The Acid Mucopolysaccharides of the Sexual Skin of Apes and Monkeys

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The process of swelling of the sexual skin of immature female rhesus monkeys (*Macaca mulatta*) induced by oestrogenic stimulation, or the process occurring in mature pig-tail monkeys (*Macaca nemestrina*) and baboons (*Papio papio*), has been shown to be associated with a general water retention (Krohn & Zuckerman, 1937), an increase in the size of the cells of the connective tissue of the sexual skin (Aykroyd & Zuckerman, 1938) and with the presence of a viscous exudate which on occasions is easily drained from cut surfaces of the tissue. The exudate has a variable but high osmotic pressure (which may exceed that of serum) and a total protein content of about 2·5% (Fisher & Zuckerman, 1937).

Ogston, Philpot & Zuckerman (1939) obtained a substance, termed mucoprotein, which was precipitated from the exudate in 1% acetic acid and which contained 2–3% of hexosamine and 12–13% of nitrogen. This material was apparently responsible for the viscous nature of the exudate. Chain & Duthie (1940) observed that extracts of testes containing hyaluronidase decreased the viscosity of the exudate. Duran-Reynals, Bunting & van Wagenen (1950) showed that the roll swellings on the sexual skin of the rhesus monkeys could be collapsed by slight pressure after the swellings had been infiltrated with testicular hyaluronidase. These authors showed that the extracellular interfibrillar region in swollen sexual skin contained a metachromatically staining material sensitive to the hyaluronidases. These observations led to the conclusions that the exudate of swollen sexual skin contained hyaluronic acid and that the production of hyaluronic acid in the sexual skin was in some way concerned with the retention of water in this tissue.

Hyaluronic acid has been known for some time as a component of the amorphous ground substance of connective tissue and has, in company with chondroitin sulphate, been isolated from skin (Meyer & Chaffee, 1941; Pearce & Watson, 1949). There are some indications that hyaluronic acid is in some way connected with water retention in tissues. Pearce & Watson (1949) showed that in pretibial myxoedema the hyaluronic acid and chondroitin sulphate content of skin of the affected part increased several times over that of unaffected skin, and that the water content of affected skin was higher than that of unaffected skin. Hyaluronic acid accompanied by serum protein occurs in synovial fluid. Oedema of the joints occurs in some arthritics and is accompanied by the increased production of hyaluronic acid, apparently in depolymerized form (Ragan & Meyer, 1949). The concentration of hyaluronic acid in the fluid from the oedematous joints was not greater than that from normal joints and there was a marked rise in serum protein in the fluid from oedematous joints. Ropes, Bennett & Bauer (1939) obtained a much higher colloid osmotic pressure from normal synovial fluids than was calculated from their albumin and globulin content. The increased osmotic pressure was considered to be due to the 'mucin' (hyaluronic acid) but this is difficult to understand as the molecular weight of synovial fluid is estimated to be in the region of 10^7 (Ogston & Stanier, 1952).

Vitreous humour, which contains hyaluronic acid together with small amounts of protein as a gel, has been injected *in vivo* with hyaluronidase in an effort to remove hyaluronic acid (Pirie, 1949). The hyaluronic acid was 'disaggregated' but not removed from the ocular cavity. There was no diminution in vitreous volume, although the intraocular tension decreased considerably. The intraocular tension was regained in 9 days whereas the hyaluronic acid did not return to its aggregate state for 6 weeks.

Ludwig, Boas & Soffer (1950) induced proptosis in guinea pigs and noted that there was an increased water and hexosamine content of the loose connective tissue of the orbit. K. Aterman (personal communication, 1952) reversed proptosis by injecting hyaluronidase into the orbit.

These examples suggest that increased concentration of hyaluronic acid may bring about the retention of fluid in a tissue. Depolymerization leads to a decreased water retention. The oedema of arthritic joints, however, could be due to the marked accumulation of serum protein in the joint rather than an effect related to altered hyaluronic acid status. It is unsatisfactory attempting to draw general conclusions since the histological

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structures of the tissues mentioned are quite different, and this influences the possible mode of entry of water into them. While it was clear that hyaluronic acid was involved in the sexual-skin phenomenon it was by no means clear whether during the turgescent phase there was an increase in hyaluronic acid concentration and whether during deturgescence there was a change in polymerization or concentration of the hyaluronic acid or both. The sexual-skin phenomenon has now been investigated to gain information on these matters. The exact nature of the material in the exudate was investigated, since apart from the determination of hexosamine (Ogston *et al.* 1939) no chemical studies have been reported. Viscosity studies were carried out to determine whether there was any depolymerization of hyaluronic acid of the exudate in any phase of the sexual-skin cycle. Measurements were also made of the changes in concentration of hyaluronic acid and chondroitin sulphate occurring in the skin in the various phases of the cycle.

MATERIALS AND METHODS

Animals. Three species of animals were used: rhesus monkeys (*Macaca mulatta*), baboons (*Papio papio*) and pig-tail monkeys (*Macaca nemestrina*). With two exceptions, the specimens obtained from the baboons were from swellings occurring in normal menstrual cycles. The baboons were used in other experiments which caused the death of all but one of them. The pig-tail monkeys, which were obtained to replace the baboons with animals of convenient size and ones with a regular and consistent sexual-skin cycle, were found on arrival from Singapore to be sexually immature. One of these animals, on one occasion only, exhibited a naturally occurring sexual-skin swelling, and it was necessary to induce sexual-skin swellings by injection of oestrogens. With two exceptions, the swellings of the rhesus monkeys were brought about by oestrogen administration.

The animals were housed in cages in a room kept at 70° F. The staple foods were bread and steamed potatoes, supplemented when available by root vegetables, cabbages, apples and oranges. Vitamin supplements were given. Water was always available in the cages.

Specimens. Each animal was numbered and the occasions on which specimens were obtained were denoted by a letter. Thus 616A and 616B denote two occasions on which specimens of tissue were obtained from animal no. 616.

Sexual-skin swellings were induced experimentally with oestrone acetate, oestradiol-3-17β dipropionate or ethinyloestradiol dissolved in arachis oil. The injections were given intramuscularly in the legs. There is no apparent reason for regarding the swellings induced by one oestrogen as different from the swelling induced by another. Tables 1–3 list the relevant details pertaining to all the specimens from the three species used in this study.

Appearance of the sexual skins. Adolescent, pubertal and almost mature sexual-skin responses were obtained in rhesus monkeys (adequately described by Zuckerman, van Wagenen & Gardner, 1938). The scrotal and pubic-lobe swellings were observed to be soft but not pitting, whereas the roll swellings of the thigh and base of tail were very firm.

The baboons exhibited consistent sexual-skin changes, in which the swelling commenced during the latter part of, or immediately after, menstruation, rose to a maximum in a week and, after a further week in the fully swollen state, collapsed rapidly (within a few days). The baboon sexual-skin swelling was red and shiny and very resilient to pressure. The experimentally induced swellings in 616 did not appear to differ from the natural swellings except that the surrounding 'bare' area was much redder in the experimental swellings.

The sexual-skin swellings induced in the pig-tail monkeys were usually pale, dull and very hard. It was possible to pit them with considerable pressure but, on one occasion, at autopsy considerable haemorrhage was seen to have developed at the site of pressure. The shape of the sexual-skin swellings in the females of these animals varied, generally consisting of pubic-lobe swellings and a circum-anal swelling which extended to the base of the tail.

Table 1. *Details of baboons (female) used in experiments*

ODP, Oestradiol-3-17β dipropionate.

Specimen no.	No. of days since commencement of sexual-skin swelling	No. of days since commencement of collapse of sexual skin	State of sexual skin
615B	3	—	Early swelling
615D	10	1	Commencing to collapse
616A	8	—	Fully swollen
616C	3	—	Rapidly increasing
616D	16	3	Flabby and almost collapsed
616E	11	0	Fully swollen
616F	8	0	Fully swollen
616G	1	—	Very early swelling
616H	6	0	Fully swollen
616J	10	3	Collapsing
(Bilateral ovariectomy was carried out between 616J and 616K)			
616K 300 μg. of ODP/3 days (900 μg. total dose)		2	Large swelling commencing to collapse
616L 400 μg. of ODP/3 days (1200 μg. total dose)		—	Large swelling still increasing in size
621A	7	—	Fully swollen
624A	15	1	Collapsing, still large

Table 2. *Details of rhesus monkeys used in experiments*

OA, Oestrone acetate; ODP, oestradiol-3-17β dipropionate; EO, ethinyloestradiol.

Specimen no.	Sex	Hormone treatment (doses in μg.) (a) Routine	(b) Total dose of hormone	No. of days since last injection	State of sexual skin
575A	M.	500 OA/day	9000	1	Swollen scrotum. Other regions were pink
584A	F.	500 OA/day	7500	0	Swollen pubic lobe and peripheral quilting
609A	M.	500 OA/day	9000	1	Enormous scrotal swelling
655A	F.	Natural pubertal swellings consisting of large pubic lobes and peripheral rolls on the legs			
673A	F.	100 ODP/day + 100 mg. of cortisone/day	2100 500 mg.	1	Large swollen pubic lobe
673B	F.	—	—	—	Entirely collapsed
675A	M.	100 ODP/day	1400	0	Considerable scrotal swelling
675B	M.	100 ODP/day	1600	7	Swelling of 675A almost collapsed
675C	M.	300 ODP/3 days	2700	2	Small scrotal swelling and quilting of back. Almost mature response
677A	F.	100 ODP/day	1400	1	Large swollen pubic lobes
677C	F.	100 ODP/day	1600	1	Reddened pubic lobes and quilting of back. Almost a mature response
680A	M.	100 ODP/3 days	1800	1	Large penile and scrotal swellings. Diffuse swelling of buttocks
693A	F.	Natural pubertal swelling with roll swellings on thighs and base of tail			
698B	M.	100 EO/day	2000	2	Large scrotal and penile swellings. Thickened buttocks

Table 3. *Details of pig-tail monkeys used in experiments*

ODP, Oestradiol-3-17β dipropionate.

Specimen no.	Sex	Hormone treatment (doses in μg.) (a) Routine	(b) Total dose of hormone	No. of days since last injection	State of sexual skin
681A	M.	300 ODP/3 days	3000	2	Large circumanal and penile swellings. Scrotum flabby
682E	F.	300 ODP/3 days	1800	7	Collapsed flushed and thickened buttock skin
683B	F.	100 ODP/day	2600	1	Large circumanal and pubic swellings
683C	F.	100 ODP/day	2600	2	683B at death 24 hr. later
685A	F.	300 ODP/3 days	1800	2	Moderate circumanal and pubic swellings
686A	M.	300 ODP/3 days	3900	1	Large circumanal and penile swellings. Scrotum flabby
687A	F.	300 ODP/3 days	1800	2	Moderate circumanal and pubic-lobe swellings
688B	F.	Naturally occurring swelling in collapsing phase			
688C	F.	300 ODP/3 days	1500	2	Moderate circumanal and pubic-lobe swelling
689D	F.	300 ODP/3 days	1200	8	Moderate circumanal and base of tail swelling. Collapsing
690A	F.	300 ODP/3 days	3000	3	Large circumanal and pubic lobe
690C	F.	300 ODP/3 days	2100	3	Large circumanal and pubic-lobe swellings increasing in size
691D	F.	300 ODP/3 days	2100	9	Moderate circumanal and large pubic swellings. Collapsing

Sometimes the circumanal and pubic-lobe swellings were separated, but on other occasions the two regions were joined by swollen vulval lips. One animal (no. 682) on all occasions responded to oestrogen treatment in an atypical manner. The first response (682A) consisted of a small pubic-lobe swelling and a diffuse pink swelling of the buttocks. The last response (682E) consisted of a small pubic-lobe swelling and a hyperaemic, slightly thickened skin over the buttocks. The male pig-tail monkeys responded slowly to oestrogen by forming hard circumanal swellings completely separate from a flabby pale scrotum and a large hard swollen penis. The appearance of the naturally occurring swelling (688B) did not differ from the experimentally induced swellings.

Biopsy technique. The animals were anaesthetized with an intravenous or intraperitoneal injection of Nembutal, supplemented if necessary with ether during the operation. The majority of the specimens were obtained with scalpel and scissors, but a few specimens were taken with the cutting needle of a diathermy apparatus. Surface blood was quickly wiped from the specimens with cotton wool.

The swollen sexual skin consisted of the epidermis and a thickened dermis, which, in the deeper layers, had the appearance of jelly. Large amounts of exudate flowed freely from some specimens, e.g. those from the large pubic and scrotal swellings of the rhesus monkeys, whereas exudate could be obtained from baboon and pig-tail specimens only by cutting into small pieces and squeezing through a fabric.

Sexual-skin exudates. The exudates were obtained by cutting the tissue into small pieces and squeezing the exudate out through muslin or Terylene fabric. The exudates from baboon and most rhesus specimens were quickly obtained in this way, whereas the exudates from the pig-tail specimens were obtained only after considerable squeezing. In view of the instability of one or more of the components of the exudate (Ogston *et al.* 1939) and in order to minimize these changes, attempts were made to obtain the exudate in the cold, but the more viscous exudates gelled. All exudates were therefore obtained at room temperature as soon as possible. The less viscous exudates were freed of particulate material by centrifuging at 7000–8000*g* for 10 min. after the addition of a small amount of Hyflo Super-Cel. This procedure did not remove all particulate matter from the highly viscous exudate from pig-tail monkeys, and some error may have been introduced into some of the analyses, especially the viscosity measurements.

Preparation of mucopolysaccharide from the exudate. Several preparations of the mucopolysaccharide fraction were made, but as small amounts only of material were available the purification was not carried very far. The products are termed MP1 etc.

MP1 was prepared at room temperature by acidifying exudate from rhesus monkeys 624A with an equal volume of 2% (v/v) acetic acid. The precipitate was redissolved in $NaHCO_3$ (1%, w/v), reprecipitated with 2% (w/v) acetic acid and washed with ethanol and ether.

MP2 was prepared from 25 ml. of exudate of 609A in the same manner as MP1, followed by one treatment with Sevag's reagent, at room temperature.

MP3 was prepared from the swollen pubic lobe of 655A, by diluting the exudate with an equal volume of water and adding 4 vol. of ethanol at −20° (Ropes, Robertson, Rossmeisl, Peabody and Bauer, 1947). The voluminous precipitate was redissolved in 0·02N-NaOH. A further ethanol precipitation at −20° was carried out and the precipitate washed in ethanol and ether. The yield after drying over P_2O_5 *in vacuo* was 23 mg.

MP4 was prepared from approx. 500 g. of coarsely-minced sexual skin of baboon 615D. After removal of exudate the skin was further extracted with 200 ml. of 0·9% NaCl at 0°. The combined exudate and extract were dialysed at 0° against water and precipitation with 4 vol. of ethanol at −20° was carried out. The bulky precipitate was washed in ethanol and dried over $CaCl_2$ *in vacuo*, after which it was extracted for 60 hr. at 0° with 80 ml. of 0·1N-sodium acetate. After dialysis against water at 0°, the mucopolysaccharide fraction was precipitated from the extract with 4 vol. of ethanol at −20°. The fine precipitate was washed in ethanol and ether and dried over $CaCl_2$. Yield was approx. 10 mg.

MP5 was prepared from 150 g. of swollen circumanal skin of pig-tail specimen 683C in a similar manner to MP4, except that the precipitate that was formed by the first ethanol treatment was not dried but redissolved in water. Yield was approx. 5 mg.

MP6 was prepared by repeatedly deproteinizing (with Sevag's reagent) 15 ml. of exudate from penis and circumanal region of 681A. Further protein was removed with Lloyd's reagent at pH 2·0–3·0 (acetic acid). The very viscous supernatant was mixed with 4 vol. of ethanol at room temperature and the precipitate washed with ethanol and dried over $CaCl_2$. This material was extracted with about 20 ml. of phosphate buffer, pH 8·0 (94·5 vol. of M/15-Na_2HPO_4 and 5·5 vol. of M/15-KH_2PO_4) and 80 ml. of ethanol was added to the extract, which was not viscous. The precipitate was dissolved in water and a further ethanol precipitation carried out. After washing with ethanol and drying over P_2O_5 43 mg. of material was obtained.

Hyaluronidase preparations. Testicular hyaluronidase I was prepared from bull's testis by the method of Freeman, Anderson, Oberg & Dorfman (1949). Its potency was not determined. Testicular hyaluronidase II (1030 turbidity reducing units/mg.) was obtained from Wyeth Laboratories Inc., Philadelphia, Pa., U.S.A. It was used at concentrations of 1 mg./ml. in 0·9% NaCl.

Streptococcal hyaluronidase was a crude enzyme fraction prepared from *Streptococcus haemolyticus* by the method of Rogers (1945). It was stored in a concentrated form in glycerol at −30°.

Hyaluronic acid. The 'reference' hyaluronic acid (Allen and Hanburys Ltd.) had been prepared from acetone-dried umbilical cords by extracting with water and precipitating from the extract with ethanol saturated with potassium acetate. The water-soluble material from this precipitate was again treated with ethanol. The preparation contained 4·3% of N, 31·9% of hexosamine and 1·6% of S; it has been examined previously by filter-paper electrophoresis (Rienits, 1953*a*).

Chondroitin sulphate. This preparation was made from bovine trachea by the method of Bray, Gregory & Stacey (1944) and contained 4·72% of N and 23·2% of hexosamine.

Filter-paper chromatography. The ascending technique was used, at 18°, with Whatman filter paper no. 54. Positions of the sugars were located with aniline hydrogen

oxalate (Partridge, 1948). The most suitable solvent system was found to be pyridine–amyl alcohol–water (7:7:6, by vol.). In contrast with the reports of Werner & Odin (1949), the hexuronic acids had R_F values of about half those of the hexosamines. Individual hexosamines and hexuronic acids were not separated in this solvent. The individual hexosamines were identified by degrading with ninhydrin and identifying the resultant pentoses (Gardell, Heijkenskjold & Roch-Norland, 1950).

Determinations of viscosity. All the viscosity determinations were carried out in the same capillary microviscometer (Hardwicke & Squire, 1952) with a flow time of 22·4 sec. for the standard buffer solution. The outer tube contained 0·8 ml. of the fluid under examination and the viscometer was immersed to a standard depth in the fluid. The readings were made at 25°.

Exudates were weighed into the outer tube and the volume was brought to 0·8 ml. with standard buffer (0·2M-NaCl and phosphate buffer, pH 7·4; as used by Ogston & Stanier, 1950). The flow time of the mixture was measured. A further 0·8 ml. of buffer was added to samples of 0·4 ml. of the mixture removed for hyaluronic acid glucosamine determination. The flow time of the fluid remaining in the viscometer was measured. Two further dilutions with buffer were carried out. A correction was made to the flow time to allow for the effect of protein in the exudate. The value of the correction was ascertained by measuring the flow times of several sera at various dilutions. The correction was calculated to be 2·5 sec. for 1 g. of protein/100 ml. of exudate or dilution.

Filter-paper electrophoresis. This was carried out as described by Rienits (1953*a*), as was the elution of material from the paper after the electrophoresis.

Chemical analyses

Hexosamine. The Elson & Morgan (1933) method, as modified by Johnston, Ogston & Stanier (1951), was used with several further modifications. Samples containing hexosamine were placed in Quickfit test tubes and evaporated to dryness with a water bath and water pump. Acidic materials (such as HCl hydrolysates) were then left *in vacuo* over NaOH overnight. If the test sample contained buffer the hexosamine standards (glucosamine hydrochloride; British Drug Houses Ltd.) were also made up in the buffer and evaporated to dryness. To this material was added 1·5 ml. of acetyl acetone reagent (0·1 ml. of acetyl acetone dissolved in 7·5 ml. of 0·5N-Na_2CO_3). The remainder of the procedure was as described by Johnston *et al.* (1951). A set of standards was put in with each batch of unknowns.

Hexuronic acid. This was determined by the method of Ogston & Stanier (1951), a correction being used for protein (Ogston & Stanier, 1952).

Nitrogen. This was determined by the Kjeldahl digestion of Hiller, Plazin & van Slyke (1948) and distillation of the NH_3 into boric acid.

Protein. Protein in the exudates was determined by a biuret method (Wolfson, Cohn, Calvary & Ichiba, 1948).

Hyaluronic acid and chondroitin sulphate. These substances were estimated in skin specimens by a colorimetric method based on the result of Pearce & Watson (1949) that each 1 g. of human skin contained sufficient hyaluronic acid and chondroitin sulphate to yield 60 μg. of free hexosamine from each compound after acid hydrolysis. About 1 g. of skin (or rather less of swollen sexual skin) was weighed into a centrifuge tube and 2 ml. of N-NaOH and 1 ml. of water were added. The mixture was incubated at 37° for 48 hr., after which the opalescent solution was brought to pH 7·0–8·0 with 2M-acetic acid and vigorously shaken with 5 ml. of Sevag's reagent. The denatured protein was compacted at the interface by centrifuging, and the supernatant fluid was adjusted to pH 5·0 (indicator paper) with dilute acetic acid. The denatured protein was washed with 1 ml. of acetate buffer, pH 5·0, and combined washing and supernatant shaken with 50 mg. of Lloyd's reagent. The Lloyd's reagent was washed with 1 ml. of the acetate buffer and combined washing and supernatant concentrated to 3–4 ml. over P_2O_5 *in vacuo*. Ethanol–ether (3 : 1, v/v; 30 ml.) was added and the mixture kept at −30° overnight. The precipitate was washed with 2 ml. of cold ethanol–ether and allowed to dry in warm air.

The hyaluronic acid contained in the precipitate was removed by enzymic hydrolysis. The precipitate was suspended in 1 ml. of enzyme–phosphate buffer (M/15-phosphate buffer, pH 7·0, in 0·1M-NaCl containing in each ml. 0·02 ml. of streptococcal hyaluronidase solution), a few drops of toluene were added and the mixture was incubated at 37° for 18 hr. The small amount of precipitate remaining was centrifuged off and washed with 1 ml. of water. To the combined supernatant and washing was added 18 ml. of ethanol–ether and precipitation completed at −30° overnight. The supernatant, together with 2 ml. of ethanol–ether washing, was evaporated to dryness and heated with 2 ml. of N-HCl at 100° for 2 hr. This fraction contained the hyaluronic acid hexosamine.

The precipitate remaining after removal of the hyaluronic acid was extracted twice with 2 ml. portions of 10% (w/v) barium acetate. The chondroitin sulphate fraction was precipitated from the combined barium acetate extracts by the addition of ethanol to 20% (v/v) concentration and leaving at 0° overnight. The precipitate was washed with 1 ml. of aq. 20% ethanol, dried in air and then hydrolysed in 3 ml. of 4N-HCl at 100° for 4 hr.

The hexosamine contents of the hydrolysates of the two fractions were determined and the hyaluronic acid and chondroitin sulphate concentrations in the skin specimens expressed as mg. of hexosamine /100 g. of skin (wet wt.). A correction was made for the contribution of hexosamine from the enzyme–buffer mixture.

The composition of the hyaluronic acid and chondroitin sulphate fractions was examined. The hyaluronic acid fraction contained no detectable acid-hydrolysable sulphate, and the hexosamine : hexuronic acid ratio varied from 0·84 to 1·39 (five determinations). The chondroitin sulphate fraction contained a molar ratio of hexosamine to uronic acid of approximately unity; the hexosamine was galactosamine, there was sulphate present after hydrolysis and paper electrophoresis showed that the hexosamine-containing material migrated at the same rate as chondroitin sulphate. Both fractions contained considerable amounts of protein. Recoveries of hyaluronic acid added at the beginning of the assay were low and variable (52, 78 and 65%). The reproducibility of the determinations on rat skin were: for hyaluronic acid $5{\cdot}96 \pm 0{\cdot}90$ mg. of hexosamine/100 g.; for chondroitin sulphate $6{\cdot}90 \pm 0{\cdot}55$ mg. of hexosamine/100 g. of skin (nine determinations). Thus the method has a low order of reproducibility but the results

obtained show that it has sufficient sensitivity to demonstrate differences in acid mucopolysaccharide content of sexual skin during its phases of turgescence and deturgescence.

Hyaluronic acid in exudates. This was estimated as mucin hexosamine. The exudates were brought to 2% with respect to acetic acid and put aside in the refrigerator overnight. The mucin was separated by centrifuging and washed with 1 ml. of 1% acetic acid. Several drops of N-NaOH were added to the mucin and the solution was transferred to a Quickfit test tube for hydrolysis in 4N-HCl for 4 hr. at 100°. Hexosamine was then determined in the hydrolysate. The hexosamine liberated in hydrolysis for 4 hr. was the same as in 6 hr. Determinations were made in duplicate and agreed to within 2%.

Sulphate. Sulphate released on hydrolysis in 2N-HCl for 2 hr. at 100° was detected turbidimetrically. The hydrolysate was centrifuged at 7000–8000*g* (for 15 min.) and to 1 ml. of the supernatant was added 1 ml. of 2% (w/v) $BaCl_2$ and 1 ml. of ethanol. After 15 min. a definite perceptible haze could be seen from material containing 50 μg. or more of SO_4^{2-} ions/ml. No attempt was made to measure the sulphate quantitatively.

RESULTS

Mucopolysaccharide of the sexual-skin exudate

Action of hyaluronidases. Exudates (0·5 ml.) from specimens 584A, 575A, 616A, 689D and 691D were diluted with 0·5 ml. of 0·9% NaCl and incubated for 18 hr. with and without streptococcal hyaluronidase (0·02 ml.) or testicular hyaluronidase (0·1 ml.). In all specimens the unstable nature of the mucin, originally observed by Ogston *et al.* (1939), was noted by the change in appearance of the mucin precipitate (on addition of acetic acid to 2%) from a clot to a turbidity after incubation without added enzyme. Incubation with either testicular or streptococcal hyaluronidase abolished the ability to form a mucin precipitate in all specimens.

Analyses of mucopolysaccharide preparations. MP1 was hydrolysed with 2N-H_2SO_4 for 4 hr. at 100°, neutralized with $Ba(OH)_2$ and brown colouring matter was largely removed with charcoal. MP2 was hydrolysed with 2N-HCl for 4 hr. at 100° and HCl removed by evaporation to dryness on the water pump. The two hydrolysates were examined for the nature of their sugar content by filter-paper chromatography. Hydrolysates from hyaluronic acid and chondroitin sulphate were also examined together with reference sugars. In both MP1 and MP2 there were only two classes of sugars present, one with an R_F that of hexosamines (0·20–0·23) and the other with an R_F of the hexuronic acids (0·08–0·10). There appeared to be much more hexosamine present on the chromatogram than hexuronic acid (owing to the destruction of the latter during acid hydrolysis). The hexosamine in MP2 was shown to be glucosamine since it formed arabinose (R_F 0·47) on oxidation with ninhydrin, in contrast with galactosamine which formed lyxose (R_F 0·67) on treatment with ninhydrin. The identity of the hexuronic acid present in MP1 and MP2 is not known since solvent systems for the separation of the individual hexuronic acids were not available.

MP6 contained less than 0·3% of total P and less than 1·5% of S (as acid-hydrolysable sulphate). Preparations MP3 to MP6 were analysed quantitatively for hexosamine, hexuronic acid and total N. The results, together with a derived value for protein, are shown in Table 4. In three of the four preparations the molar ratio of hexosamine to hexuronic acid does not differ greatly from unity. Hyaluronic acid contains equimolar quantities of hexosamine and hexuronic acid (Meyer, 1958).

Preparations MP3 and MP6 were examined by filter-paper electrophoresis. The results are shown in Fig. 1. MP6 appears to contain a single compound containing hexuronic acid but there was not sufficient material to repeat the electrophoresis with hexosamine as a basis for locating the mucopolysaccharide. MP3 appeared to consist of two compounds each containing hexosamine and hexuronic acid. Neither of these compounds migrated as far as chondroitin sulphate, which under the same conditions would have migrated approx. 32 cm. The appearance of the two peaks may have

Table 4. *Quantitative analyses of MP3–MP6*

	N (%)	Hexosamine (%)	Hexuronic acid (%)	Molar ratio Hexosamine / Hexuronic acid	Protein (%)
MP3	9·75	3·46	2·96	1·26	59·4
MP4	6·70	4·58	3·10	1·59	42·5
MP5	6·88	5·30	5·44	1·05	39·1
MP6	10·25	7·56	9·17	0·90	62·2

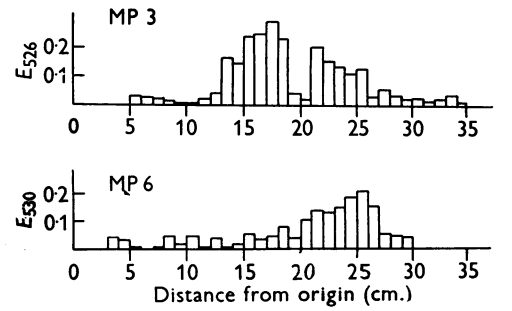

Fig. 1. Filter-paper electrophoresis diagrams of mucopolysaccharide preparations from exudates. MP3: electrophoresis in 0·1M-phosphate buffer, pH 7·0; hexosamine determinations on eluates. Chondroitin sulphate would have migrated approx. 32 cm. under these conditions. MP6: electrophoresis in 0·1M-acetate buffer, pH 5·0; hexuronic acid determinations on the eluates.

been due to faulty extraction of mucopolysaccharide from the filter-paper strips subsequent to electrophoresis or may have been due to two distinct polymer sizes of hyaluronic acid. It was not possible to decide between these possibilities owing to lack of material.

Relative viscosity measurements and hyaluronic acid of exudates

The ratio (called the 'viscosity concentration ratio' = $1/c.\log \eta/\eta_{solvent}$, where $\eta/\eta_{solvent}$ is the relative viscosity) was determined on a number of exudates and used as an indication of the degree of polymerization of the hyaluronic acid in exudates. [A similar quotient was used by Ragan & Meyer (1949) in studies on synovial fluids.] There was considerable variation observed in the value of the ratio, ranging from 0·0014 to 0·035, although about 80 % of the values fell within the range 0·003–0·01. The greatest variation occurred in the material from the baboons and rhesus monkeys where the hyaluronic acid concentration is lowest. It was not possible to discern any species differences in the viscosity concentration ratio nor was any relationship detected between the ratio and the state of the sexual-skin swelling, i.e. the degree of swelling or the phase of the cycle. There did not seem to be any relationship between the concentration ratio and the concentration of the hyaluronic acid in the exudate.

Concentrations of acid mucopolysaccharides in sexual skin

In Tables 5–7 are set forth the results of analyses for water, chondroitin sulphate and hyaluronic acid together with the exudate hyaluronic acid levels from the sexual skins of the three species. The water content of the sexual skin increases enormously when the tissue swells and reaches well over 90 % in some instances. In all the species the hyaluronic acid concentration of the swollen skin is greater than that of unswollen sexual skin or ordinary skin. The reverse is seen with the chondroitin sulphate concentrations. When the concentrations of the acid mucopolysaccharides are expressed in terms of dry weight of tissue it can be seen that the chondroitin sulphate concentration is, with few exceptions (e.g. 616F, 688C and 690A), slightly greater in swollen sexual skin than in unswollen skin. This increase in concentration occurs despite the increase during swelling in solid matter of the sexual skin due to an influx of electrolyte and serum protein; thus the total amount of chondroitin sulphate present in a sexual skin increases when it swells.

There are marked species differences in the concentrations of hyaluronic acid in the swollen sexual skins, and also there are marked differences in the exudate hyaluronic acid concentrations from the three species. The concentrations of hyaluronic acid in swollen sexual skin and exudate may determine to some extent the turgidity of the skin. Thus in general the swollen sexual skins of the pig-tail monkeys were very hard and these had high concentrations of hyaluronic acid in both whole sexual skin and exudate. The specimens 682E and the scrotal swelling of 681A (Table 7) were not hard (see Materials and Methods section), and the hyaluronic acid concentrations in exudates from these two tissues were lower than exudates from other pig-tail monkey sexual skins. In addition, the total tissue hyaluronic acid values were lower than most other pig-tail specimens of sexual skin. In the rhesus monkeys (Table 6) the one hard swelling (the roll swelling from the thigh of 693A) gave much higher values for exudate hyaluronic acid and total tissue hyaluronic acid than the other specimens which were of the softer, more flabby variety. Within the rhesus group (Table 6) specimens 675A, B and C illustrate the manner in which the sexual-skin response of rhesus monkeys alters with successive courses of oestrogen, and 677A and C illustrate the same point in a less complete fashion. The oedema is less, and less hyaluronic acid is produced. The results from 673B and perhaps 677C may be taken as indicating that the acid mucopolysaccharide content of unswollen sexual skin is not different from that of ordinary skin.

In all species there was a relationship between the water content of the tissues and their hyaluronic acid contents. With the baboons there is a suggestive, but insignificant, correlation between the water and the hyaluronic acid concentrations, but there is a significant relationship between the water concentration and the logarithm of the hyaluronic acid concentration (r 0·645; P lies between 0·05 and 0·02). The analyses from the rhesus monkeys show similar relationships, although in this series there was a highly significant correlation between the water content and the logarithm of the hyaluronic acid concentration (r 0·5975; P lies between 0·01 and 0·001). The analyses from the sexual skins of the pig-tail monkeys reveal that there is a highly significant correlation between the water content and the hyaluronic acid concentration or its logarithm (r 0·786 and r 0·923 respectively with P less than 0·001 in both cases).

The data of Table 5 show that in the baboon the level of hyaluronic acid in the exudate is much lower in the turgescent phase than during the deturgescent phase. Thus values for the deturgescent phase are 5·1, 7·9 and 11 and for the turgescent phase are 0·9, 2·3, 2·6, 1·8, 3·0, 2·4 and 3·5. The lowest value for exudate hyaluronic acid is from a very early swelling (616C) and the highest from an

almost collapsed swelling (616D). Similar differences between the turgescent and deturgescent phases were not seen in the exudates of the other species studied.

From the data in Table 5 it is obvious that in the sexual skin of the baboon with the exception of 616D only a proportion of the tissue hyaluronic acid could be in the exudate. To illustrate this a hypothetical 'hyaluronic acid space' was calculated to represent the volume of exudate required by each 100 g. of tissue if the hyaluronic acid of the tissue was solely present in the exudate. Values of

Table 5. *Hyaluronic acid and chondroitin sulphate concentrations in circumanal sexual skins from baboons*

Specimen no.	Condition of sexual skin	Water content (%, w/w)	Concn. in skin: Hyaluronic acid (mg. of hexosamine/100 g. of tissue) Wet	Hyaluronic acid Dry	Chondroitin sulphate Wet	Chondroitin sulphate Dry	Concn. of protein in exudate (g./100 ml.)	Concn. of hyaluronic acid in exudate (mg. of glucosamine 100 ml.)	Hyaluronic acid space* (ml./100 g.) of tissue)
615B	Slightly swollen	90·7	6·0	64	1·6	17	—	—	—
616C	Very early swelling	90·8	5·1	56	2·1	23	3·3	0·9	590
616G	Rapidly increasing in size	88·3	4·6	39	2·5	21	3·5	2·3	210
616L	Late turgescent phase	—	—	—	—	—	3·3	—	—
616K	Almost fully swollen	94·0	18	300	—	—	2·0	2·6	690
616F	Fully swollen	93·3	13	200	0·8	12	3·8	1·8	740
616H	Fully swollen	93·8	14	230	2·0	32	5·4	3·0	480
616E	Fully swollen	92·9	11	150	2·4	34	4·2	2·4	450
621A	Fully swollen	—	—	—	—	—	—	3·5	—
615D	Commencing to collapse	95·7	8·7	200	2·1	49	2·8	5·1	170
616J	Collapsing	92·5	12	160	2·9	39	1·8	7·9	160
616D	Almost collapsed	83·4	6·2	37	3·5	21	2·6	11	60

* Hyaluronic acid space = $\frac{\text{Concn. of hyaluronic acid in skin}}{\text{Concn. of hyaluronic acid in exudate}}$.

Table 6. *Hyaluronic acid and chondroitin sulphate relationships in skin from rhesus monkeys*

Specimen no.	Condition of sexual skin	Water content (%, w/w)	Concn. in skin: Hyaluronic acid (mg. of hexosamine/100 g. of tissue) Wet	Hyaluronic acid Dry	Chondroitin sulphate Wet	Chondroitin sulphate Dry	Concn. of hyaluronic acid in exudate (mg. of glucosamine/100 ml.)	Hyaluronic acid space* (ml./100 g. of tissue)
673A	Swollen pubic lobe	82·4	14	81	0·8	4·5	—	—
673B	Collapsed pubic lobe	51·0	5·8	10	4·2	8·6	—	—
675A	Large swollen scrotum	—	15	—	1·2	—	—	—
675B	Swollen scrotum: collapsing	—	7·1	—	0·8	—	—	—
675C	Skin of chest	69·1	4·4	14	—	—	—	—
675C	Scrotum: almost collapsed	71·7	16	—	—	—	—	—
675C	Base of tail swelling	86·5	8·5	63	—	—	5·5	160
675C	Quilted skin of thighs	77·1	10	44	—	—	no exudate	—
677A	Large swollen pubic lobe	90·4	12	128	1·4	15	—	—
677C	Skin of chest	50·9	6·0	12	—	—	—	—
677C	Flushed pubic lobe	68·5	5·4	17	—	—	—	—
677C	Slightly quilted thigh skin	71·4	4·6	16	—	—	—	—
677C	Small swelling at tail base	70·2	9·4	32	—	—	—	—
680A	Skin of chest	64·0	3·8	11	7·6	21	—	—
680A	Thickened skin of buttocks	92·6	8·1	110	2·2	30	1·5	530
680A	Grossly swollen scrotum	93·2	9·5	139	2·5	37	1·4	680
693A	Hard roll swelling	84·3	33	210	2·7	17	99	33
698B	Skin of chest	63·0	5·2	14	6·2	17	—	—
698B	Swollen penile sheath	—	11	—	1·0	—	5·6	200
698B	Thickened skin of flanks	80·0	14	72	3·2	16	10	140
698B	Flabby scrotal swelling	82·0	11	63	6·1	34	2·0	560

* See footnote to Table 5.

more than 100 ml./100 g. of tissue show that all the hyaluronic acid could not possibly be present in the exudate. In all species this calculation demonstrates that part of the tissue hyaluronic acid is not contained in the exudate. Attempts were made to fractionate the tissue hyaluronic acid into that which was easily extractable and that which resisted extraction. Several methods of extraction were used and these together with the results obtained are presented in Table 8. In all the specimens a proportion of the hyaluronic acid was not extracted. This may have been due partly to incomplete extraction by the methods used but is probably an indication of the hyaluronic acid which

Table 7. *Hyaluronic acid and chondroitin sulphate relationships in skin from pig-tail monkeys*

Specimen no.	Condition of sexual skin	Water content (%, w/w)	Concn. in skin: Hyaluronic acid (mg. of hexosamine/100 g. of tissue) Wet	Hyaluronic acid Dry	Chondroitin sulphate Wet	Chondroitin sulphate Dry	Concn. of hyaluronic acid in exudate (mg. of glucosamine/100 ml.)	Hyaluronic acid space* (ml./100 g. of tissue)
681A	Skin of chest	57·4	4·4	10	8·5	20	—	—
681A	Large circumanal swelling	94·9	60	1200	2·8	55	69	87
681A	Swollen penis	96·2	62	1600	1·2	32	63	98
681A	Large flabby scrotum	96·6	21	620	1·8	53	12	170
682E	Skin of chest	60·0	4·3	11	7·9	20	—	—
682E	Thickened circumanal region	77·8	14	63	5·2	23	1·8	770
682E	Swollen base of tail	88·8	16	140	5·7	51	11	150
683B	Large circumanal swelling	93·9	48	790	2·1	34	93	52
683B	Large pubic swelling	91·5	38	450	2·1	25	55	69
685A	Large circumanal swelling	91·5	60	710	1·5	18	50	120
685A	Large pubic swelling	93·6	50	800	1·2	19	29	170
687A	Large circumanal swelling	92·8	47	640	2·4	33	23	210
687A	Large pubic swelling	91·9	42	510	1·6	20	23	180
688B	Moderate circumanal swelling	—	59	—	5·5	—	—	—
688C	Skin of chest	50·0	3·9	78	6·1	12	—	—
688C	Large circumanal swelling	92·9	72	1000	0·9	13	—	—
688C	Large pubic swelling	92·0	51	630	1·3	16	—	—
689D	Skin of chest	60·0	7·9	20	—	—	—	—
689D	Moderate circumanal swelling	93·2	41	600	—	—	85	49
689D	Small pubic lobe	82·5	27	150	—	—	—	—
690A	Large circumanal swelling	95·1	47	960	1·6	33	130	36
690A	Large pubic swelling	94·6	60	1100	2·0	37	130	46
690C	Skin of chest	67·0	5·2	16	5·5	17	—	—
690C	Large circumanal swelling	93·8	41	660	3·2	52	59	69
690C	Large pubic swelling	91·3	47	540	3·0	35	62	76
691D	Skin of chest	62·1	6·1	16	—	—	—	—
691D	Moderate circumanal swelling	91·1	22	250	—	—	18	120
691D	Fair pubic swelling	92·3	18	240	—	—	33	55

* See footnote to Table 5.

Table 8. *Effect of various extraction procedures on acid mucopolysaccharide levels in sexual skin*

Specimen no.	Concn. of acid mucopolysaccharide: Before extraction: Hyaluronic acid hexosamine	Before extraction: Chondroitin sulphate hexosamine	After extraction: Hyaluronic acid hexosamine	After extraction: Chondroitin sulphate hexosamine
	(mg./100 g. original wet wt. of tissue)			
615D*	8·7	2·1	5·5	1·7
616H*	14·3	2·0	13·2	1·3
677A†	12·3	1·4	9·3	1·2
676A†	9·3	1·2	4·2	1·0
686A‡	40·2	2·3	6·2	0·9

* Chopped into small pieces and extracted with 0·9% NaCl until no more acid-precipitable material was obtained.
† Dried in acetone, ground with sand and extracted with 10% (w/v) sodium acetate.
‡ Homogenized in a stainless-steel Potter–Elvehjem homogenizer in 0·9% NaCl. The supernatant, on centrifuging at 7000*g* for 15 min., was still turbid and may have contained finely divided particulate matter.

was bound to insoluble structures in skin. In all cases the level of chondroitin sulphate fell upon extraction and this was probably due to the loss of particulate matter upon extraction.

DISCUSSION

The properties of the mucopolysaccharide in the sexual-skin exudate are consistent with the material being hyaluronic acid. This confirms the expectations based on the observations of Chain & Duthie (1940) and Duran-Reynals *et al.* (1950), who showed that a hyaluronidase-sensitive material was present in exudate and extracellular spaces of swollen sexual skin. In addition hyaluronic acid is a component of rat and human skin (Meyer & Chaffee, 1941; Pearce & Watson, 1949). Swollen sexual skin, however, differs from ordinary skin in that part of the hyaluronic acid appears in a soluble form and is not all bound in a form which resists extraction except with drastic procedures such as dilute alkali. Results in Tables 5–7 show that this exudate hyaluronic acid represents only a part of the general increase in this substance which occurs in sexual skin when it swells. The same tables also permit of the conclusion that during turgescence the total quantity of chondroitin sulphate also increases, although only slightly, in sexual skin when it swells. None of this chondroitin sulphate was found in the exudate and the role of chondroitin sulphate in the sexual-skin swelling was not further investigated.

Results in Tables 5–7 demonstrate a relationship between the hyaluronic acid content of sexual skin and its water content. This appears to be the first time that such a relationship has been obtained for any tissue with a semi-quantitative analytical method. As noted in the section on Materials and Methods the analyses for the mucopolysaccharides were not very satisfactory but were, however, sufficient to demonstrate gradations of hyaluronic acid content existing between unswollen and swollen sexual skin. The reason for this association between total water content of the tissue and its hyaluronic acid content is not understood and is made difficult by the fact that the sexual-skin oedema is both intra- and extra-cellular in composition (Aykroyd & Zuckerman, 1938), and hyaluronic acid is found extracellularly by histochemical techniques (Duran-Reynals *et al.* 1950). It would seem that hyaluronic acid production is but one factor in bringing about oedema in the sexual skin. The following considerations refer only to a possible mechanism whereby hyaluronic acid could play a part in regulating extracellular-fluid volume.

Among the animals examined in this study only the baboon exhibited regular and consistent sexual-skin cycles. In the baboon the cycle was consistently characterized by a moderately slow turgescence followed by a sudden deturgescence. The cycle in the other species, which had to be experimentally induced, was drawn out and irregular. It is only in material from the baboons that analyses show any regular pattern (Table 7). During the turgescent phase the protein content of the exudate increased and the level of hyaluronic acid in the exudate remained low. Immediately after the onset of deturgescence and subsequently, the exudate protein concentration fell and the hyaluronic acid content of the exudate rose to much higher levels than during the turgescent phase. The concentration of protein found in the exudate of the turgescent phase suggests that the permeability to serum protein of the blood capillaries in the sexual skin is increased during the turgescent phase. However, preliminary experiments (Rienits, 1953*b*) did not show any significant variation during the various phases of the sexual-skin cycle of the rate of transfer of ^{131}I-labelled serum protein from blood to exudate.

These observations are consistent with a hypothesis which assumes that the hyaluronic acid which is not present in the exudate, and which gives evidence of being 'bound' or in an insoluble form, exerts a control on the passage of plasma proteins from the vascular to the lymph capillaries. During the turgescent phase the increasing quantities of this 'bound' hyaluronic acid would cause an accumulation of plasma proteins in the extracellular phase by hindering their access to the lymph capillaries. These proteins would attract fluid to satisfy the new osmotic relationship with the blood (in accordance with the Starling concept) and exudate would appear. The removal of hyaluronic acid from its non-exudate location would allow the removal of the plasma proteins in the exudate to the lymph and deturgescence would follow. Deturgescence in the baboon is a rapid process and the high exudate hyaluronic acid concentration during this phase is suggested as being derived from the bound or insoluble hyaluronic acid which has been liberated rapidly into the exudate but not removed from the exudate at such a rapid rate.

The hypothesis does not require any changes in capillary 'permeability' to occur during the sexual-skin cycle. In addition to the observations of Rienits (1953*b*) mentioned above, the experiments of Duran-Reynals *et al.* (1950) suggest that there is no altered permeability of sexual skin during turgescence. These authors caused the collapse of certain regions of swollen sexual skin by slight pressure after infiltration with hyaluronidase. The swellings did not swell again until the next sexual-skin cycle. Hyaluronidase is not known to affect

capillaries more than transiently (Zweifach & Chambers, 1950) and the effect is to increase permeability.

Day (1952) has produced evidence that hyaluronic acid is a prime factor in the regulation of the passage of fluid through membranes made of sheets of subcutaneous fascia. Hyaluronic acid was regarded as the natural material which reduced the pore size in the meshwork of fibres embedded in a ground substance of protein and mucopolysaccharides. Admittedly the results of the experiments of Day (1952) performed *in vitro* are difficult to transpose to conditions *in vivo*, but it seems possible that hyaluronic acid could also regulate the flow of proteins as well as fluid through extracellular regions.

The failure to find any consistent patterns of changes in the 'viscosity concentration ratio' during the sexual-skin cycle may indicate the lack of importance of exudate hyaluronic acid in maintaining extracellular-fluid volume. On the other hand, the work of Johnston (1955) suggests that intrinsic viscosity measurements, which are probably better measurements than those employed here, are not sensitive indicators of changes in molecular size, etc. of hyaluronic acid.

The analyses of the material from the pig-tail and rhesus monkeys revealed no pattern of events such as occurred in the baboons. It is felt that this is due to the irregular responses obtained with these species and also to the slowness with which deturgescence took place on ceasing oestrogen administration, allowing a state approaching equilibrium to be obtained in the exudate. The rapidity of deturgescence in the baboon probably resulted in the constituents of the tissue being far from an equilibrium and hence the differences observed between the concentration of the various constituents during turgescence and deturgescence. An additional factor is that deturgescence of a naturally occurring swelling is not simply due to a lack of oestrogen but that there is an effect of progesterone produced after ovulation, and this substance promotes deturgescence (Gillman, 1940; Gillman & Stein, 1941).

SUMMARY

1. An examination was made of the 'mucoprotein' present in the exudates of oedematous sexual skins of rhesus monkeys (*Macaca mulatta*), pig-tail monkeys (*Macaca nemestrina*) and baboons (*Papio papio*). The mucoprotein was susceptible to the action of both testicular and streptococcal hyaluronidase and, when partially purified, was found to contain considerable amounts of protein together with glucosamine and a hexuronic acid (presumed to be glucuronic acid) in approximately equimolar amounts. The partially purified material contained no detectable sulphate. From these observations and behaviour on paper electrophoresis it was concluded that the mucopolysaccharide in sexual-skin exudates was hyaluronic acid and that chondroitin sulphate was not present.

2. Determinations were made of the hyaluronic acid and chondroitin sulphate concentrations in sexual skin during the swelling and collapsing stages. There were considerable species differences in amounts, but in all three species swelling was accompanied by considerably increased hyaluronic acid concentration and decreased chondroitin sulphate concentration (expressed on a wet-weight basis). It was apparent that the swelling of sexual skin was accompanied by a vast increase in the absolute amount of hyaluronic acid in the tissue and calculations on a dry-weight basis revealed that the total amount of chondroitin sulphate increased slightly during the sexual-skin swelling.

3. Calculation, and extraction experiments, revealed that a considerable proportion of the hyaluronic acid of swollen sexual skin is not contained in the exudate.

4. Significant relationships were found to exist in all species between the total hyaluronic acid content of sexual skins and their water content.

5. Viscosity measurements did not reveal any evidence of any consistent changes in the degree of polymerization of the exudate hyaluronic acid during the sexual-skin cycle or reveal any relationship of degree of polymerization to hyaluronate concentration in the exudate.

6. The relationship of the changes in hyaluronic acid concentration to the oedema formation is discussed.

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The Mechanism of Action of Hydrocortisone on Mitochondrial Metabolism

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Many studies on the metabolic effects of steroids have been reported. The literature has been adequately reviewed by Gordan, Bentinck & Eisenberg (1951), Hayano & Dorfman (1951) and Lieberman & Teich (1953). Steroids have been shown to inhibit the metabolism of hexoses, phosphorylated glycolytic intermediates, tricarboxylic acid substrates and amino acids and the activity of hexokinase, tyrosinase, urease, ascorbic acid oxidase, D-amino acid oxidase, hyaluronidase, lipase and transaminase.

This study was concerned particularly with the influence of steroids on oxidative metabolism. In general, steroids inhibit the activity of enzymes requiring pyridine nucleotides for electron transfer (Guidry, Segaloff & Altschul, 1952; Sourkes & Heneage, 1952; Cochran & Du Bois, 1954). The succinoxidase system, which does not include a pyridine nucleotide, is unaffected by most steroids.

The aims of this study were to investigate the effect of 17-hydroxycorticosterone (hydrocortisone) on mitochondrial oxidation systems and to determine the mechanism of inhibitions observed. A preliminary account has been published (Gallagher, 1958).

METHODS

Enzyme preparations. Rats were killed by a blow on the neck and sheep by severing the cervical vessels. Tissues were removed rapidly and immersed in ice-cold 0·25 M-sucrose. Homogenates were prepared in 0·25 M-sucrose with glass homogenizers and fractionated by differential centrifuging at 0° (Schneider, 1948). Mitochondria were resuspended in 0·25 M-sucrose unless otherwise indicated.

Hexokinase was prepared from yeast by the Cross, Taggart, Covo & Green (1949) modification of the method of Berger, Slein, Colowick & Cori (1946). The fraction precipitated between 25 and 40% (by vol.) of ethanol was dissolved in aq. 1% glucose and stored in a frozen condition. The optimum addition of this solution for oxidative phosphorylation as determined by assay was 0·01 ml./1·5 ml. final volume.

Reagents. Adenosine triphosphate, adenosine 5-monophosphate, cytochrome *c*, diphosphopyridine nucleotide (DPN), nicotinamide, reduced glutathione (GSH), citrate, L-glutamate, succinate, L-malate, octanoate, ethylenediaminetetra-acetic acid, semicarbazide hydrochloride and inorganic reagents were obtained commercially. Succinic acid was recrystallized three times from hot water, then dissolved, neutralized and precipitated as the sodium salt with ethanol. The sodium succinate was then redissolved and crystallized from 80% ethanol. Octanoic acid was purified by distillation *in vacuo*. Cytochrome *c* was