to 33°C. Above this temperature the rotation became more positive, but as this is associated with the formation of opalescence, i.e. of heat precipitate, it cannot be interpreted. Gelling caused complete opacity at 33°C.

Conclusion:- the specific rotation of neutral salt soluble collagen at pH 7.0 is -378° from 0° - 25°C .

C.10.4. The variation of specific rotation of neutral salt soluble collagen with temperature at pH 3.7.

The solution of neutral salt soluble collagen in acetate pH 3.7, made in (C.9.6.) was used. The specific rotation was first measured at 0°C (-377°), then at 34° and 37°C , and after keeping the solution some time at this temperature it was returned to 0°C . Little change had occurred and it was reversible. The results are shown in (Fig.34) as a plot of specific rotation against time, with temperature indicated, and in (Fig.35) are plotted specific rotation against temperature with the various stages, so far corresponding to (1) and (2), indicated.

The solution was now brought to 39.5°C and a rapid change occurred, which was hastened at 40°C , the final specific rotation being -142° (stages (3) and (4) in (Fig.35)).

A partial reversal seemed to occur at 0°C and so the solution and cell were left in the refrigerator (4°C) overnight (12 hours, stage (5) in Fig.35.). This reversal was only partial, the specific rotation at 0°C being -294° . On warming the solution to 34°C the specific rotation rapidly decreased (numerically) again, the change being completed at 40°C to a final specific rotation of -46° (stage (6) in Fig.35). The dependence on temperature now appeared to be reversible and this was substantiated by a return to a specific rotation of -300° on storing overnight in the refrigerator (16 hour; stage (7) in Fig.35). It is interesting that intermediate changes of temperature from 40°C to 23°C and 10°C caused corresponding changes in specific rotation during this last stage (see Fig.34).

The final solution was dialysed against phosphate pH 7.0 in the usual way and its gel ratio determined: hardly any heat precipitate formed and the gel ratio had the nominal value of 4%.

Conclusions:-

- (1) The specific rotation of neutral salt soluble collagen at pH 3.7 and 0°C was the same as at pH 7.4 : -377° .
- (2) Warming to $34 - 37^{\circ}\text{C}$ at pH 3.7 affected neutral salt soluble collagen very little (or very slowly) and what change there was in specific rotation ($+13^{\circ}$) was reversible.

(3) Warming to 39.5°C at pH 3.7 caused an irreversible change; that this consisted of conversion of the collagen to gelatin was indicated by (a) subsequent polarimetric behaviour (b) lack of any appreciable amount of heat gellable material which could subsequently be recovered.

(4) The product into which neutral salt soluble collagen had been changed at 39.5°C and pH 3.7, showed a reversible variation of specific rotation with temperature, being -144° at 40°C and -297° at 0°C . This corresponds to the change in specific rotation found for bovine hide gelatin by Robinson 112: -130° at 35°C and 260° at 18°C , also -325° at 8°C ; and to the gelatin formed from ichthyocol by Cohen²²: (only approximate values were given): ca. -110° hot, ca. -290° at 2°C .

C.10.5. The variation of specific rotation of acid soluble collagen with temperature at pH 3.7

While I knew of Dr. Cohen's experiments by indirect, personal communication. I had no access to her results at the time. For comparison I repeated the previous experiment with acid soluble collagen from rabbit skin, made up in acetate buffer.

The results are shown in (Fig.34). It is clear that the behaviour of the two collagens was closely similar. Thus acid soluble collagen had a specific rotation of -382° (cf. neutral salt soluble -377°) and this altered little until an irreversible change occurred at 39.5°C (cf. neutral salt soluble collagen 39.5°C) to a specific rotation -142° (cf. neutral salt soluble collagen -142°). On standing in the refrigerator this recovered to -309° (cf. neutral salt soluble collagen -294°), and on subsequent warming to 34°C changed to -155° (cf. neutral salt soluble collagen -156°).

Conclusions:

- (1) Acid soluble and neutral salt soluble collagen at pH 3.7 are closely alike in that:
 - (a) specific rotations were practically identical (-382° and -377°);
 - (b) no irreversible change occurs at 36° , but an irreversible change occurs at 39.5° ;
 - (c) The specific rotations and their variation with temperature are practically the same for the two products (at 40°C : -142° and -144° ; at 0°C -309° and -297°).
- (2) Unless the acid soluble collagen used contained appreciable impurities of low rotary power, the identity of specific rotations with neutral salt soluble collagen suggests that either:

(a) fractions A, B and C (C.5.7.) of neutral salt soluble collagen all have a closely similar, high negative rotation, in which case fraction B would have a specific rotation indicative of a collagen, or:

(b) fraction B has a low specific rotation and fractions (A and C) have a correspondingly higher specific rotation than the sample of acid soluble collagen.

- in a brief investigation, inaccurate due to high dilution, the specific rotation of fraction B was -280° at room temperature.

Cohen²² only gives an approximate value for the specific rotation of acid soluble collagen (ichthyocol) at $350^{\circ} \pm$ Ca 30° .

C.11. PHYSICAL MEASUREMENTS

C.11.1. Routine procedure

Solutions were exhaustively dialysed against appropriate buffer and cleared on the ultracentrifuge (Rotor 40, 39,000, 1 hour) unless this had already been done. Concentration was determined refractometrically and then suitable dilutions made up, usually five dilutions 0.08 - 0.015 g./100 ml, 7 ml. of each. The "gel ratio" of the parent solution was determined (C.5.2) Couette viscosity, flow birefringence and ultracentrifuge measurements were made on each sample as described in the experimental sections. Fresh solutions were used, as a rule, for each measurement and then discarded. Solutions were kept below 10°C throughout, except for refractometry and ultracentrifuge measurements, when immediately prior to use the solutions were warmed to within 1° of room temperature by immersion for 5 minutes in a water thermostat running at room temperature.

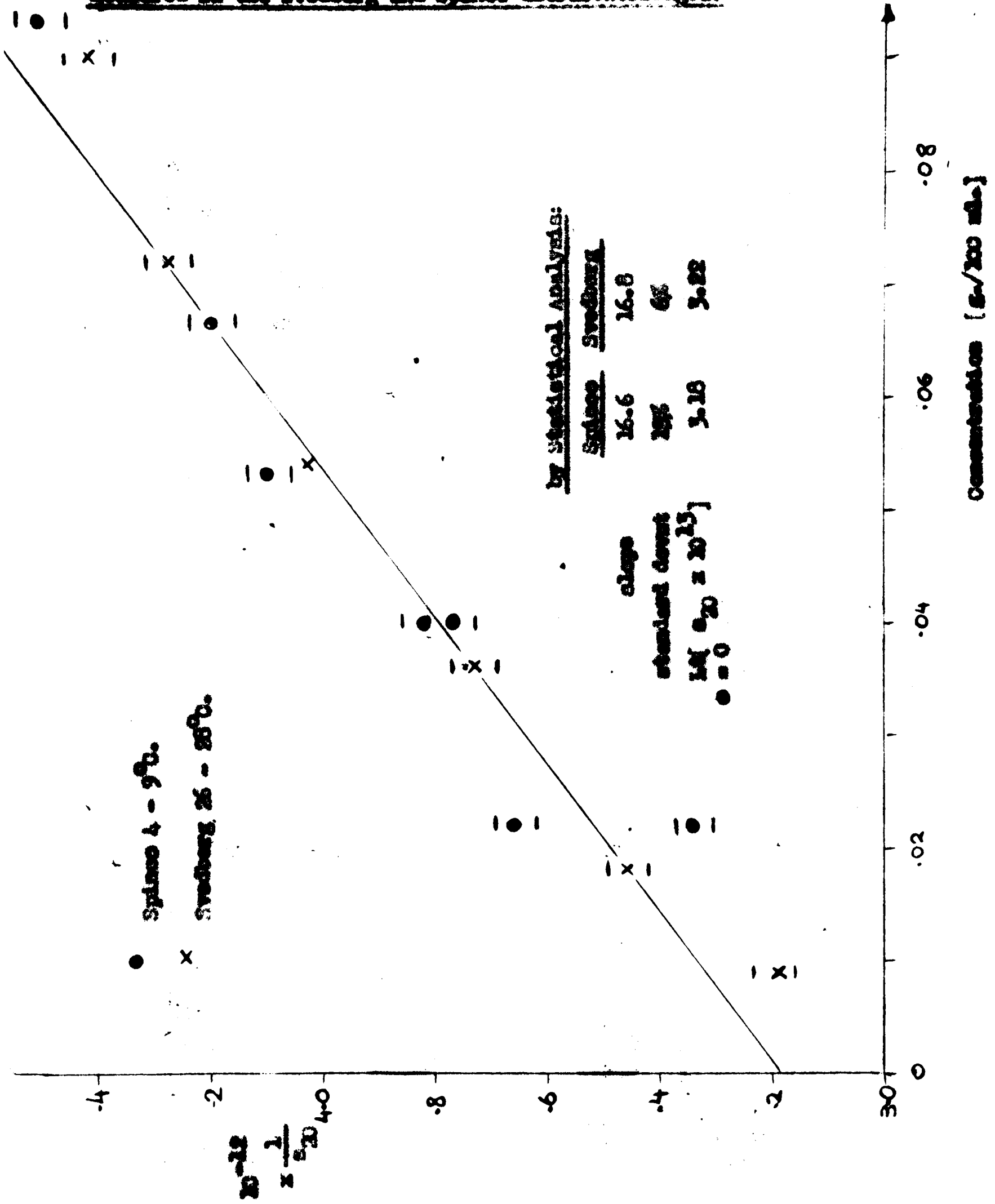
Additional refractometric, gel ratio or polarimetric measurements were made as required.

C.11.2. Ultracentrifuge measurements

The Svedberg oil ultracentrifuge at Oxford cannot be run much below 26°C (solution temperature). During preliminary ultracentrifuge runs at pH 6.8 "heat precipitation"

Fig. 3.

Variation of the reciprocal of the corrected sedimentation coefficient with concentration, for a sample of neutral salt soluble collagen measured in the Svedberg and Spinco ultracentrifuges.



had occurred. After stable conditions, at pH 7.4 had been found (C.4.6.) it was necessary to check (1) that no "heat precipitation" took place during the measurements at room temperature, (2) that they were representative of the material.

These checks were carried out on solution 131 sample V. Refractive increments of all solutions were measured before and after ultracentrifuging; samples were taken from stirred contents of analytical cell at the end of an ultracentrifuge run. Within 2% no difference was found between pairs of measurements. Also no particles of "heat precipitate" could be recovered from the bottom of the cell. This verified (1).

To check (2), the Spinco analytical ultracentrifuge at the National Institute for Medical Research, Mill Hill was used, run at $4^{\circ} - 9^{\circ}\text{C}$. A correction for adiabatic stretching of the Spinco rotor⁹ was applied to the derived sedimentation coefficients. Plots of $\sqrt{s_{20}}$ against concentrations are given in (Fig.36) as obtained from the two machines. Statistic analysis of the two linear plots showed: (a) that the results obtained with the Svedberg, machine have a lower scatter (standard deviation $\pm 6\%$) than those obtained with the Spinco machine (standard deviation $\pm 19\%$). This is probably chiefly due to the more accurate determination of temperature in the Oxford

machine, and also due to the better photographic material which was available.

(b) Extrapolation to zero concentration gave the sedimentation coefficients corrected to water at 20°C: 3.22S (Svedberg) and 3.18S (Spinco), which differ by 1.2%. (In the correction of the sedimentation coefficients to water at 20°C, the partial specific volume of gelatin¹⁴⁶, 0.71, was used and it was assumed to vary by 0.005 per 10°C, a variation given by Svedberg & Pedersen¹²⁹, Appendix II, for an average protein). The difference is of the order of experimental error.

Conclusions:- Results obtained with the Oxford Svedberg oil ultracentrifuge used at room temperature on solutions of neutral salt soluble collagen at pH 7.4 were identical with those obtained at 4-8°C in a Spinco analytical centrifuge; measurements made with the Oxford machine under such conditions are therefore representative and can be used in conjunction with physical measurements made below 10°C.

C.11.3. Materials investigated

Physical measurements were made on three different preparations of neutral salt soluble collagen (P19, P57, P71), on the three fractions A, B, and C into which one of these samples (P71) was separated, and on a control sample (P87) which was prepared by heat gelling P71, dissolving in 1% acetic acid

and dialysing back into phosphate solution. One sample (P19) was also investigated with sulphite present in solution (PS19). All solutions were made in M/15 phosphate buffer pH 7.4.

When the first sample (P19) was investigated the preparative Spinco ultracentrifuge was not available for preliminary clarification of the solutions, and correspondingly the properties of this sample differed somewhat from those of the others.

The fractions A, B and C were prepared as follows: 11 ml. of the clarified solution 172 were heat gelled (37°C, 1 hr.) in a Spinco rotor 40 centrifuge tube, kept in the refrigerator for 62 hrs. and after fragmenting the residual gel centrifuged (39,000 r.p.m. for 20 mins.). The clear supernatant containing fractions A and B was similarly gelled (37°C, 1 hr.), the gel fragmented and fraction B separated as clear supernatant after centrifuging for 10 mins. at 35,000 r.p.m. in Spinco rotor 40 equilibrated to 33°C. The residue of the first centrifugation was fraction C, and of the second was fraction A. Each was dispersed in about 10 ml. of 1% acetic acid, taking care to keep the temperature of everything near freezing point. Each fraction was dialysed overnight against 1% acetic acid. A little fine granular material formed in each, and an appreciable part of fraction C did not dissolve. The solutions were then exhaustively dialysed against three changes of phosphate buffer

Table 8.

Summary of dynamic measurements made on
neutral salt soluble collagen.

<u>Sample</u>	<u>Concentration</u> [g./100 ml.] $\times 10^2$	<u>η_{sp}</u> $\times 10^{13}$	<u>η_0</u>	<u>G</u> [sec ⁻¹]	<u>$\chi_{c \rightarrow 0}$</u>
P 19	9.0 7.2 5.4 3.6 1.8 0.9	2.26 2.34 2.48 2.68 2.89 3.14	5.2 4.2 5.5 3.1 1.6 1.9	$\left\{ \begin{array}{l} 57 \\ 110 \\ 253 \\ 370 \\ 540 \\ 725 \end{array} \right.$	40 38 36½ 35 33 31
P 57	10.3 8.0 5.7 3.4 2.3 1.1	2.32 2.27 2.54 2.57 2.69	3.5 2.8 2.1 1.5 1.2	$\left\{ \begin{array}{l} 100 \\ 227 \\ 490 \\ 940 \end{array} \right.$	40½ 36½ 35½ 35
P 71	8.0 6.0 4.0 2.5 1.5	2.37 2.41 2.59 2.67 2.64	5.6 4.6 3.0 2.4 1.3	$\left\{ \begin{array}{l} 142 \\ 291 \\ 616 \\ 930 \\ 1240 \end{array} \right.$	40 38 36 34 31½
P 71 A	8.0 6.0 4.0 2.5 1.5	2.25 2.46 2.61 2.56	4.4 2.3 1.8 1.3 1.3	$\left\{ \begin{array}{l} 465 \\ 930 \\ 1790 \end{array} \right.$	40 35 30
P 71 B	3.8 2.5 1.5	2.88 2.89 2.94	3.6 2.1 1.8		
P 71 C	8.0 6.0 4.0 2.5 1.5	2.28 2.48 2.58 2.55	4.2 4.5 2.3 1.5 1.3	$\left\{ \begin{array}{l} 217 \\ 465 \\ 930 \\ 1790 \end{array} \right.$	40 36½ 32 26½
P 87	8.0 6.0 4.0 2.5 1.5	2.40 2.42 2.63 2.63	3.6 3.1 2.1 2.0 1.2		
PS 19	7.2 5.4 3.6 1.8	2.51 2.87	2.6 2.2 1.8 1.3		

pH 7.4 and centrifuged (Spinco rotor 40, 39,000 r.p.m. for 1 hr.) There was an appreciable residue from fraction C; the supernatants were used for physical studies.

Sample F87 was made by heat gelling (37°C, 1 hr.) 11 ml. of solution F71 enclosed in a dialysis sac and immersed in phosphate buffer. The procedure used was that of section C.9.4., as was the subsequent dialysis via 1% acetic acid back into phosphate buffer. The final solution was crystal clear and was not centrifuged before use.

Sample F819 was made by adding to a solution of F19 a concentrated solution of sodium sulphite (Analar grade) in phosphate buffer, to a final sulphite concentration 0.02M.

C.11.4. Results and discussion of results

C.11.4.1. Ultracentrifuge measurements

There was some difficulty in measuring up the schlieren records of the ultracentrifuge when the concentration of the solution of neutral salt soluble collagen was 0.025 g./100 ml. or less. When necessary, the best straight line was fitted by the method of least squares, for the plot of (distance moved by boundary versus time) used in the calculation of sedimentation coefficients¹⁷

The sedimentation coefficients measured are tabulated in Table 8. For each material plots of $1/S_{20}$ versus concentration were made, as illustrated in Fig.36; the values of $[d(1/S_{20})/dc]$ and of the sedimentation coefficient extrapolated

Table 9

Parameters calculated for particles of neutral salt soluble collagen

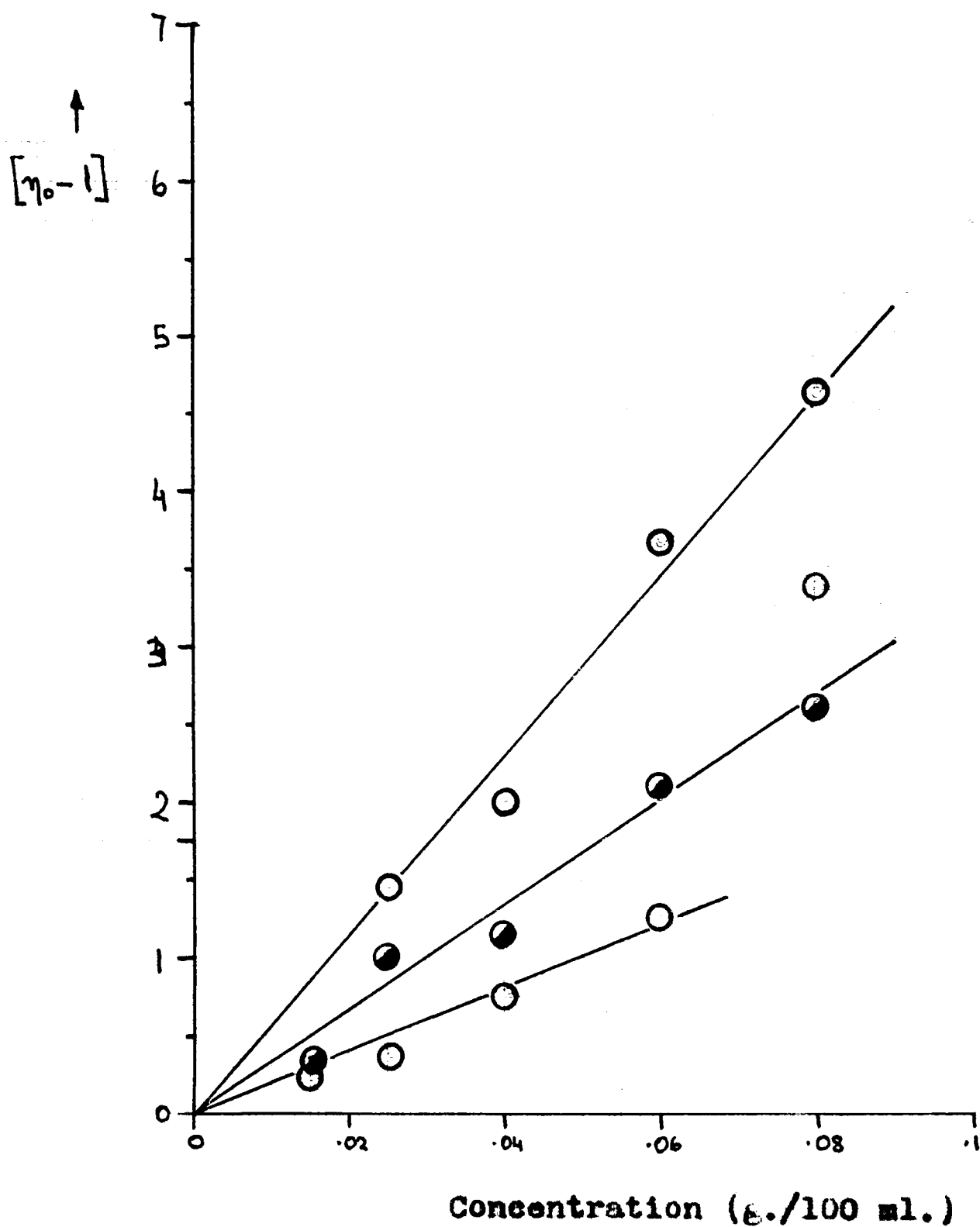
Sample	$10^{13} \times S_0$	$10^{-13} \times \frac{d(1/S)}{d\phi}$	$[\eta]_0$	Θ	J axial ratio	V [*] (ml/g.) hydra- tion	$10^{-5} \times H$ partic- le wt.	$2a$ (Å) length (1)* (2)*	$2b$ (Å) width (1)* (2)*
(Native)									
P19	3.23	1.68	47	220	42	36	5.0	5,800 3,400	140 80
P57	1.76	1.07	28		43	19	5.3	4,200	100
P71	1.70	0.81	57	360	83	14	6.2	5,700 3,100	70 40
Fraction A P71B	2.07	1.00	20	140	35	21	4.1	3,300 2,600	95 75
Fraction B P71B	2.97	0.55	60		158	5	5.9	6,100	40
Fraction C P71C	1.84	0.99	33		50	19	5.1	4,200	85
"Re-dissolved" P87	2.86	0.94	36		55	18	5.1	4,400	80
"Sulphite" P819	3.23	1.68	20		22	43	7.1	3,600	160

(1)* - calculated from plug theory only

(2)* - calculated from flow birefringence, using the axial ratio obtained by plug theory

Fig. 37.

Specific viscosity $\left[\frac{\eta_0 - 1}{c}\right]$ of some samples
of neutral salt soluble collagen.



- sample P 71.
- ◐ sample P 87.
- sample P 71 A.

to zero concentration which were thereby obtained are given in Table 9.

A representative schlieren record of a solution at 0.06g/100 ml. is shown in Phot.16.

C.11.4.2. Viscosity measurements

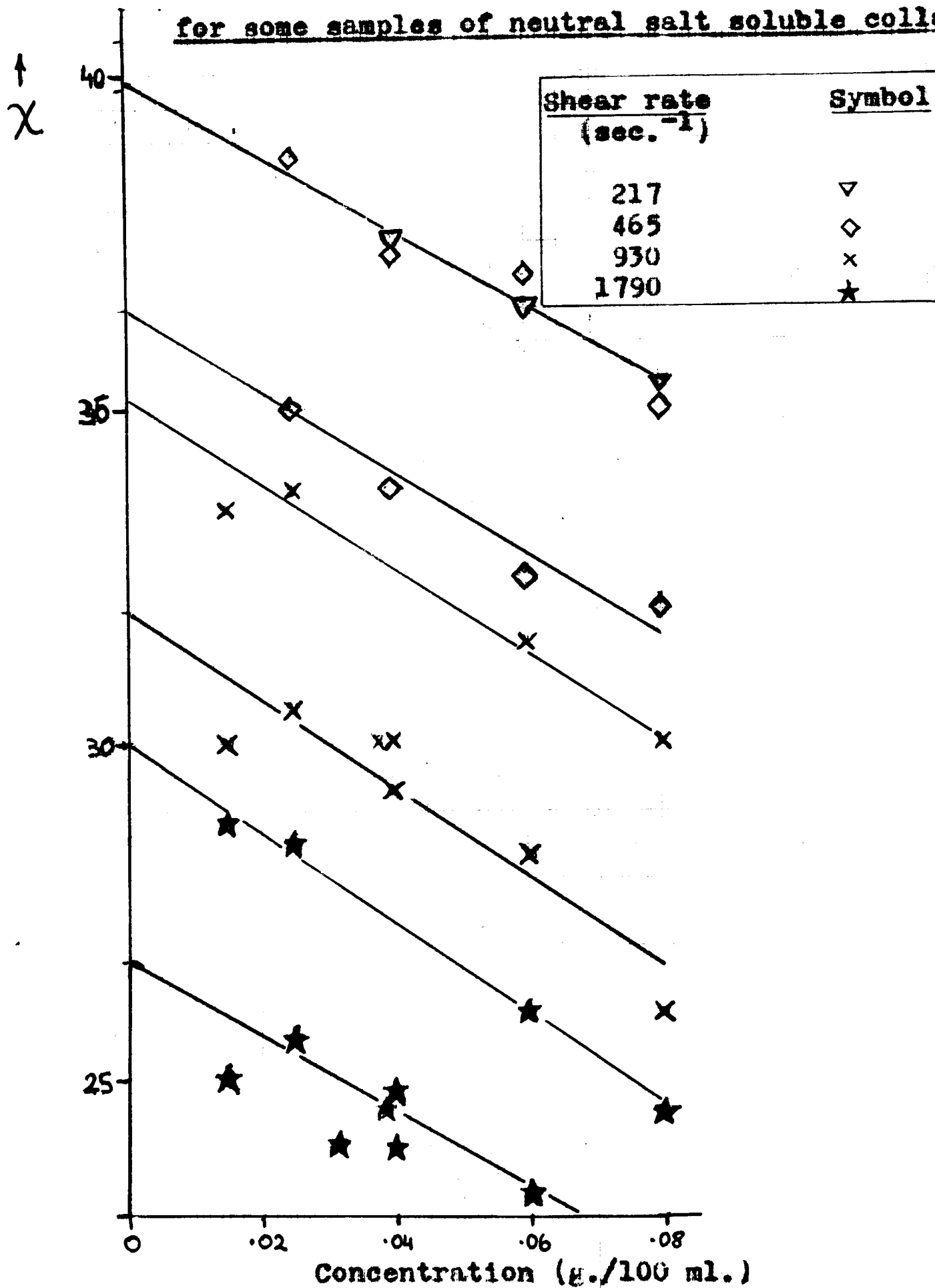
These were made with the Couette viscometer as described in section A.3.2. Relative viscosities at zero shear rate $(\eta_R)_0$, were obtained by plotting $[1/(\eta_R)_G]$ against shear rate G and extrapolating to $G = 0$. Plots were similar to that shown in Fig.9, though at higher dilutions η_R was independent of shear rate except at rather low shear rates. The specific viscosity $\frac{[\eta]_c - 1}{c}$ was found to be independent of concentration at concentrations of neutral salt soluble collagen less than about 0.08g/100 ml. as illustrated in Fig.37.

The values of η_0 obtained are given in Table 8, and of intrinsic viscosities $[\eta]_0$ in Table 9.

The use of a fine torsion wire (Eureka wire, B.WG45) enabled good plots of η_R versus shear rate to be made except for the most dilute solutions at low shear rates. The limiting factors were the tolerance in the machining of the containing pot, and surface tension effects at the interface of liquid and the surfaces enclosing it.

Fig. 38.

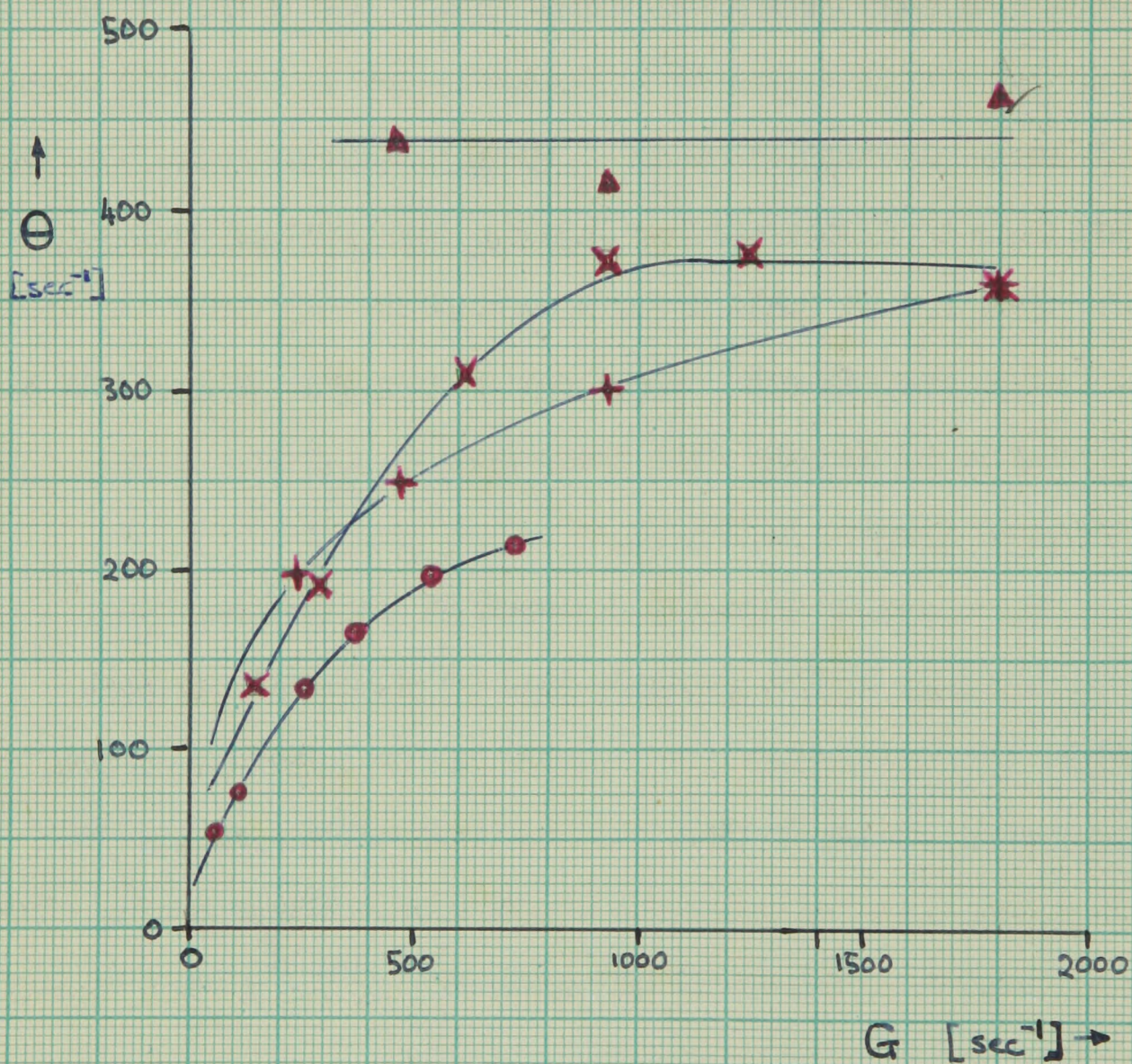
The variation of the angle of isocline χ
with concentration, at different shear rates
for some samples of neutral salt soluble collagen.



- — sample P 71 A.
- — sample P 71 B.
- — sample P 71 C

Fig. 39.

Variation of rotary diffusion coefficient Θ
with shear rate G , of some samples of neutral
salt soluble collagen.



- sample P 19
- × sample P 71
- + sample P 71 C
- ▲ sample P 71 A.

C.11.4.3. Flow birefringence measurements

The angle of isocline, χ , was measured as described in section A.5.4. At a given shear rate, χ was found to vary with the concentration of the solution of neutral salt soluble collagen. Extrapolation of χ to zero concentration was therefore made as illustrated in Fig.38. The extrapolated values are tabulated in Table 3 and were used to calculate the rotary diffusion coefficient Θ by means of equation 17 (section A.5.3.) Θ was found to vary with shear rate G , and this is shown for different samples in Fig.39.

The cross of isocline observed in the machine becomes less distinct as the concentration of particles and their degree of orientation with respect to the polarised light, are decreased. Owing to this effect, measurements on dilute solutions could be made only at the higher shear rates. The uncertainties involved in extrapolation of χ to zero concentration are clear from Fig.38.

C.11.4.4. Polarimetry

A brief polarimetric study was made on a solution of fraction B. The results are only approximate because the method of preparation of fraction B gave only a dilute solution (0.03 g./100 ml.). The mean measured specific rotation was -300° , and no change was observed on warming the solution (in phosphate buffer pH 7.4) to about 35°C .

C.11.5. Calculation of molecular parameters

Particle weight, hydration, axial ratio and length were calculated by the methods given in section A.5.

For these calculations there were assumed (1) a partial specific volume of 0.71, and (2) a specific refractive increment of 0.00184 refractive index units per g./100 ml. These values were assumed because no experimental method was available which would have yielded more reliable values. The determination of each requires a measurement of dry weight of neutral salt soluble collagen and this is hindered by (1) the low concentration of all reasonably purified solutions (2) the high ratio of weight of buffer salts to weight of protein in solution (3) the slimy precipitate formed on dialysis against water (4) the limited quantities of material available.

The partial specific volume assumed is that of gelatin¹⁴⁶, the specific refractive increment is the value usually taken to be representative of proteins¹⁴⁵. It is unlikely that any appreciable error is introduced by these assumptions, in view of the anomalous behaviour of the solutions, the approximate theoretical treatments available, and the probable heterogeneity of most of the solutions.

The derived parameters are given in Table 9.

The calculation of molecular parameters by the combined treatments of Sirha, Perrin & Scheraga (A.5.3.) was made for

axial ratios of 50 and 100. Agreement between observed and calculated values of Θ was poor and depended on shear rate. Qualitatively the values obtained by 'plug' theory (A.5.2.) were supported.

C.11.6. Discussion

A survey of Table 9 shows that the kind of model found applicable to neutral salt soluble collagen is a highly elongated particle of axial ratio of the order of 50, a few thousand Angstrom units long and 50-100A wide, with a particle weight of some 500,000 and a hydrated volume of the order of 20 ml./g.

To what extent is it likely that such a model gives a true representation of those unknown particles in solution, and how much importance may be attached to differences in the parameters of the model for different samples?

Evidence comes from two sources: (1) internal evidence from the methods used here and (2) information obtained by other methods, in particular by electron microscopy.

First, the solutions are not homogeneous, (with the possible exception of fraction A of P71) and the derived parameters are therefore some form of average of the values which would have been obtained if the constituents could have been measured separately. While the single schlieren peak of the sedimentation diagram (phot. 16) excludes gross

heterogeneity, it does not indicate homogeneity. The variation of Θ , the apparent rotary diffusion coefficient calculated from flow birefringence, with shear rate G , as shown in Fig.39, is a fair indication that the material is not homogeneous. The partial formation of gels on warming to 37°C and their stepped resolution is indicative of heterogeneity, as are the electron micrographs of P67, of P71B (Phot.21 and 22) and of P71C (Phot.23 and 24). Only fraction A of P71 appears fairly homogeneous, both by the constancy, within experimental error, of Θ (Fig.39) and of the uniform appearance of what must be artefacts in the electron-micrographs (Phot.23). Fraction B of P71 is contaminated with fibres from the heat gel (Phot. 21 and 22), in spite of centrifuging for 10 mins. at 35,000 r.p.m., and fraction C of P71 with clusters of only partly disintegrated gel ("cobwebs", Phot.23 and 24). The presence of such "cobwebs", in spite of centrifuging for 1 hr. at 39,000 r.p.m., is itself indicative of its sedimentation behaviour. If a chain is assembled by an end to end arrangement of long units, each with its own mass and frictional coefficient, then the sedimentation of the chain will be little different from that of the constituents, provided that their frictional interaction with solvent has not been affected by the polymerisation. Such may be the case for particles of neutral salt soluble collagen polymerised to "cobwebs"; in addition the relative movement of "cobwebs" and

solution is hindered by the high viscosity of solutions of neutral salt soluble collagen. Thick fibres formed by lateral association of finer units should sediment faster than their constituent parts, and the contamination of fraction B of P71 was probably due to insufficient centrifugation.

These sub-microscopic contaminants of the solutions will have influenced measurements made at very low shear rates, more so than other measurements. The measured intrinsic viscosities will therefore be slightly too high and the rotary diffusion coefficients too low. Estimating the influence of changes in these values on the parameters given by plug theory indicates that the given axial ratio, length and molecular weight may be slightly too high and the volume of hydration too low. Only rotary diffusion coefficients measured at high shear rates, where contaminants will have the smallest effect, were used for calculation of lengths of particle.

The effect on the derived parameters of a continuous distribution of particle sizes, which some of the electron micrographs may be taken to indicate, cannot be predicted.

Considerable particle-particle interaction is indicated by (1) the shape of the schlieren peak in sedimentation diagrams and (2) the variation of the angle of isocline at a given shear rate with concentration (Fig.38). The effects of this interaction on the derived parameters should have been made small by extrapolating all values to zero concentration,

though of course assumptions and errors are implicit in the process of extrapolation.

The approximations and assumptions made in the derivation of plug theory have been considered earlier (A.5.2. and B.7.4.)

The best values of length of particle are probably those calculated from measured rotary diffusion coefficients Θ , assuming an axial ratio as given by plug theory. Θ is a sensitive measure of the particle length, as it varies inversely as the cube of this, while it is only dependant upon a logarithmic function of the axial ratio.

The model of a greatly elongated particle in solution seems likely from the ease with which one-dimensional arrays, fibres, are formed from these solutions. In fibres of appreciable width there is, of course, also lateral aggregation, but splayed out or tapered fibres are often seen under the electron microscope, and in these fibrils the widths approach those of molecules. The high viscosities in dilute solution also support the concept of elongated particles, and the marked birefringence must mean that the particles are strongly asymmetric. Whether the elongated particles are more like stiff rods or like flexible random chains, is another matter. Given the lengths calculated, the hydrated volumes found are rather small for a random coil, and the axial ratios found, also indicate an elongated particle which stays elongated, that is, is not very flexible.

A length of the order of 2,000-4,000Å is reasonable for a particle which can give rise to structures which have regular repeat distances of that order of magnitude (Phot.13).

There is little external evidence for the order of particle weight. Two diffusion measurements were kindly made for me by Dr. J. R. Hall with the Oxford Gouy diffusimeter, for which the material is unsuitable because of its high viscosity, small diffusion coefficient and the low concentrations used. The translational diffusion coefficient of sample P19 at 0.08 g./100 ml. (the effective limit of concentration for the instrument) was $0.55 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$. If it is assumed that the same frictional coefficient holds at that concentration for sedimentation, a particle weight of 470,000 is calculated from the equivalent sedimentation coefficient and the Svedberg equation:

$$M = \frac{R.T.s}{D(1-\bar{v}\rho)}$$

where M = particle weight

S = corrected sedimentation coefficient

D = translational diffusion coefficient

$\bar{v}$ = partial specific volume of solute

ρ = density of solvent

T = absolute temperature

R = gas constant

This particle weight is in agreement with the general order of magnitude. Molecular weights of 300,000 have been found

by various methods for another soluble collagen, ichthyocol¹³.

Differences in the parameters calculated for the different samples, are on the whole not likely to be significant. The largest particle, of weight 9.8×10^5 , was found for a solution P19 which had not been clarified on the preparative ultracentrifuge and the next particle in size, of weight 6.2×10^5 was measured on a sample (P71), which in spite of prolonged preparative ultracentrifuging retained a little opacity. Due to this, it is also uncertain whether importance should be attached to the smaller particle (P87) found for the same material after heat gelling and solution in acetic acid. It is interesting that after this treatment the solution was quite clear, without centrifuging. Probably this treatment does have some effect as indicated by the changed intrinsic viscosity, and this conclusion is supported by electron microscopic evidence (C.8.2.1.) The somewhat smaller parameters calculated for fraction A of P71 may be due to the removal of larger material present in the other solutions. The high axial ratio and length of particle found for fraction B of P71 are no doubt too great, as even a few large fibres (as found by the electron microscope) would have an appreciable effect in the dilute solutions available (0.04-0.01 g./100 ml.).

The effect of sulphite on solutions of P19 was quite definite, halving the intrinsic viscosity, and will be discussed later.

C.12.

DISCUSSION AND CONCLUSIONS

Originally the investigation was undertaken to characterize by some standard procedures of physical chemistry a new material isolated from skin extracts. Within the limitations imposed by the nature of both substance and methods, this has been done. There are two other interesting aspects however: the place of this substance in the path of collagen formation and its relationship to the general problem of aggregation of proteins.

The overall properties of the whole material indicate it to exist in solution as elongated particles some thousands of Angstrom units long, of the order of 100A wide, and within that space entraining a fair, though not very large, volume of solvent; the material itself having a mass of some 5×10^5 molecular weight units. At the same time this material was shown to be heterogeneous.

An investigation of Jackson's method of isolation showed up several points of interest. First, that purine and pyrimidine containing material, absorbing light of wavelength 250 μ -270 m μ , which was also found by the American workers in their extracts, is an impurity which can nearly be eliminated by repeated precipitation of the collagenous material with NaCl. Secondly, that purified neutral salt soluble collagen has a definite absorption at 275 m μ , and this is most probably due to

tyrosine contained in the substance. Thirdly, that the material, as isolated by this procedure, contains a small, yet definite amount of phosphorus (0.01%). Although this may easily be due to an impurity: (1) this impurity passes step by step with neutral salt soluble collagen through the stages of isolation; (2) it remains in the same proportion to neutral salt soluble collagen when this is separated into a soluble and an insoluble part by warming to 37°C; (3) it is most unlikely to be adsorbed inorganic phosphate; (4) it is incorporated into "neutral salt soluble collagen" in the animal body at much the same rate as is C^{14} labelled glycine. Lastly, it became clear that ultra-centrifugal clarification of all solutions is necessary, and that even this is inadequate to remove some of the aggregated material. This is in agreement with the findings of Boedtker & Doty¹³ on the related substance ichthyocol, and casts a shadow of doubt on all analyses of material which was not treated in this way.

The heterogeneity of the material may be conceived of in three parts. First, material which is chemically quite unrelated to the collagens, such as purine and pyrimidine containing substances. Such material has probably been mostly removed during isolation, as the analyses of Eastoe would indicate. Jackson has at certain times found small amounts of hexoseamine. Secondly, there may be a heterogeneity solely of different states of aggregation, in solution, of particles having identical

chemical compositions. Thirdly there may be a heterogeneity due to particles which have slightly different chemical compositions and physical properties leading in particular to slightly different behaviour of aggregation.

What is the interpretation then, which is to be placed on the separation of neutral salt soluble collagen into the fractions A, B and C? The methods employed for separation (heating to 37°C and solution via 1% acetic acid) either do not affect the properties of the material, or do so only to a small extent. Thus specific optical rotation and 'gel ratio' remain constant, there appears to be a slight decrease in the calculated parameters of particle weight, axial ratio and particle length (P71 and P87 in Table 9) the intrinsic viscosity is changed, and the appearance in the electron microscope is changed (even though this may only indicate a change in some properties responsible for the formation of a given artefact, as for fraction A).

Whether the process of separation affects the material or not, provided the separation is real, it must indicate some differences of the material in solution; (unless the first process of warming to 37°C has caused some splitting of a homogeneous material, but as it is known that the material is physically heterogeneous, it is more simple to regard the process as separating that which was originally heterogeneous; both processes may also occur). I believe that the process of separation is real both as judged from the constancy of gel

ratio (Fig.18) and of the various curves of re-solution of gel on standing in the cold for various lengths of time (Fig.24). Thus closely similar gel ratios were obtained on different samples and under such different conditions of centrifuging as: Spinco rotor 40, 35°C, 35,000 r.p.m. for 20 mins. and a bench small angle centrifuge at 17°C, 8,000 r.p.m. for 10 mins. Although the electron microscope showed fraction B to be contaminated with fibres from fraction A (Phot.21), yet there was a considerable background of different, small material. Again there was a striking difference between the ease of solution of precipitated fraction A in 3% acetic acid and the difficulty experienced with fraction C.

Chemical measurements were not made in this investigation, but the measurements of specific ultraviolet absorption made on the different fractions do indicate a difference. The specific ultraviolet absorption is arbitrarily defined as logarithmic absorption per unit concentration of 0.1 g./100 ml. At 275 mμ the values found for the various fractions were:

Fraction B	0.395
Fraction C	0.224
Fraction A	0.123

Combining the values of fraction C and A in the proportions in which these fractions are found in the total gellable material:

$$\frac{40}{100} \times 0.224 + \frac{60}{100} \times 0.123 = 0.164$$

This value is similar to that found for total gellable rat ^{neutral} ~~gelable~~ salt soluble collagen (Fig. 29) as is the value for fraction B, and hence also is their ratio: $B/(A + C)$, 2.4 as compared with 2.3. At present I am not able to interpret these values in any way other than that they simply indicate differences between the fractions.

One might suggest various hypotheses which would explain the fractionation purely in physical terms of gel formation and re-resolution. Thus it might be postulated that the fibril formation during gellation proceeds from various nuclei scattered through the solution and that when polymerisation has effectively stopped monomers or oligomers are left trapped in the interstices, and that these particles form the supernatant fraction B on centrifuging. Similarly that fraction C is composed of residual cores of such centres of polymerisation. Such hypotheses would not explain the fact that on repeated heat gelling of fraction A the proportion of fraction B is not increased, and that similarly a sample of isolated fraction A had a gel ratio of 95%; also it would fail to explain the complete and rapid resolution of fraction A in the cold, after repeated heat gelling.

I conclude therefore, that the three fractions have at least to a small degree different chemical compositions. Within each fraction there may be different states of aggregation, and different ranges of aggregation may perhaps also be characteristic of each fraction. Whether the initial warming to 37°C produces

some chemical change or not, I do not know. It does not cause a degradation to gelatin. That fraction B is not a gelatin is shown by: (1) optical rotation does not vary with temperature in buffer at pH 7.4; and (2) prolonged heating to 37°C does not produce more B from A and C. I regard fraction B as also being a soluble collagen, because of its closely similar behaviour to fractions A and C in all respects, including a high specific optical rotation, except the formation of a heat gel.

What is the place of these neutral salt soluble collagens in relation to the other collagens? First it is clear that they are not identical with the acid soluble collagens, such as ichthyocol, although they are no doubt closely related. Repeated attempts to dissolve neutral salt soluble collagen in M/15 citrate buffer pH 3.5-3.8 failed, and this is the very buffer which is regularly used by investigators studying acid soluble collagen. Conversely acid soluble collagen obtain from rabbit skin is, without addition of other substances, not soluble in M/15 phosphate buffer pH 7.4. There are also the investigations of Harkness et al. showing different rates of incorporation of ^{14}C glycine into the two substances.

The similarities are striking: near-identical specific optical rotation and conversion into similar gelatins under closely similar conditions; molecular parameters which are rather close considering the errors involved e.g. particle weights of 3 and 5×10^5 ; and common ability to be precipitated as typical

collagen fibres, and closely similar amino acid compositions, though these may differ in tyrosine content.

Probably Orekhovitch's view is applicable: that there is a series of closely related soluble collagens, all on the pathway of fibrogenesis. Thus one may arrange the substances in a series of decreasing solubility: fraction B, fraction A, fraction C, acid soluble collagen. There is an alternate view, recently put forward by Jackson, that some of the materials studied are artefacts, in that they have been changed during isolation. While Jackson, tentatively, thought of acid soluble collagen in this way, one might apply the same reasoning to neutral salt soluble collagen. The mammalian body is at near 37°C, a temperature at which fractions A and C of neutral salt soluble collagen are insoluble. What is their state in the living skin then? I suspect that they are fibres, but unstable ones, so that they are soluble at cold room temperatures. This brings us to the problem of the function of other substances in relation to neutral salt soluble collagen and to the more general problem of protein aggregation as instanced in this specific example.

Different reversible states of aggregation are now known for a number of proteins, such as insulin, and in the cases of lactoglobulin, casein, and a fragment of elastin, the state depends on the temperature, with the higher aggregation occurring at the higher temperature.

With collagen the property of alignment of particles is striking and a regular cross banding can be maintained over considerable widths of fibre. Under the particular conditions this state must have a lower free energy than that of the corresponding isolated particles. What have the present investigations shown? First, that there is an appreciable energy barrier to the change, a high energy of activation characteristic of protein denaturation. Secondly that the electrical charges present on the molecules and in their environment have a considerable effect on the change. Thus below a pH of about 5.5 no macroscopic aggregates are formed, and this value of pH coincides with the pH value at which the gelatin made from neutral salt soluble collagen alters its direction of migration in an electric field. At higher pH values it was shown that the rate of change is directly proportional to the hydrogen ion concentration. Negatively charged ions such as sulphite, versene, cyanide and chondroitin sulphate, greatly decrease the speed of aggregation under given conditions, while positively charged ions such as "TRIS", α -picoline and collidene accelerate the process. Here should also be mentioned the effect of A.T.P. which carries a negative charge and enables acid soluble collagen to dissolve at neutral pH in the cold, and at this pH acid soluble collagen then similarly forms fibres on warming to 37°C. (other strongly negatively charged ions may have the same property). It would appear that some negatively

charged particles favour a separation of the particles and some positively charged ones an aggregation. It is in this connection that the experiments with sulphite are of interest (see Table 9) Sample P819 was calculated to have a lower axial ratio (half of P19) and to be shorter. The measured quantity which was more than halved was the intrinsic viscosity $[\eta]_0$. In view of the heterogeneity of these solutions, comparisons of particle parameters is of doubtful value, but a halving of $[\eta]_0$ does indicate that a significant change has occurred, that some interaction between particles has been altered, reduced.

Repeated attempts to find a substance which might be keeping neutral salt soluble collagen in solution in the animal body, have failed. There may be conditions under which chondroitin sulphate and substances related to it, not only retard the formation of heat precipitate, but completely prevent it. There is also the problem of what the effective, local pH value might be at microscopic sites within connective tissue. The concept of pH may have little meaning left when applied to such a system, and it seems unlikely that the acid groups of various polysaccharides could reduce such a pH value to less than 5.5, the limit of "heat" aggregation. Certainly bulk connective tissue showed physiological pH values when tested with indicators. At least it is reasonable that acidic mucopolysaccharides could interfere with fibrogenesis, and there are pathological conditions when good collagen fibres are not

formed and when such mucopolysaccharide is present. One cannot help wondering whether these matters are not connected with the occurrence of such very fine collagen fibres in umbilical cord and in the vitreous humor ("vitreosin").

A possible view in these forms of protein aggregation is that the changes of free energy are due to changes of energy of interaction of water molecules with each other and with protein. Thus it could be that at 37°C the total free energy is less when water molecules are linked to each other by hydrogen bonds than when they are linked to protein, and that water molecules are set free when the protein particles aggregate to form fibres. Whatever the forces involved, one does at least obtain the impression that quite good and complicated collagen fibres can be formed without the intervention of a "mucopoly-saccharide cement", which is so frequently invoked. This does not mean that other substances may not be necessary to insure the stability of the macroscopic, physiological fibre unit.

Lastly, aggregation may be thought of in two forms: end to end aggregation of units and lateral aggregation. From the various electron micrographs it would appear that there can be quite stable end-to-end aggregates of the width of single molecules or little more. The lateral aggregation appears to be disrupted more easily. Heterogeneity of some of the samples may be due to differences in this end-to-end aggregation. What might happen to the peptide chains during aggregation is quite

unknown. Striations characteristic of some ordering continue in fibres down to very fine widths (Phot. 21).

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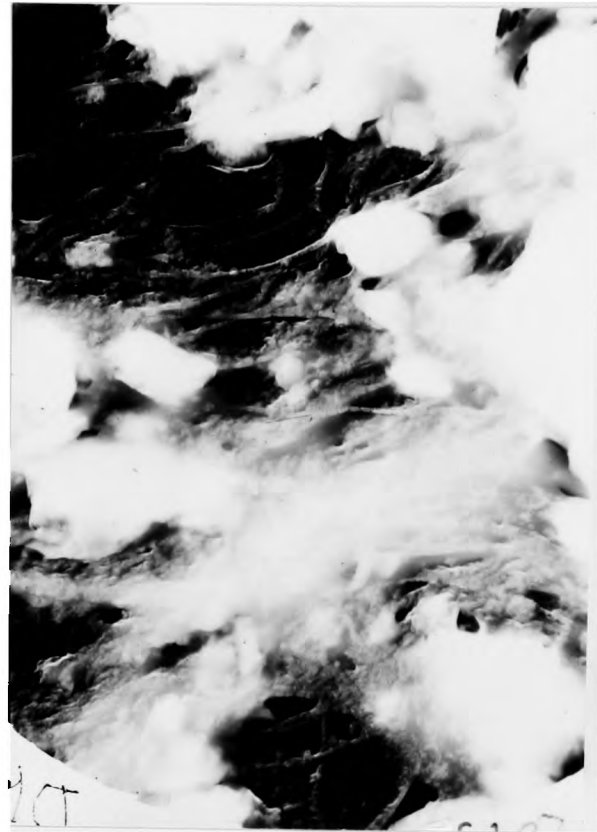
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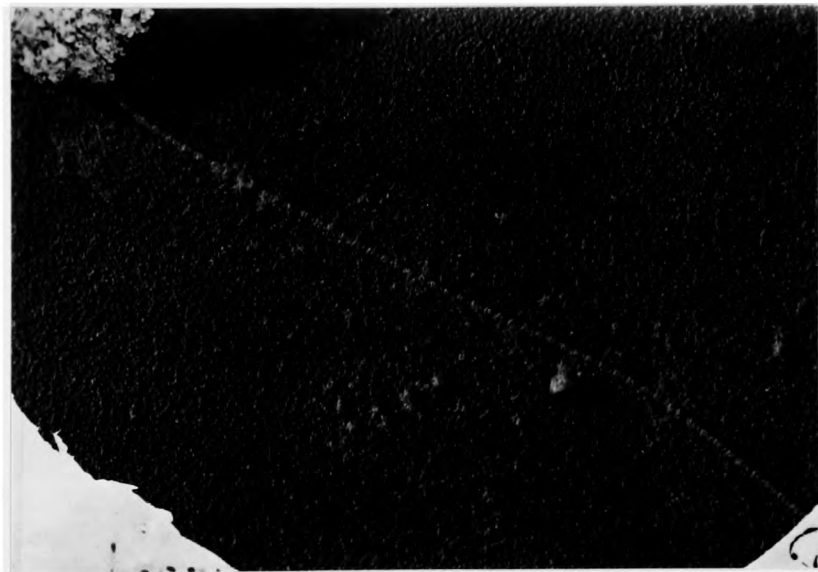
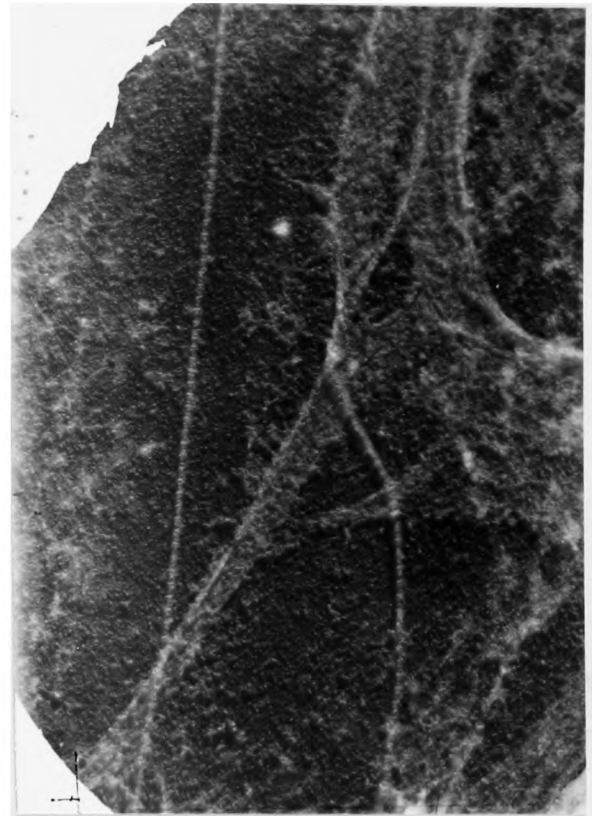
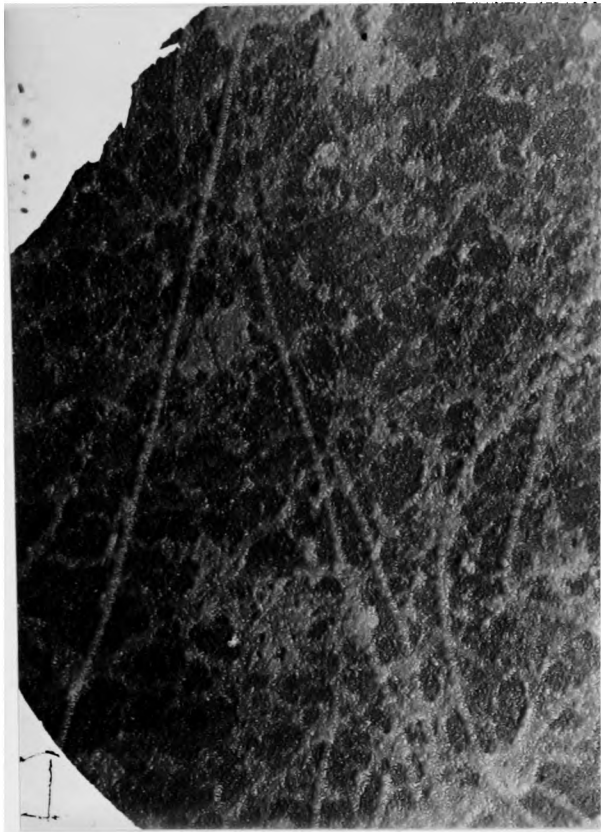
Phot. 1.



Wharton's jelly, homogenised, formalin fixed
and phosphotungstate stained; shadowed.

Magnification: $\times 22,000$ (approx.)

Phot. 2.



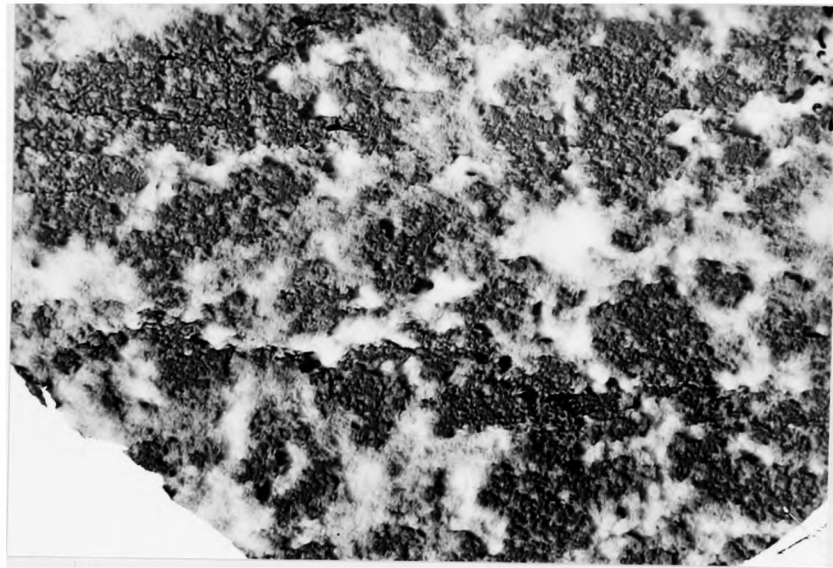
Typical fibres from Wharton's jelly of 24 week old foetus. Grid touched against side of an incision in the cord; phosphotungstate and shadowed. Magnification: x 16,000 (approx.)

Phot. 3.



Extract of Wharton's jelly, after ultra-
centrifugation (Spinco rotor 40, 30,000 r.p.m.
for 25 minutes), phosphotungstate and shadowed.
Magnification x 10,000 (approx.)

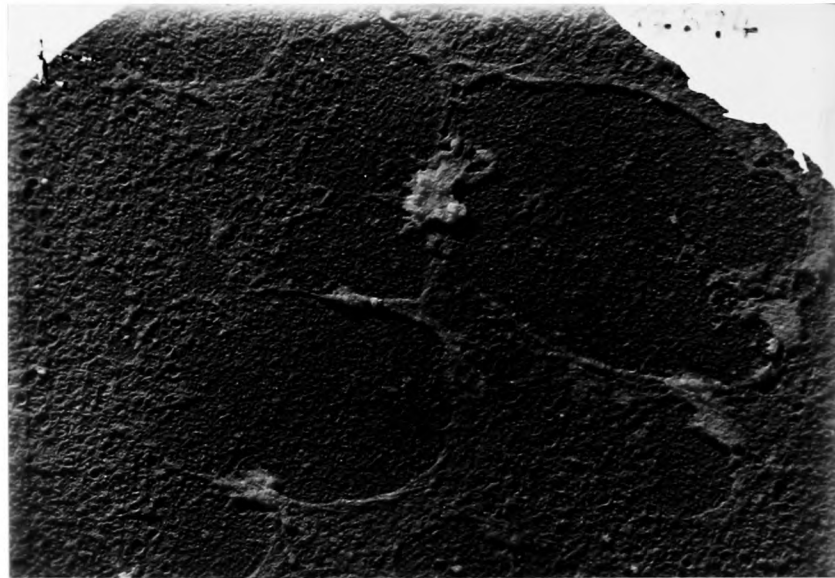
Phot. 4.



Bovine synovial fluid, centrifuged,
phosphotungstate and shadowed.

magnification: x 15,000 (approx.)

Phot. 5.



Precipitate obtained from a solution of
bovine U.F.E. by ultracentrifugation,
(Spinco rotor 40, 39,000 r.p.m. for 1 hr.);
phosphotungstate and shadowed
Magnification: x 16,000 (approx.)

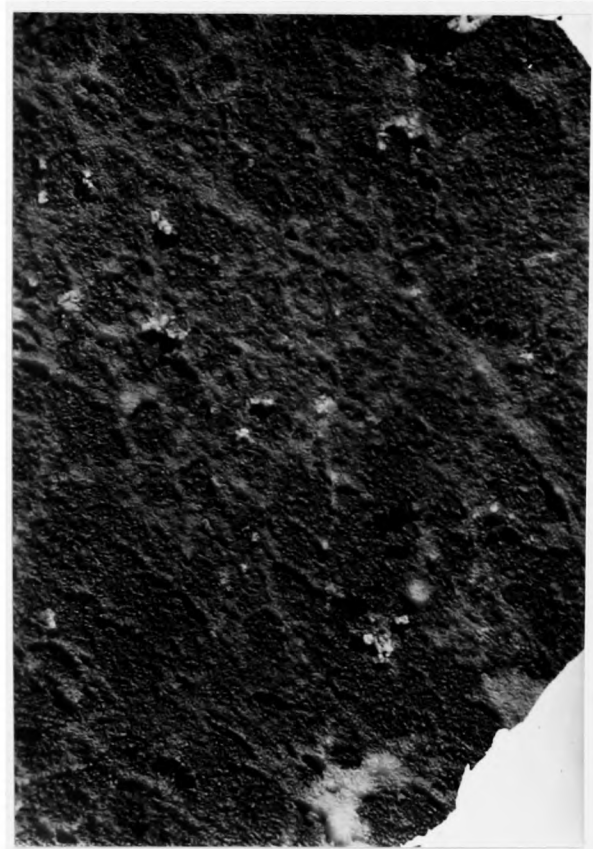
Phot. 6 and 7.

6.



$\frac{1}{2}$ hr.

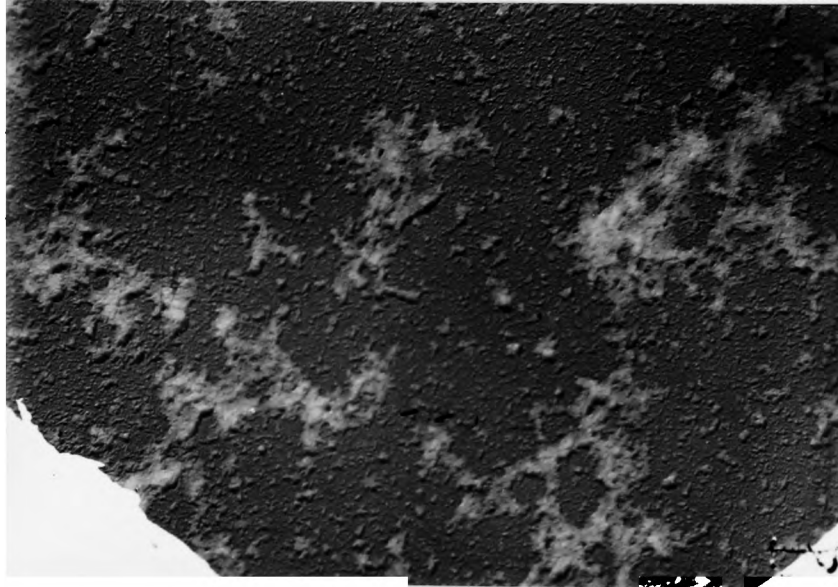
7.



$3\frac{1}{2}$ hr.

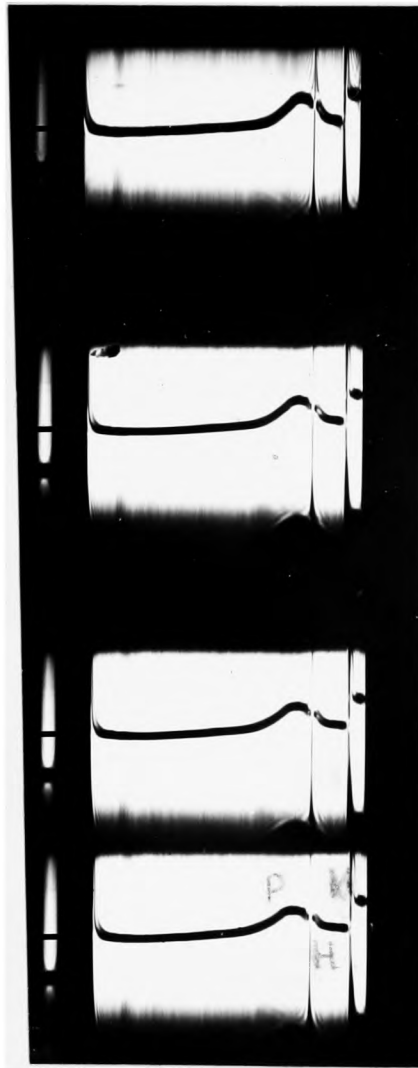
Bovine U.F.R. after $\frac{1}{2}$ hr. and after $3\frac{1}{2}$ hr.
incubation with hyaluronidase (37°C. , 100 Bengel
units of hyalase per ml.); phosphotungstate and
shadowed. Magnification: $\times 16,000$ (approx.)

Phot. 8.



Bovine synovial fluid after treatment with
hyaluronidase. Typical picture obtained when
the material is uniformly distributed and
not too dense; phosphotungstate and shadowed.
Magnification x 16,000 (approx.)

Phot. 9.



Ultracentrifuge schlieren record of umbilical
cord extract. 920 r.p.s., 5 min. interval between
photographs, bar angle 20° ; M - meniscus,
H - hyaluronic acid boundary, P - protein boundary.
Direction of sedimentation from M to P.

Phot. 10.



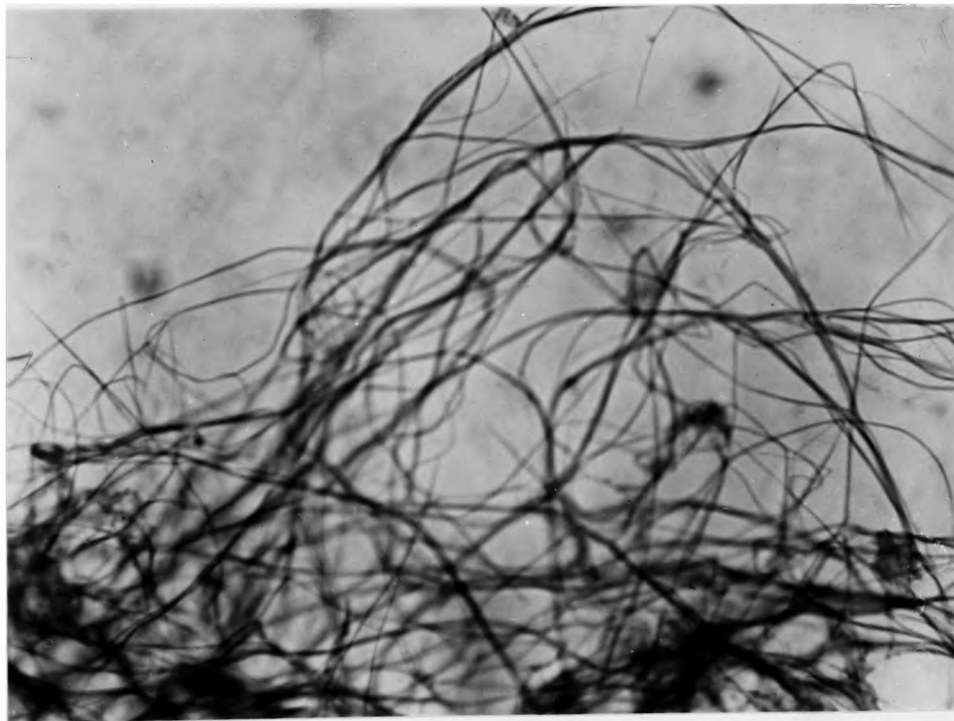
Ultracentrifuge schlieren record of human
synovial fluid. 910 r.p.s: 5 min. interval
between photographs: M - meniscus, H - hyaluronic
acid boundary, P - protein boundary; Direction
of sedimentation from M to P.

Phot. 11.



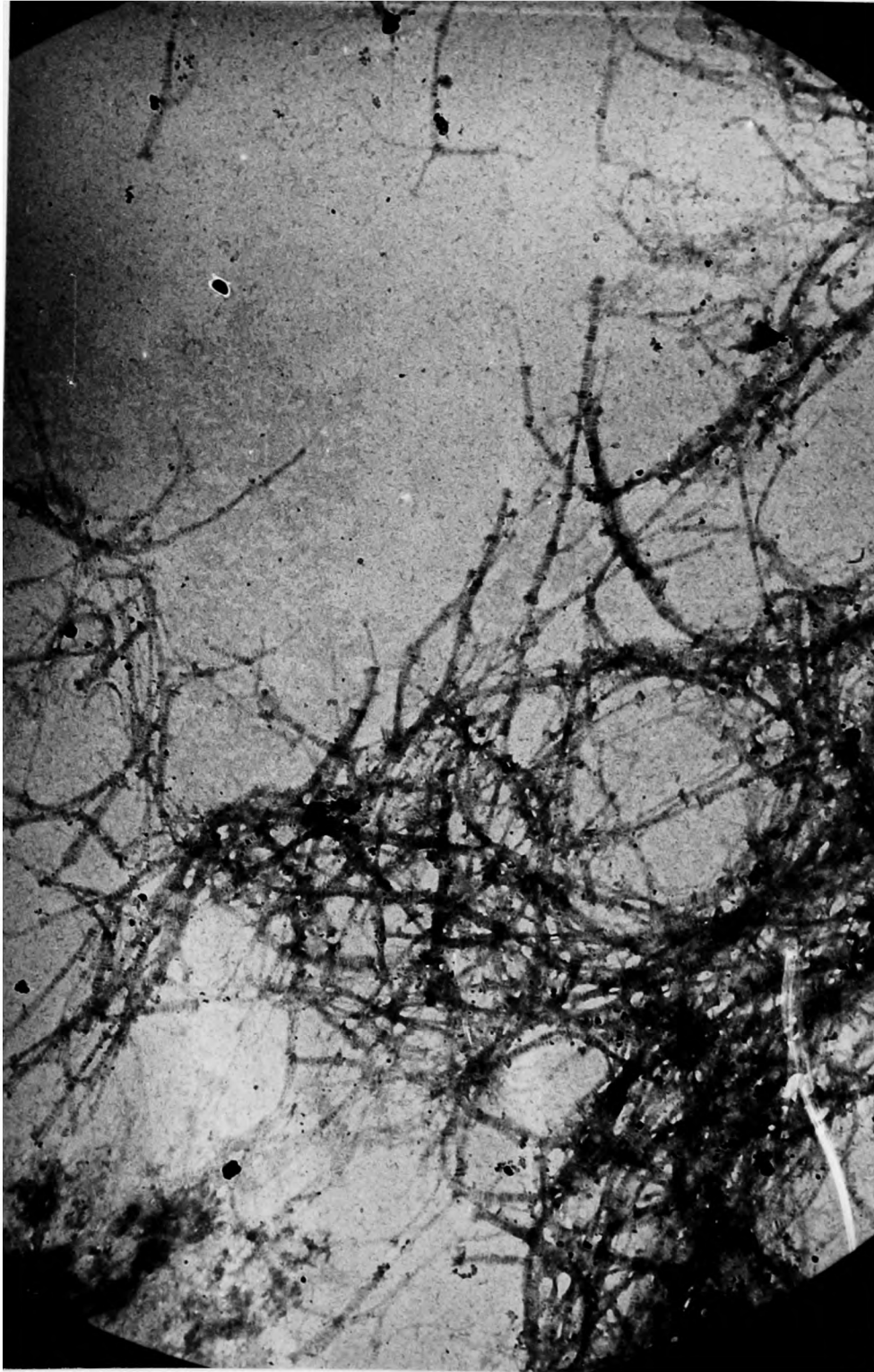
Ultracentrifuge schlieren record of hyaluronidase
degraded U.F.R. 920 r.p.s: 5 min. interval
between photographs; M - meniscus, H - hyaluronic
acid boundary, F - fast component.

Phot. 12.



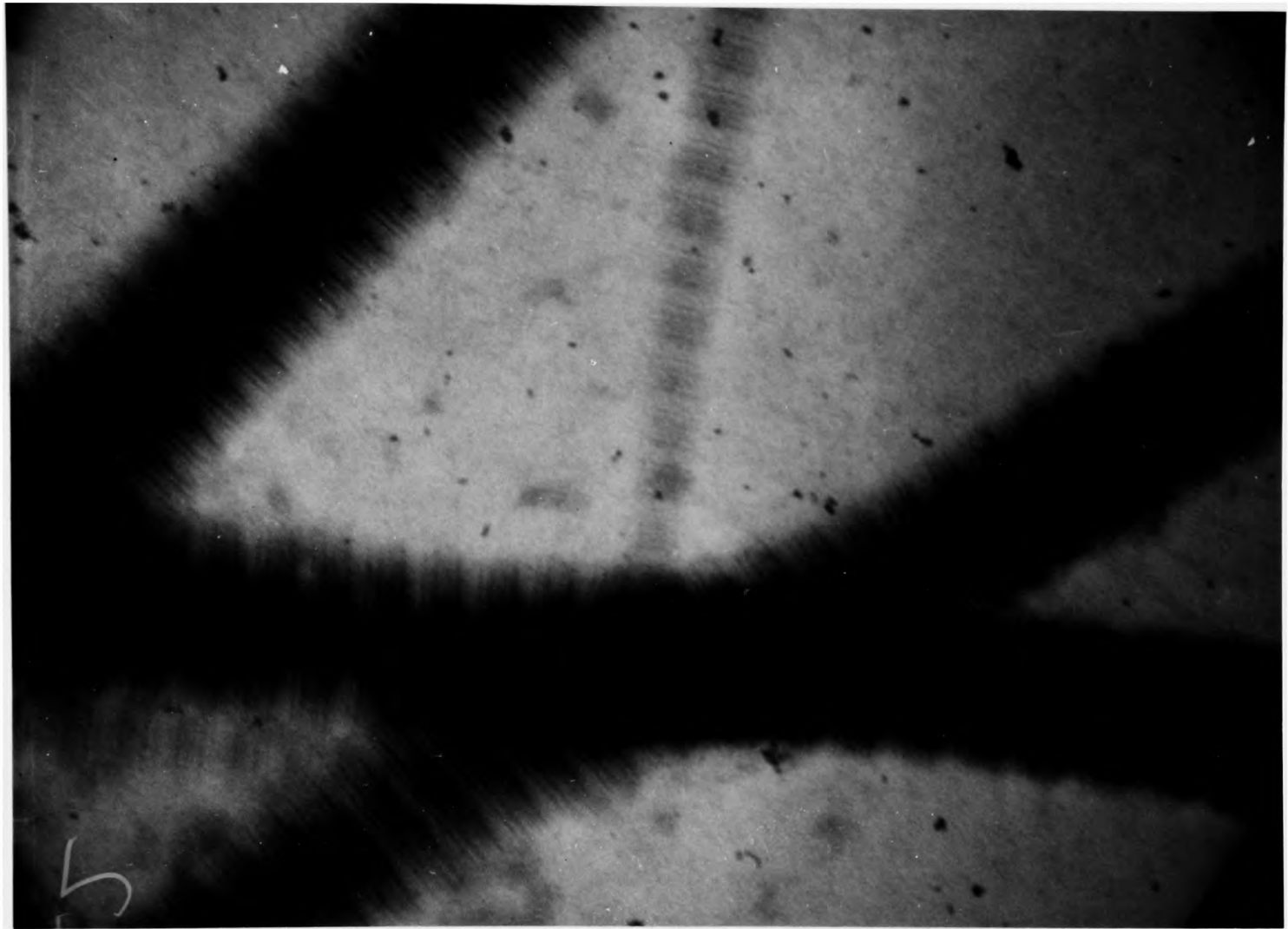
Heat precipitate of neutral salt soluble
collagen; fixed in formalin and stained
with van Gieson. Optical micrograph:
Magnification x 900 (approx.)

Phot. 13.



Neutral salt soluble collagen; fixed in formalin,
disintegrated by ultra-sound, stained with
phosphotungstate. Magnification x 26,000

Phot. 15.



"Heat precipitate" from neutral salt soluble
collagen; phosphotungstate.

Magnification: x 165,000.

Phot. 16.



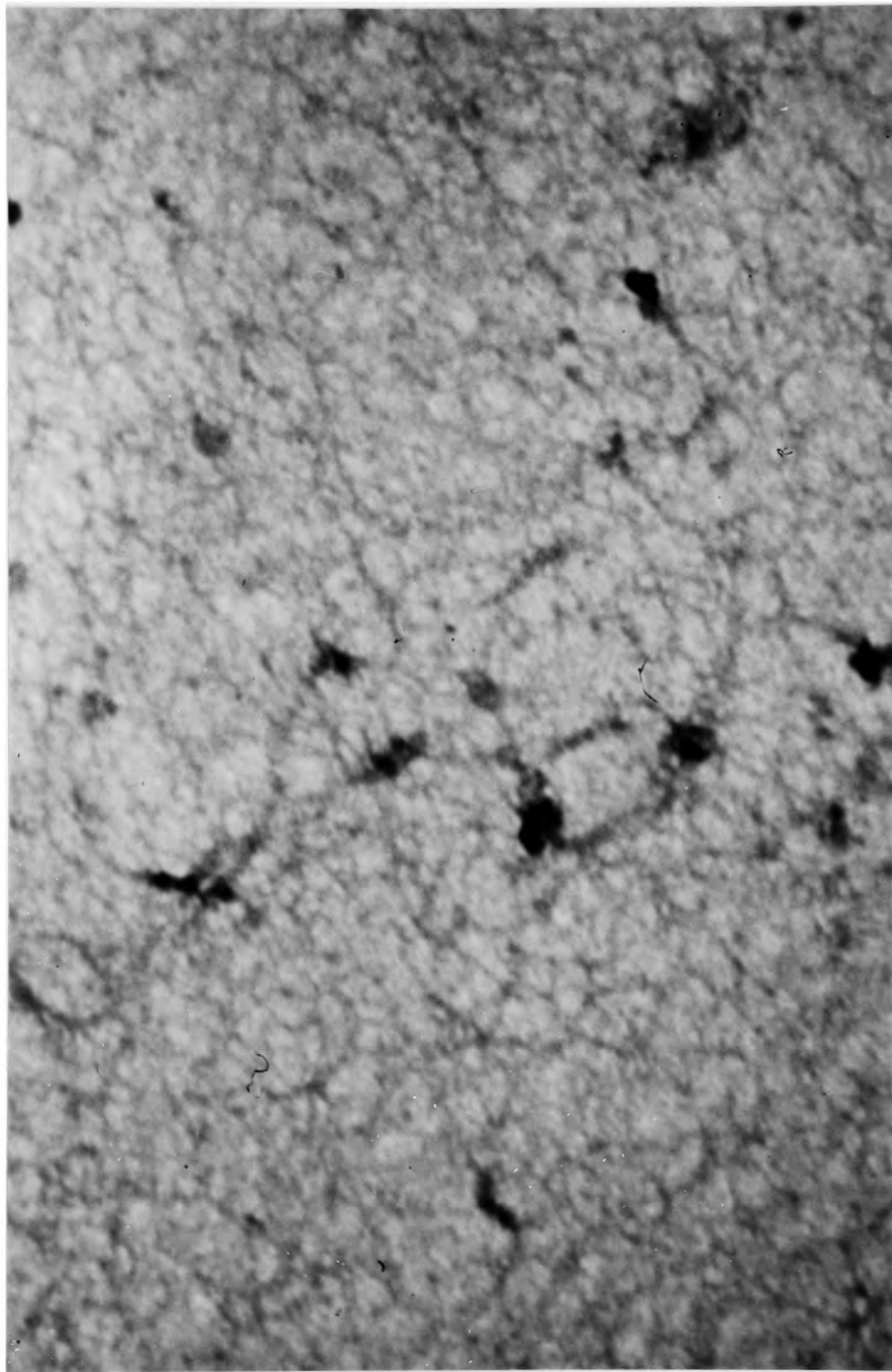
Ultracentrifuge schlieren record of neutral salt soluble collagen. 900 r.p.s; 5 min. interval between photographs. M - meniscus, C - boundary of neutral salt soluble collagen. Direction of sedimentation from M to C.

Phot. 11.



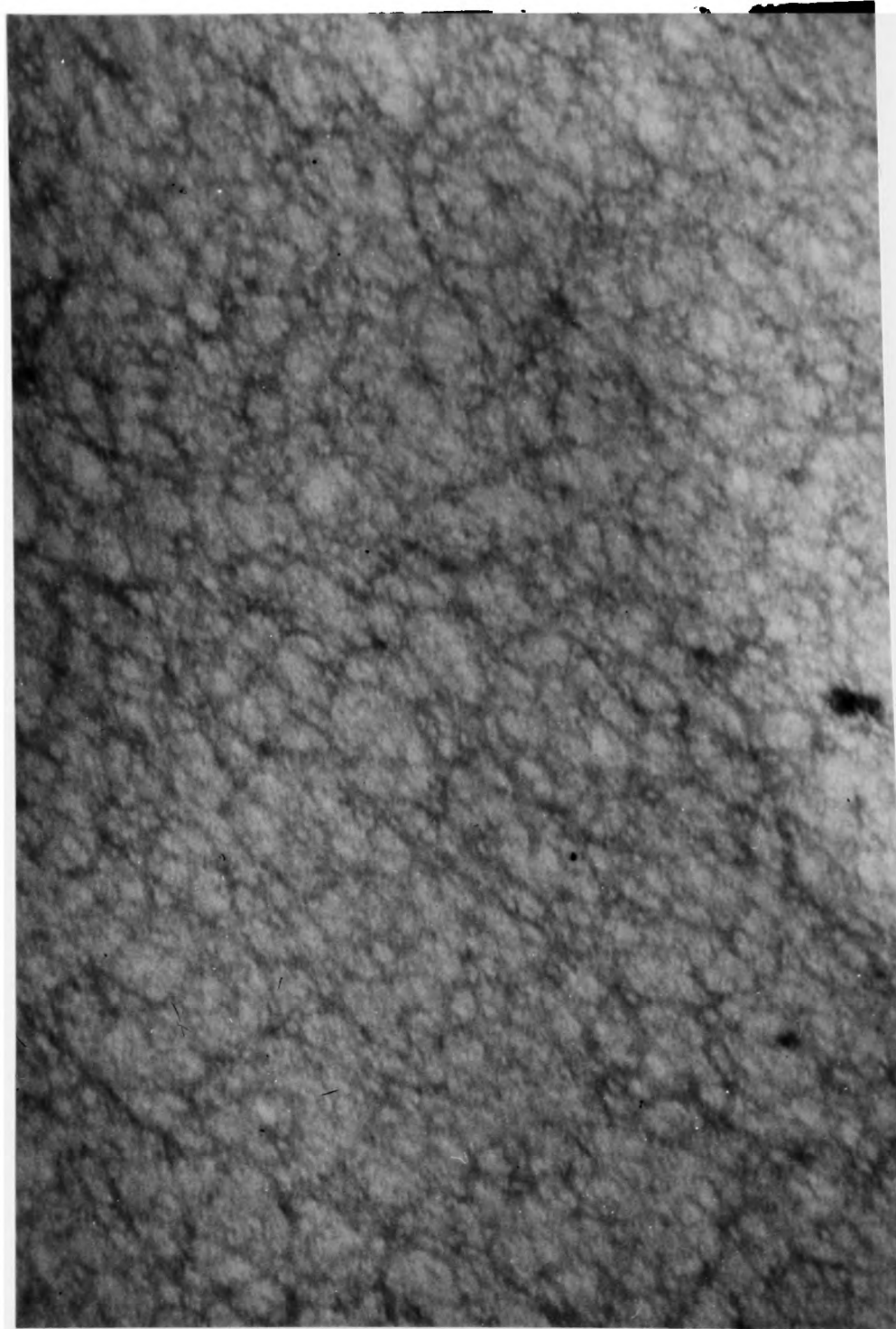
Ultracentrifuge schlieren record of U.F.R. after exhaustive digestion with hyaluronidase, showing fast components. 920 r.p.s.; 5 min. interval between photographs; M - meniscus. H - hyaluronic acid boundaries; F1, F2.- boundaries of fast components. Direction of sedimentation from M to F.

Phot. 18.

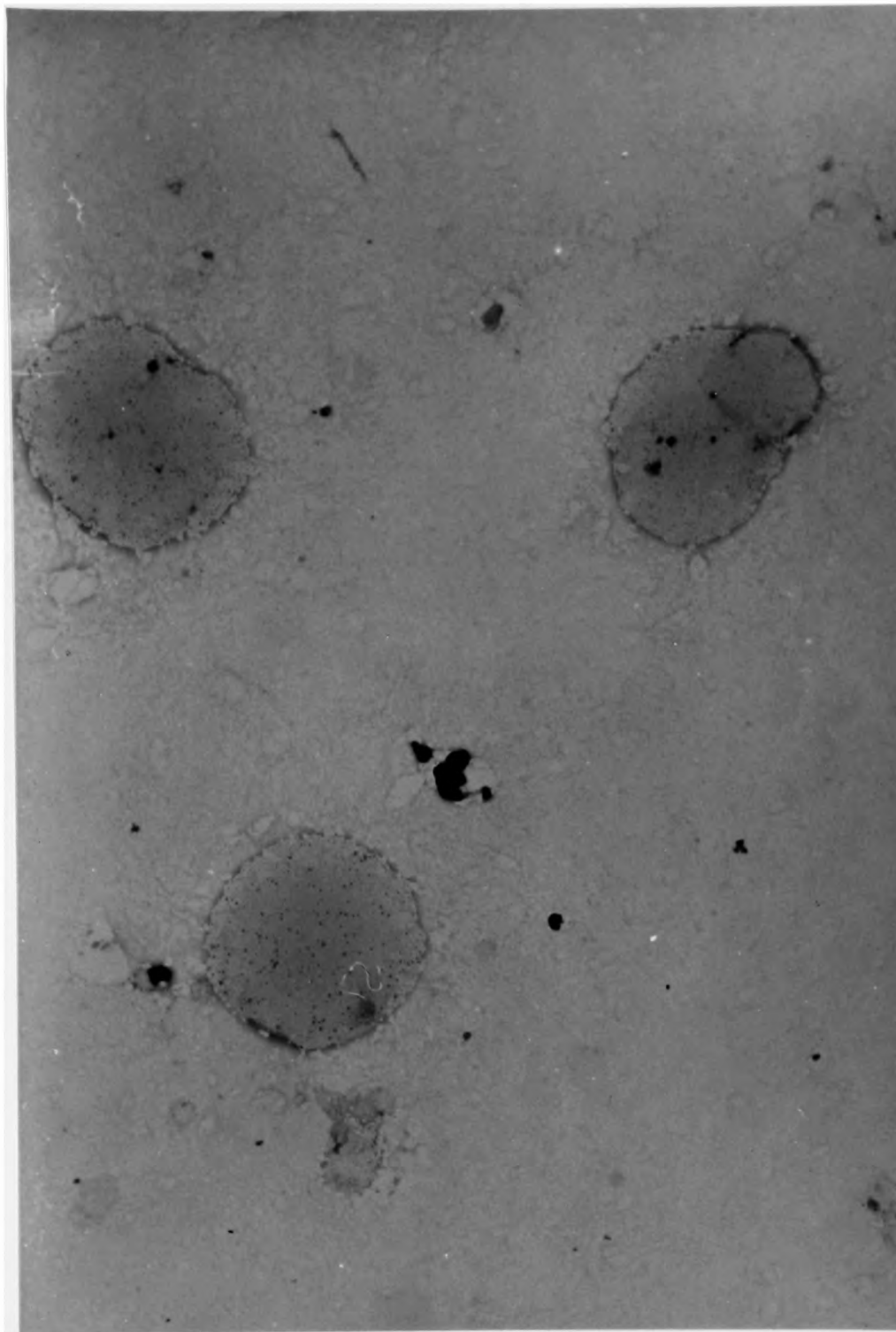


Neutral salt soluble collagen, sample P 71;
osmium fixed, phosphotungstate
Magnification: x 130,000.

Phot. 12.



Neutral salt soluble collagen, sample P 71;
osmium fixed, phosphotungstate.
Magnification: x 130,000.



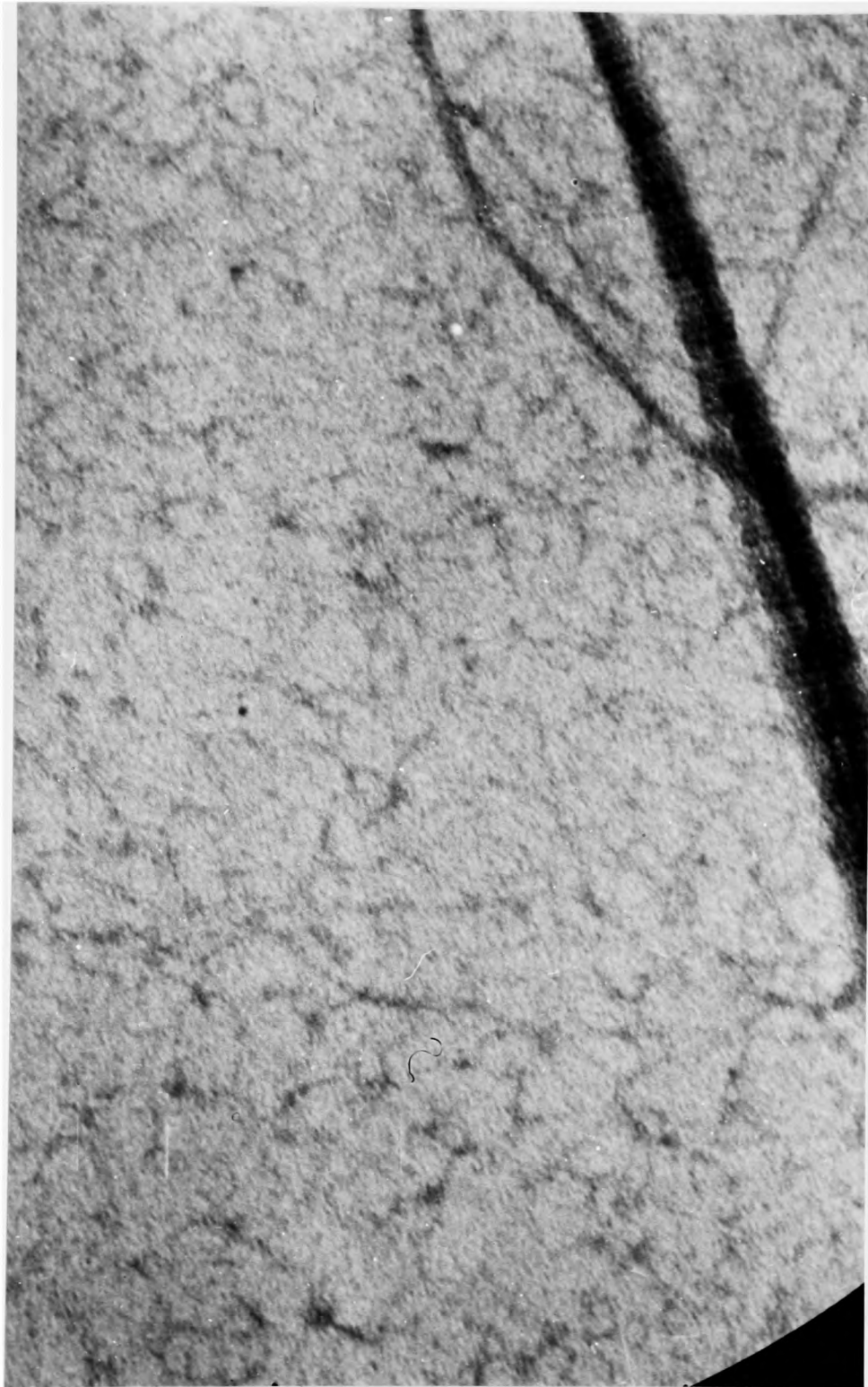
Fraction A of neutral salt soluble collagen.
sample P 71 A; osmium fixed, phosphotungstate.
Magnification: x 34,000.

Phot. 21.



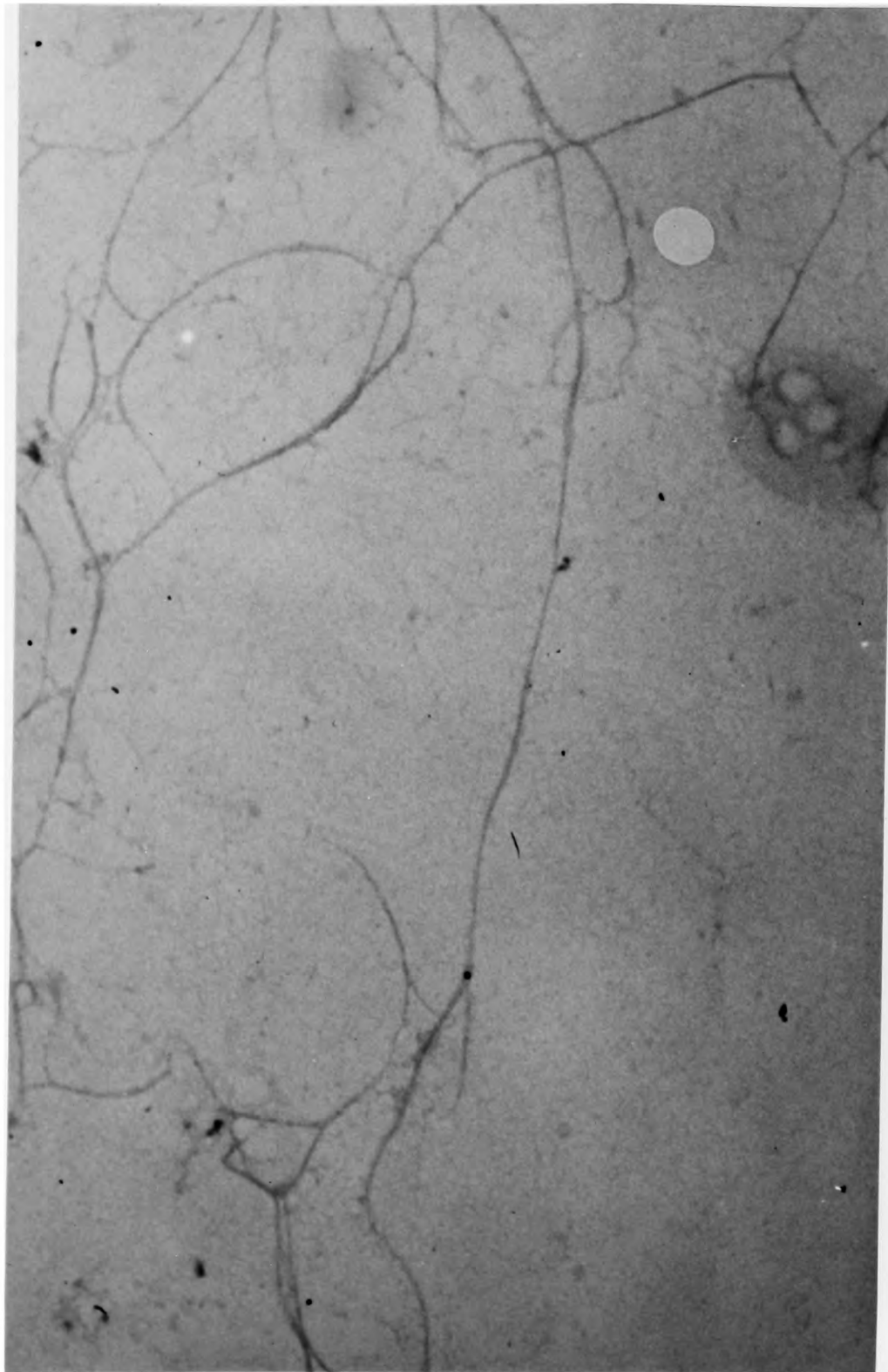
Fraction B of neutral salt soluble collagen,
sample P 71 B: osmium fixed, phosphotungstate.
Magnification: x 33,000.

Phot. 22.



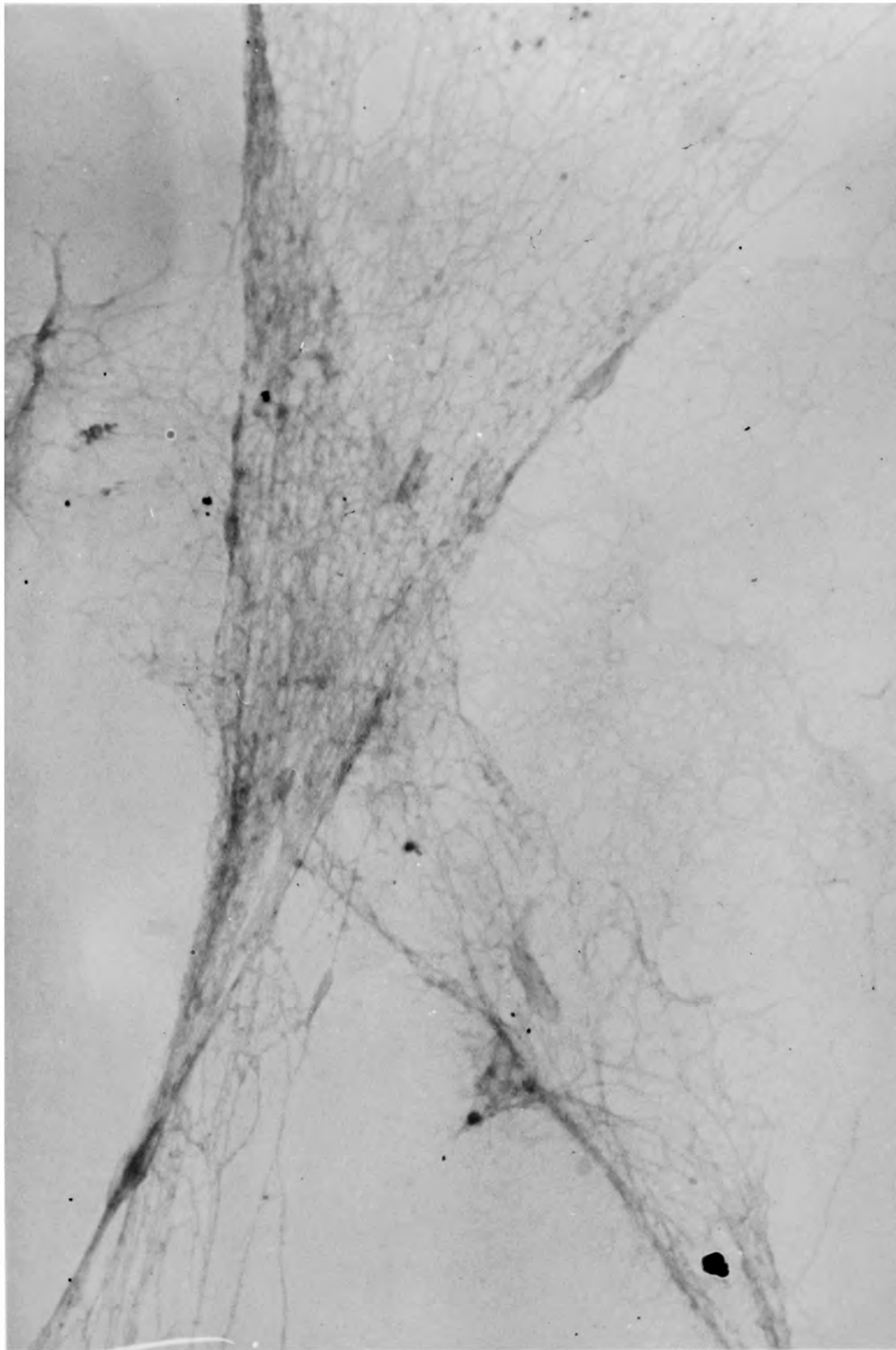
Fraction B of neutral salt soluble collagen,
sample P 71 B; osmium fixed, phosphotungstate.
Magnification: x 135,000.

Phot. 23.



Fraction C of neutral salt soluble collagen,
sample P 71 C; osmium fixed, phosphotungstate.
Magnification: x 44,000.

Phot. 24.



Fraction C of neutral salt soluble collagen,
sample P 71 C; osmium fixed, phosphotungstate.
Magnification: x 33,000.