SWELLING AND TRANSPARENCY OF CORNEA AND SCLERA AS COMPARED WITH A MODEL SYSTEM COMPOSED OF PIGSKIN GELATIN AND MUCOITIN SULPHATE*

by

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INTRODUCTION

From previous investigations[1] it was concluded that collagen and mucopolysaccharide are important for the transparency of the cornea. It was surmised that these are not physico-chemically independent but are reciprocally related, possibly in a complex system as described by BUNGENBERG DE JONG[22]. In continuation of these investigations, the transparency of the bovine cornea and the relation of this to its water content have been further studied under various physico-chemical conditions. Comparative investigations on cornea and sclera have also been carried out, using cornea and sclera of albino rats in order to avoid difficulties due to pigment on the sclera. Finally, "model" experiments were carried out with a complex of pigskin gelatin and potassium mucoitinsulphate.

EXPERIMENTAL PART

Methods

The technique** is essentially the same as that used by VAN WALBEEK AND NEUMANN[1]. In addition to pieces of bovine cornea (diameter 13 mm) cut out with a hand trephine, we also used round pieces (dry weight 1–3 mg) cut out with scissors from cornea and sclera of albino rats. All the fragments were dried and kept in a vacuum desiccator over conc. sulphuric acid. The determinations of hydratation and transparency were carried out as described in (1), *i.e.* the transparency again by means of a photocell connected to a galvanometer. The pH of the buffers was checked by measurements with a Beckman pH meter. Acetic acid/sodium acetate and McIlvaine (Na_2HPO_4/citric acid) buffers were used. Since the concentration of salts has a marked influence on the swelling behaviour of connective tissue, this was kept constant in the buffer, *i.e.* 2 meq./l for Na acetate and 10 meq./l for Na_2HPO_4. The pH of the buffers was thus regulated by varying the concentration of acid. The swelling of the tissue fragments took place in approx. 50 ml of buffer.

INFLUENCE OF pH AND SALTS ON SWELLING AND TRANSPARENCY OF CORNEA AND SCLERA

Influence of pH on swelling and transparency of bovine cornea

A series of bovine corneae were placed some in acetic acid/Na acetate and some in McIlvaine buffer. As in previous investigations[1], we again found a minimum of swelling at pH 3.9–4.1. With prolongation of the experiment the pH for minimum

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References p. 481.

swelling shifted somewhat (Figs. 1A and 2A). This shift of the point of minimum swelling in the pH/swelling curve is a result of the washing out of salts present in the tissue[2]. The transparency of the tissue fragments was least at pH below 3.9, increased with rising pH and showed above pH 6 a tendency to decrease when the swelling was pro-

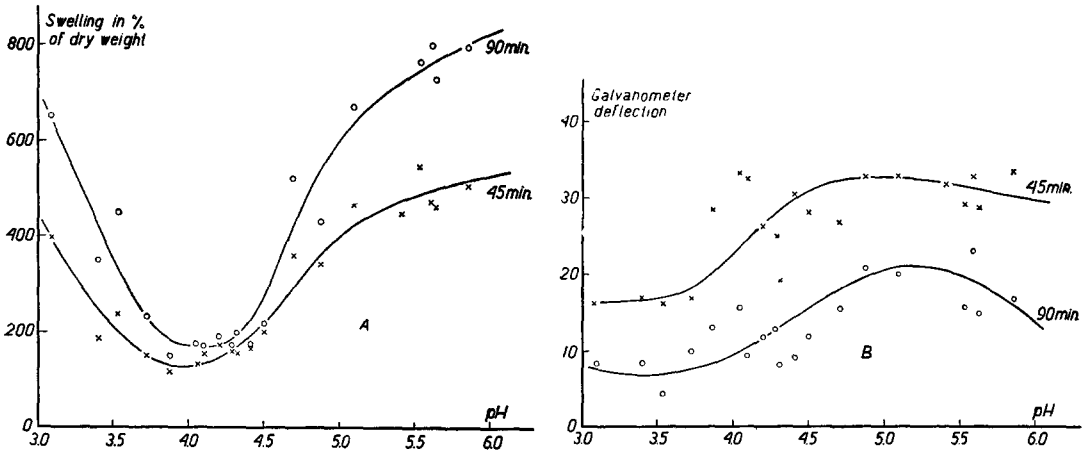

Fig. 1. Influence of pH on swelling (A) and transparency (B) of bovine cornea; in acetic acid/acetate buffer.

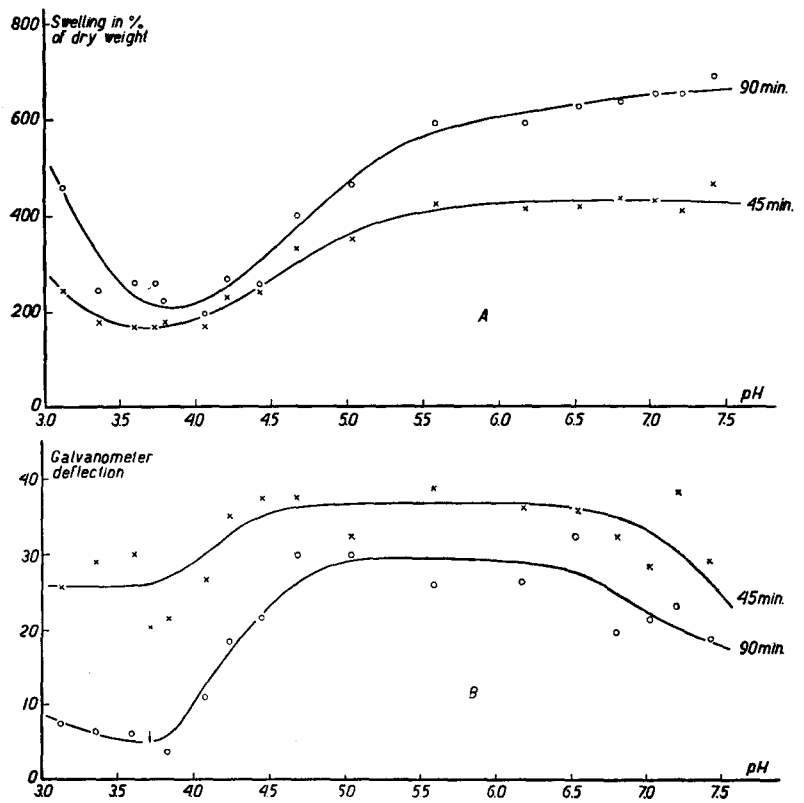

Fig. 2. Influence of pH on swelling (A) and transparency (B) of bovine cornea; in McIlvaine buffer.

References p. 481.

longed, probably in consequence of the considerable increase in volume (Figs. 1B and 2B). Attempts to study the transparency at the physiological water content of 350% of the dry weight, however, met with a difficulty in that in the pH range 3.5–4.65 this physiological water content was not reached in the time covered by our experiments. The transparency values over this range can, however, be extrapolated with a high degree of probability from Figs. 1 and 2 and thus are very low (dotted line on the graphs of Figs. 3 and 4). Still more striking than in Figs. 1 and 2 is here the extreme decrease of transparency at the pH of minimum swelling. For higher values the transparency remains more or less constant at the physiological water content.

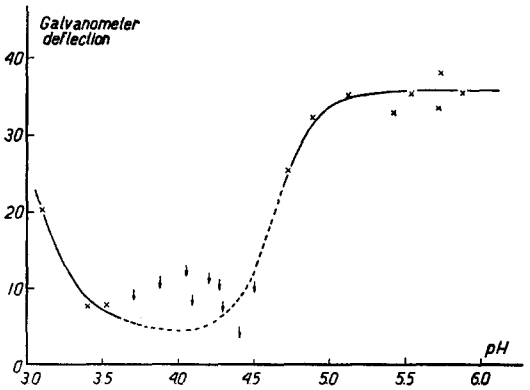

Fig. 3.

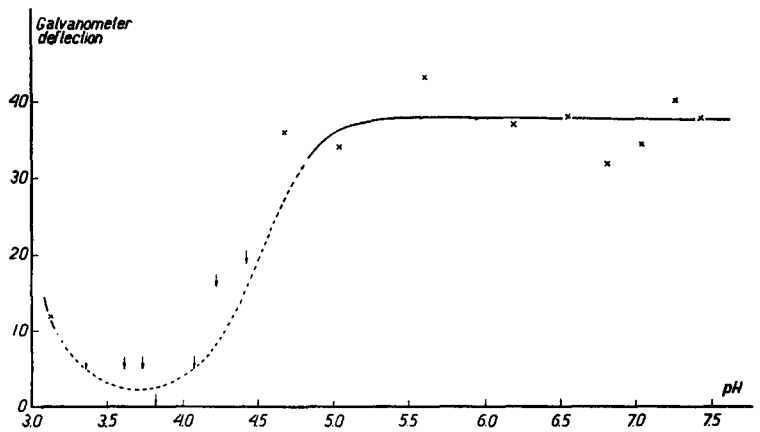

Fig. 4.

Influence of pH on transparency of bovine cornea at the physiological water content; in acetic acid/acetate buffer (Fig. 3) and in McIlvaine buffer (Fig. 4).

Influence of pH on swelling and transparency of rat cornea

The pH/swelling curves (Fig. 5A) are essentially similar to those for bovine cornea, except that the shift of the point of minimum swelling in prolonged swelling is more pronounced. The pH/transparency curve after 45 and 90 min does not give a clear picture, as the degree of swelling here has a much greater influence than is the case with bovine cornea (Fig. 5B). The pH/transparency curve at the physiological water content of 285% of the dry weight (Fig. 7) shows an unmistakable minimum of transparency in the pH range of minimum swelling, as was the case with the bovine cornea, but here the pH range in question is higher because the washing out of salts occurs much faster than with the bovine cornea.

Influence of pH on swelling and transparency of rat sclera

In agreement with LEYNS AND GAULHOFER[3] and GRAUBERT[4], we found the swelling

References p. 481.

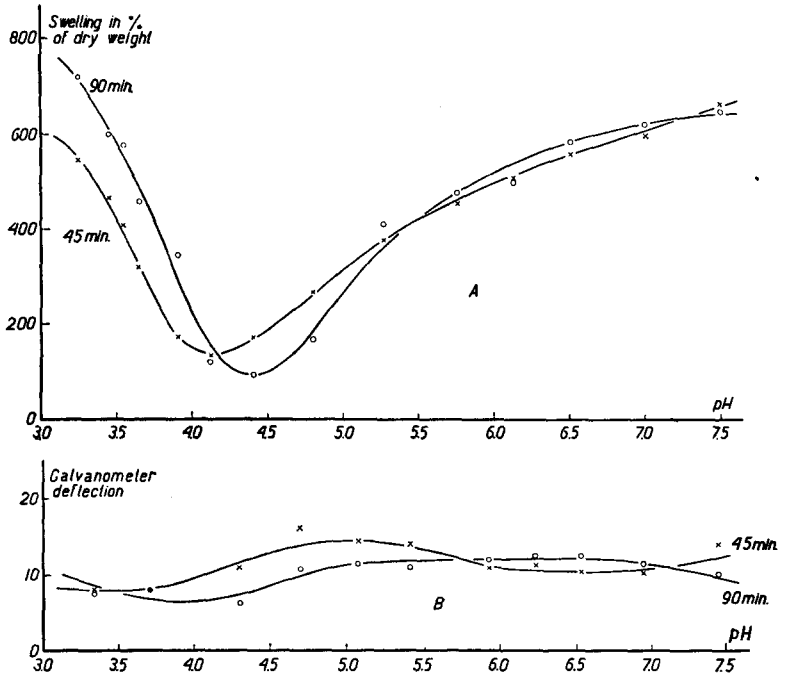

Fig. 5. Influence of pH on swelling (A) and transparency (B) of rat cornea; in McIlvaine buffer.

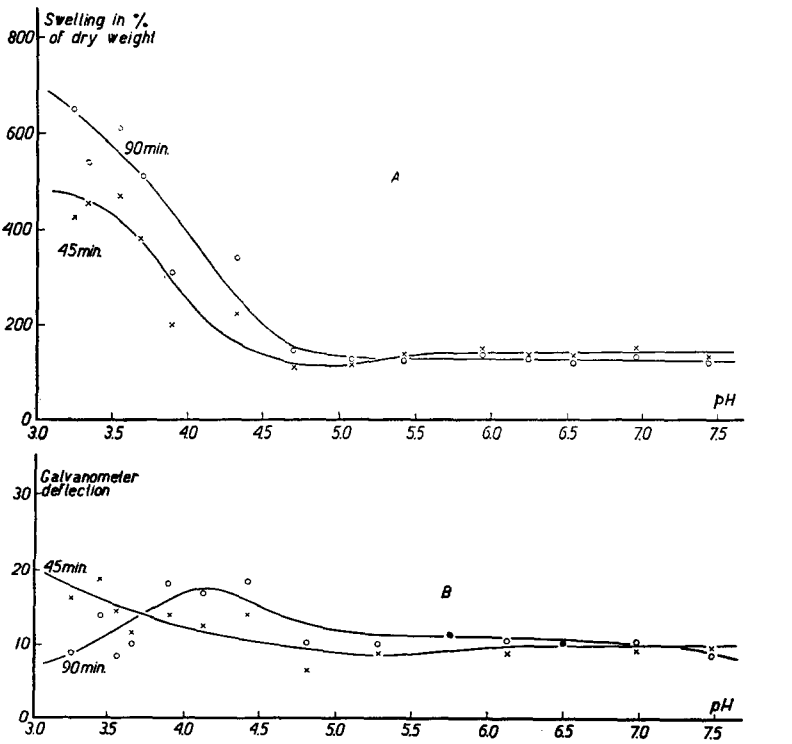

Fig. 6. Influence of pH on swelling (A) and transparency (B) of rat sclera; in McIlvaine buffer.

References p. 481.

at pH below 4 to be the same as with the cornea (Fig. 6A). Above pH 4 we noted a marked difference between cornea and sclera; the increase of swelling over this range observed with cornea was absent with sclera. The pH/transparency curve after 45 and 90 min also failed to show any definite features (Fig. 6B). The pH/transparency curve at the physiological water content of 178% of the dry weight (Fig. 8) was quite different from that for the cornea, in that no definite minimum of transparency was seen. There was a suggestion of a maximum at pH approx. 4.

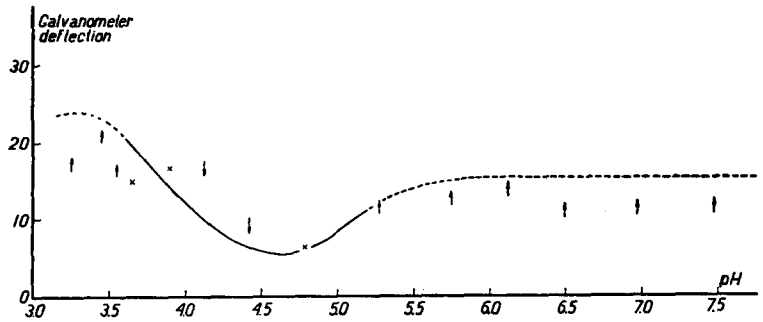

Fig. 7.

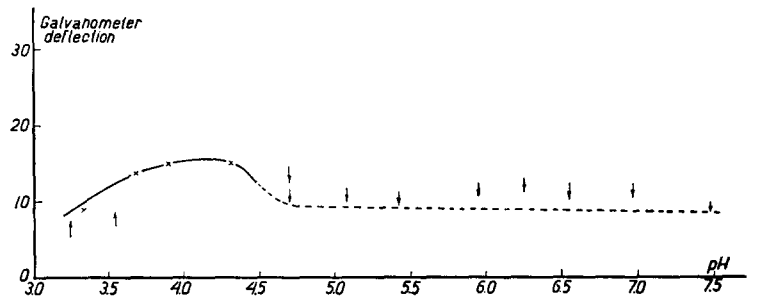

Fig. 8.

Influence of pH on transparency of rat cornea (Fig. 7) and sclera (Fig. 8) at the physiological water content; in McIlvaine buffer.

Effects of salts on swelling and transparency of bovine cornea in McIlvaine buffer at pH approx. 7.0

A preliminary investigation of the effects of salts on the swelling and transparency of bovine cornea was carried out in McIlvaine buffer at pH approx. 7.0. The anions Cl^-, SO_4^{-2}, $Fe(CN)_6^{-3}$ and $Fe(CN)_6^{-4}$ were found to be identical in their effects on swelling (Fig. 9A). The scatter of the curves after 90 min was within the experimental error of approx. 5%. The effect of salts on the transparency at the physiological water content was also the same for all the anions (Fig. 11). All curves showed a maximum transparency at log conc. approx. 0.75. Only the $K_3Fe(CN)_6$ curve showed a much higher transparency than those for the other salts. We are unable to account for this.

In contrast to the uniform effect of the uni- and polyvalent anions, the cations behaved very differently (Fig. 10). All salts caused a considerable decrease of swelling after 90 min. This decrease was proportional to the valency of the cations (Fig. 10A). The discrepancy in the position of the $CaCl_2$ transparency curve (Fig. 10B) is probably due to precipitation of Ca phosphate in the cornea. The effect of these cations on the transparency at the physiological water content (Fig. 12) also increased with the valency

References p. 481.

of the ion. Whereas the maximum transparency with KCl was found at log conc. 0.7, for $CaCl_2$ it was at log conc. about 0.0 and for $Co(NH_3)_6Cl_3$ at a log conc. of less than —1.0.

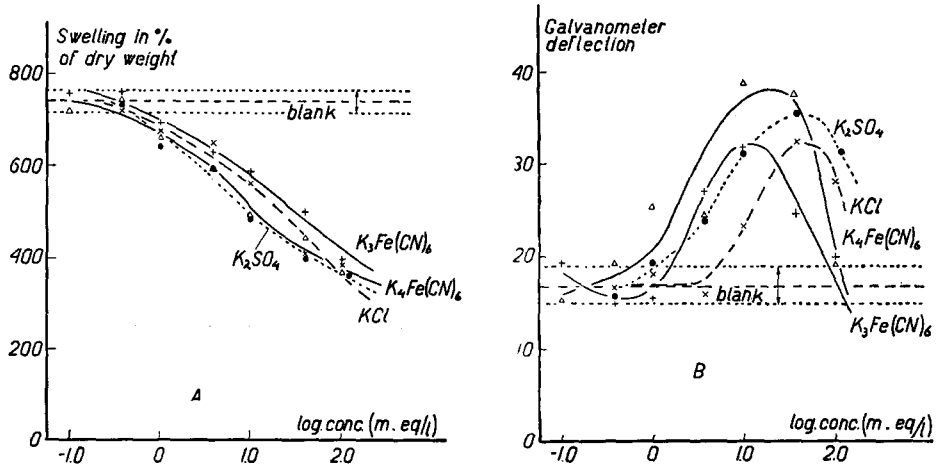

Fig. 9. Effects of anions on swelling (A) and transparency (B) of bovine cornea; in McIlvaine buffer at pH approx. 7.0.

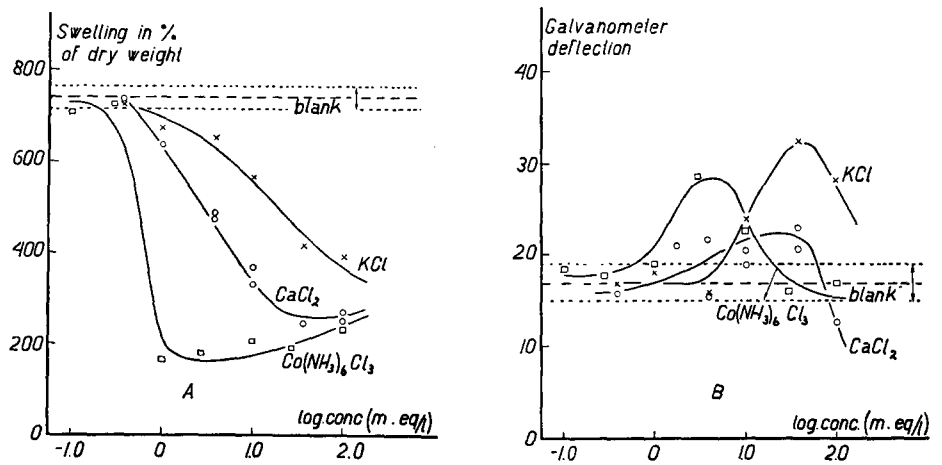

Fig. 10. Effects of cations on swelling (A) and transparency (B) of bovine cornea; in McIlvaine buffer at pH approx. 7.0.

INFLUENCE OF pH ON SWELLING AND CLOUDING OF GELS OF PIGSKIN GELATIN WITH x % OF POTASSIUM MUCOITINSULPHATE

Since collagen itself is unusable for model experiments on account of its insolubility, we sought a soluble protein with properties as similar as possible to those of collagen. The so-called pigskin gelatin, which is prepared from collagen by acid treatment, was found the most suitable. This substance can be obtained simply by heating collagen in water to above 70° C; it owes its name to the fact that it was originally obtained from the skins of pigs. As soon as collagen is treated with alkali—as in the preparation

References p. 481.

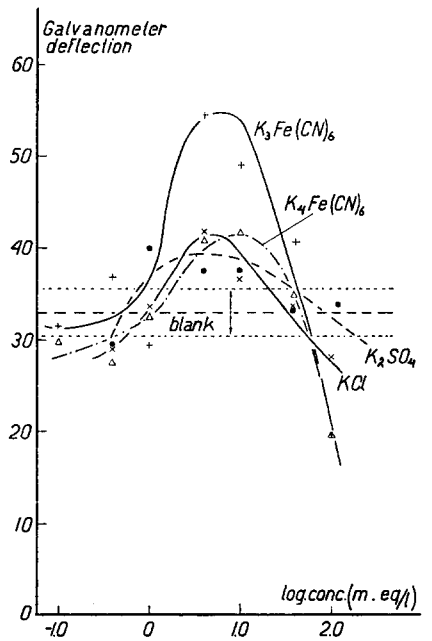

Fig. 11. Effects of anions on transparency of bovine cornea at the physiological water content; in McIlvaine buffer at pH approx. 7.0.

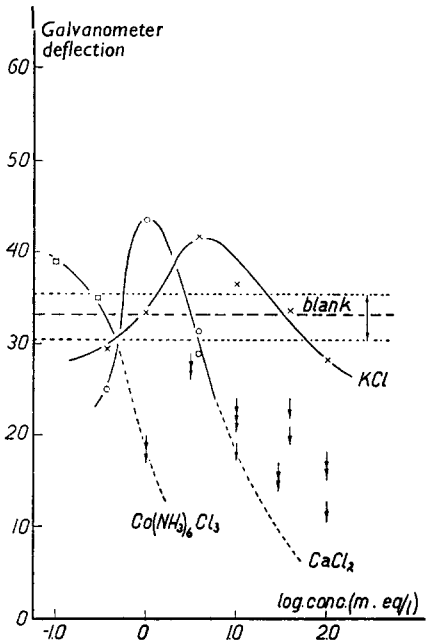

Fig. 12. Effects of cations on transparency of bovine cornea at the physiological water content; in McIlvaine buffer at pH approx. 7.0.

of ordinary commercial gelatin—products are obtained with iso-electric points lower than that of collagen itself, owing to the splitting-off of amide NH_2 groups from the partially amidated glutamic and aspartic acids. This is the reason why the literature gives so many different isoelectric points of collagen preparations. These values range from that of native collagen (*ca.* 9) to that of completely limed gelatin (*ca.* 5; AMES[5]). The titration curve of the pigskin gelatin used by us was found to agree very well with that of skin collagen (BOWES AND KENTEN[6]; LOEVEN[2,24]).

In our model experiments, 10% gels were prepared from the pigskin gelatin by dissolving it in bidistilled water at 60° C, pouring into Petri dishes and allowing to set in a refrigerator at 0° C.

Cubes with a side of 0.5 cm were then cut from the gels, weighed and placed in the buffer solutions. When gels containing a certain proportion of mucopolysaccharide were prepared, the weighed ingredients were dissolved together as described above.

Mucoitinsulphuric acid was obtained as described by PIRIE[7] by extraction of bovine corneae with 10% NaCl. The product was purified by the method of EINBINDER AND SCHUBERT[8] (treatment with kaolin and precipitation with glacial acetic acid in presence of potassium acetate).

Table I shows the composition of the potassium mucoitinsulphate obtained, with the amounts of the components theoretically to be expected if the mucopolysaccharide has the elementary composition $(C_{14}H_{19}NSO_{14}K_2 \cdot 4H_2O)_n$ and a weight per unit of 607.7[9,10].

Glucosamine was determined chiefly by the method of GUNNAR BLIX[11], a modification of the colorimetric method of ELSON AND MORGAN[12]*. Sulphur was determined by the titrimetric method

* For the modifications in the method of BLIX[11] introduced in this laboratory, see LOEVEN[2,24].

References p. 481.

of ALICINO[13] as adapted by MEYER, ODIER AND SIEGRIST[14] for determinations in mucopolysaccharides. Nitrogen was determined by the micro-Kjehldahl method and water by heating to constant weight above 100° C. It is evident that this preparation is not absolutely pure. It appears from the literature, however, that the absolute values for nearly all the constituents are always found to be 10–20% lower than the calculated values (EINBINDER AND SCHUBERT[15]).

TABLE I

COMPOSITION OF THE POTASSIUM MUCOITINSULPHATE PREPARATION

Compound or element	*Theoretical values in % of total unit weight*	*Our preparation*	
		% found	*% of theoretical values*
glucosamine	29.5	21.1	72
glucuronic acid*	31.9	27.4	86
sulphur	5.27	4.04	77
nitrogen	2.31	2.42	105
water	11.9	12.0	101

* Our sincere thanks are due to Mr. G. J. M. HOOGHWINKEL who carried out the determination of glucuronic acid in our preparation.

These experiments were carried out in the McIlvaine buffer. The swollen gel cubes were weighed after 3 days and the amount of water taken up was expressed as a percentage of the dry weight. The dry weight was determined by drying a piece of the gel to constant weight at 110° C. In these experiments the error introduced by removal of adherent water was very small. Triplicate determinations showed this to be not more than 2.5–3%. Fig. 13 shows the influence of pH on the swelling of 10% gels of pigskin gelatin in which a certain proportion of the gelatin has been replaced by K-mucoitinsulphate. Table II shows the pH values at which marked complex relations occur in the gel—as shown by its becoming turbid. The degree of turbidity is arbitrarily denoted in the table by the number of crosses. The swelling behaviour of pigskin gelatin greatly resembles the curve of swelling against pH for collagen, as given in the literature and extensively studied by BOWES AND KENTEN[16]. In the acid pH range a marked swelling occurs, this falls off rapidly up to pH 5.3–5.4 and then decreases slowly in the higher pH range investigated. Partial replacement of the gelatin by potassium mucoitinsulphate up to about 6% does not appreciably alter this swelling behaviour, except that the swelling is considerably reduced, especially in the acid range. Above 6% of mucopolysaccharide an inflection in the curve begins to appear in the pH range examined (at pH 5.1–5.2 with 10% of potassium mucoitinsulphate). Increase of the proportion of potassium mucoitinsulphate in the gel leads to (a) an increasingly marked

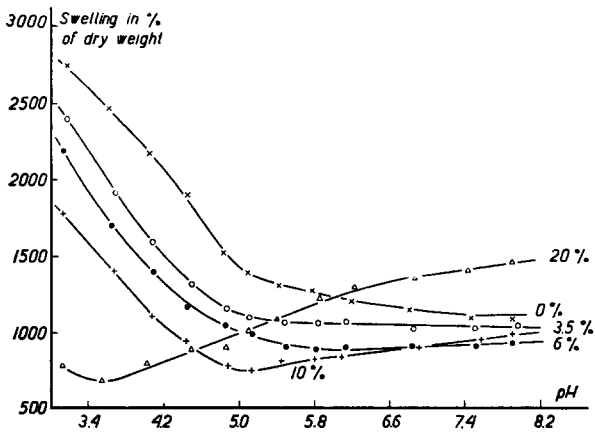

Fig. 13. Influence of pH on the swelling of gels of 10% gels of pigskin gelatin containing x % of potassium mucoitinsulphate; in McIlvaine buffer.

References p. 481.

inflection of the pH/swelling curve, which (b) shifts towards lower pH, while (c) this shift of the whole pH/swelling curve is accompanied by a large increase of swelling at pH values above the point of inflection.

TABLE II

INFLUENCE OF THE pH ON THE TURBIDITY OF GELS OF PIGSKIN GELATIN WITH x% POTASSIUM MUCOITINSULPHATE IN McILVAINE BUFFER

pH	*Pigskin gelatin*	*3.5% muc. sulph.*	*6% muc. sulph.*	*10% muc. sulph.*	*20% muc. sulph.*
3.15			—	—	× ×
3.60			—	—	× × ×
4.05			—	?	× ×
4.45			—	×	×
4.85			—	× ×	×
5.10	no	no	?	× × ×	?
5.45	turbidity	turbidity	?	× ×	?
5.80			×	×	?
6.15			×	?	—
6.85			?	?	—
7.50			?	—	—
7.90			—	—	—

DISCUSSION

Although for most investigators the influence of mucopolysaccharides in the connective tissue is no longer a disputed point, this does not apply to the nature of the protein-mucopolysaccharide linkage. Some investigators consider it to be a salt linkage (MEYER[9,17]; PEARCE AND WATSON[18]; PARTRIDGE[19]; WOODIN[20]). Others are more inclined to believe that the mucopolysaccharide is bound in a protein complex (EINBINDER AND SCHUBERT[8,15]; BLIX[21]). MEYER and especially PARTRIDGE both explain their experimental observations with the aid of hypotheses which attempt to regard the nature of the protein-mucopolysaccharide bond from a purely physico-chemical point of view. PARTRIDGE'S ideas are akin to those of BUNGENBERG DE JONG[22] on complex formation in colloid systems, although he uses a quite different terminology. By complex colloid systems we understand colloid systems in which opposite charges are present. A characteristic is the presence of Coulomb interactions (= intensity of the complex relations). With the establishment of these complex relations there is generally a tendency to a decrease of the solubility of the colloid. In gels this will be shown by decrease of swelling power and by the appearance of turbidity. This will occur especially where the total positive charge is equal to the total negative charge.

BUNGENBERG DE JONG AND SENGERS[23] studied the complex gel of gelatin (isoelectric point *ca.* 4.8) and gum arabic. Here the intensity of the complex relations depends on the proportions of the two colloids in the gel and on the pH. Since the turbidity was chosen as the measure of the complex relations, the results of this investigation are comparable in some degree with ours as given in Table II. Although in this table the degree of turbidity is arbitrarily denoted by the number of crosses, we see that with constant proportions of pigskin gelatin and potassium mucoitinsulphate the greatest degree of turbidity occurs around the point of inflection of the pH/swelling curve (Fig. 13). At constant pH the degree of turbidity depends on the proportions

References p. 481.

in which the two colloids are mixed. Lowering of pH causes a shift of the maximum turbidity towards a gel containing a higher proportion of potassium mucoitinsulphate.

The influence of complex relations in the gels on the swelling gives a picture corresponding to that observed with the turbidity: minimal swelling (= greatest degree of turbidity) with the strongest binding between pigskin gelatin and potassium mucoitinsulphate. The swelling is also dependent on the proportions in which the colloids are mixed and on the pH: conditions with which a complex colloid system of an amphoteric and a negatively charged colloid must comply.

It is now possible to compare the results of these experiments on a model system with those of the experiments on connective tissue. The shape of the pH/swelling curve for the pigskin gelatin corresponds, as already pointed out, to that for its parent substance collagen. With collagen also there is a marked swelling in the acid pH range and a very long range of minimum swelling instead of a point of minimum swelling. Not until a pH above 10 has been reached does the second limb of the curve appear with collagen. Small amounts of mucopolysaccharide have practically no influence on the shape of this curve. This is seen in the swelling behaviour of the rat sclera, which contains about 1.3% of chondroitinsulphuric acid*. It is also shown by the model experiments, so that the sclera corresponds to a pigskin gelatin gel containing about 6% of potassium mucoitinsulphate, both with respect to the influence of pH on the swelling behaviour and with respect to the degree of turbidity at the physiological water content. Increase of the proportion of mucopolysaccharide in the tissue leads to a change from the range of minimum swelling and maximum turbidity to a definite point of minimum swelling and maximum turbidity, which shifts towards lower pH values at higher concentrations. This is clearly shown in the influence of pH on the swelling and turbidity of bovine and rat corneae, containing about 4.4 and about 3.2% of mucoitinsulphuric acid respectively*. These phenomena are also clearly in evidence in the model experiments. Both as regards its swelling behaviour and as regards its turbidity phenomena, the cornea might be compared with a gel of pigskin gelatin containing about 15% of potassium mucoitinsulphate.

From these considerations we may conclude that the hypothesis according to which corneal and scleral connective tissues are regarded as physico-chemical systems, *i.e.* as complex colloid systems as defined by BUNGENBERG DE JONG, with collagen and mucopolysaccharide as the two essential constituents, is highly probable.

SUMMARY

In continuation of previous work, the influence of pH (and salts) on the swelling and transparency of the cornea and sclera was studied. The influence of pH was then compared with that observed on a model system of pigskin gelatin containing x % of potassium mucoitinsulphate. On the basis of these results it is considered highly probable that in connective tissue a complex relation, similar to that described by BUNGENBERG DE JONG and co-workers, exists between the collagen and mucopolysaccharide.

* The chondroitinsulphuric acid content of the rat sclera and the mucoitinsulphuric acid content of the bovine and rat cornea were calculated from the hexosamine content determined by the method of BLIX (see footnote on pag. 477).

The assumption is here made that the composition of these mucopolysaccharides is that given by MEYER[9,10], *viz.*: $(C_{14}H_{21}NSO_{14})_n$ with a unit weight of 459.4 and composed of N-acetyl-D-hexosamine; D-glucuronic acid and sulphuric acid. The hexosamine (glucosamine in the case of mucoitinsulphuric acid and galactosamine = chondrosamine in that of chondroitinsulphuric acid) amounts to 38.9% of the total mucopolysaccharide.

References p. 481.

RÉSUMÉ

Poursuivant leurs travaux antérieurs, les auteurs ont étudié l'influence du pH (et des sels) sur le gonflement et la transparence de la cornée et de la sclérotique. L'influence du pH a été comparée à celle exercée sur un modèle constitué par de la "pigskin" gélatine renfermant x % de mucoïtin sulfate de potassium. Les résultats obtenus suggèrent que, dans le tissu conjonctif, une relation complexe, analogue à celle décrite par BUNGENBERG DE JONG et ses collaborateurs, existe entre le collagène et les mucopolysaccharides.

ZUSAMMENFASSUNG

In Fortzetzung früherer Arbeiten wurde der Einfluss von pH (und Salzen) auf die Quellung und Transparenz von Cornea und Sklera untersucht. Weiter wurde der Einfluss des pH verglichen mit dem auf ein Modell aus "pigskin"-Gelatine, die x % Kalium-Mukoitinsulfat enthielt. Auf Grund dieser Ergebnisse wird es für sehr wahrscheinlich gehalten, dass im Bindegewebe eine Komplexbeziehung zwischen Kollagen und Mucopolysacchariden besteht, ähnlich der von BUNGENBERG DE JONG und Mitarbeitern beschriebenen.

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