INFLUENCE ON CORNEAL PERMEABILITY OF BATHING WITH SOLUTIONS OF DIFFERING REACTION AND TONICITY*

BY

D. M. MAURICE

*Medical Research Council, Ophthalmological Research Unit, Institute of Ophthalmology,
University of London*

THE commonest method of investigating the permeability of the cornea is to bring a solution of the test substance into contact with its epithelial surface and to measure how much penetrates into the aqueous humour or into the stroma of the cornea itself. Clearly, it is desirable to know what range of properties of the test solution may be tolerated without causing changes in the property that is being measured. A related question arises in the preparation of pharmaceutical agents for instilling into the eye where maximum action should be obtained without causing any damage to the tissues.

One such property, about which disagreement exists, is the tonicity of the solution. Nakamura (1923) carried out experiments which suggested that both hypotonic and hypertonic solutions caused an increase in the permeability of the cornea as a whole to ionic substances. Potts (1953) reached similar conclusions and located the tonicity which gave the minimum epithelial permeability at the equivalent of 1.35 per cent. NaCl, which he inferred was the tonicity of tears. Many investigators, however, have used test solutions made isotonic with blood, that is the equivalent of 0.9 per cent. NaCl. The conclusions drawn by both Nakamura and Potts did not seem in every case to have a satisfactory experimental basis, and it was decided to re-investigate the problem by determining the penetration of ^{24}Na into the eye from solutions of differing tonicity.

The experiments described here show no evidence of an increase in epithelial permeability over the range of tonicity 0.9–10 per cent. NaCl. Hypotonic solutions did, however, cause the rise in permeability described by Nakamura and Potts. It was also found that an increase in the tonicity of the bathing solution in the neighbourhood of 0.9 per cent. NaCl caused an increase in the permeability of the corneal endothelium, that is, of the cellular layer covering the cornea on the side remote from the solution. Whether this increase was a result of damage to the layer or an active process was not determined.

The reaction of the bathing solution is an influence which has remained virtually uninvestigated as far as ionized substances are concerned. For poorly dissociated compounds the changes in penetration have been cor-

*Received for publication April 18, 1955.

related with changes effected by the *pH* on the degree of association of the substance rather than on corneal permeability (Swan and White, 1942; Cogan and Hirsch, 1944). The measurement of the permeability of the corneal epithelium to ^{24}Na when exposed to buffered solutions of differing *pH* forms another object of this investigation. It was found that it remained unchanged within the range *pH* 4–10, but rose outside these limits.

Methods

Procedure.—The experimental technique was similar to that previously described (Maurice, 1951) but differed sufficiently in detail to warrant a fresh description. Adult rabbits, not selected for size, were anaesthetized with intraperitoneal urethane and their lids trimmed of hair. Each cornea was then tested for the absence of gross epithelial damage by instilling two drops of 1 per cent. fluorescein in normal saline and allowing them to remain on the eye for one minute, after which they were gently flushed away with 1 ml. saline. If any staining of the cornea was visible to the naked eye it was rejected. The animal was laid on its side and the skin covering the lids was gripped at four points by sharp-pointed clips. These clips were suspended by rubber bands from a frame standing a few inches above the head so that the lids were lifted up and away from the eye. The test solution, warmed to body temperature, was instilled into the basin formed by the conjunctival sac so as to cover the cornea; its level was maintained from time to time if loss occurred through the naso-lacrimal system.

After $9\frac{3}{4}$ min. the solution in the basin was withdrawn into a syringe, the eyelids freed from the clips and the eye subluxated; its surface was washed with 20 ml. saline at 10 min. from the first instillation. The corneal surface was quickly dried with the end of a rolled filter paper, and a 20-gauge needle mounted on a 1-ml. syringe was pushed through the cornea into the anterior chamber, the aqueous humour mixed by withdrawing a little and returning it, and then the anterior chamber evacuated as completely as possible. The cornea was gripped in a pair of forceps and was cut free with a border of sclera attached using sharp-pointed scissors. It was then freed from lens and iris, blotted on filter-paper and the surrounding ring of sclera trimmed off. The preparation of the cornea from washing to drying regularly took from $\frac{3}{4}$ –1 min.

Directly the experiment on the one eye was completed the animal was turned on its other side and the second eye was treated similarly. The animal's head lay over a 2-in. square hole in the table while the first eye was being operated upon in order to avoid damaging the second cornea.

The radioactivity of the aqueous humour, cornea, and test solution was determined in the manner previously described. An F 10 liquid counter (20th Century Electronics), in which the sample fills an internal helix of capacity 0.3 ml., was used for the assay.

Test Solutions.—The ^{24}Na was received in the form of NaCl or NaHCO_3 and was made up in distilled water to the equivalent of 0.9 per cent. NaCl . To the bicarbonate a few drops of bromothymol blue were added and it was neutralized with HCl . To each 100 ml. of the solution was added 1 ml. of 10 per cent. fluorescein solution. This served to locate splashes and to determine when the corneal epithelium was altered sufficiently by the test solution to stain visibly with fluorescein, the usual clinical test for damage.

To prepare the solutions of differing tonicity the radioactive solution was mixed with an equal volume of one of a series of NaCl solutions, from 19 per cent. to distilled water. The final solution varied in strength, therefore, from 10–0.45 per cent. NaCl ; to every 10 ml. of this was added 0.5 ml. of M/5 phosphate buffer at *pH* 7.4.

In a separate set of experiments, the effect of 1 ml. of the radioactive solution in 9 ml. 0.9 per cent. NaCl was compared with the effect of 1 ml. in 9 ml. isotonic (5.2 per cent.) glucose solution. Also determined separately was the influence of NaCl solutions more

dilute that 0.45 per cent., these being made up by mixing the radioactive solution with the appropriate volume of distilled water.

The solutions of differing reaction were made by mixing the radioactive solution with an equal volume of 0.3 M glycine, citrate, or borate buffer. A few in the neighbourhood of the physiological pH were made up with phosphate buffer in the proportions used for a solution of tonicity 0.9 per cent. NaCl in the previous series. The reactions of the test solutions were measured before instillation with a Cambridge pH-meter and miniature type glass-electrode, an 'Alki' electrode being used for the most alkaline solutions. The solution removed from the conjunctival basin was generally tested also. As a rule, it had moved a few tenths of a pH unit towards neutrality from its original value, and an average of the two readings was taken as being representative of the whole period.

Generally, three or four animals were operated on in a day and with each animal the test solutions used for the two eyes were of different pH or tonicity; any one solution was tested as often on second eyes as on first eyes whenever possible. The test solutions were chosen from, as a rule, three stock solutions of different pH or tonicity which were selected without systematic order and prepared each day.

Results

General.—The manner in which ^{24}Na penetrates the eye when it is allowed to remain in contact with the outer surface of the cornea has been described in detail elsewhere (Maurice, 1951; 1953). After sufficient time has elapsed for it to diffuse across the corneal stroma, its penetration is controlled by the resistance of the two limiting cellular layers of the cornea. The ^{24}Na passes very slowly across the epithelium into the stroma and then about one hundred times more readily across the endothelium into the aqueous humour.

Under comparable conditions, the total quantity of ^{24}Na which enters the cornea and aqueous humour is proportional to the epithelial permeability. For a short experimental period, t , before a considerable fraction has been lost across the endothelium, the concentration of ^{24}Na in the cornea a_c , is determined by:

$$K_{ec} = \frac{a_c}{a_e} \frac{M_c}{A} \frac{1}{t}$$

where a_e is its concentration in the test solution K_{ec} the permeability of the epithelium, and A and M_c the superficial area and mass of the cornea. Since the factor M_c/A varies only slightly from eye to eye, K_{ec} is given by a_c if the values of a_e and t are fixed.

For experimental periods of the order of 10 min., long compared with the time taken for the ion to cross the corneal stroma but sufficiently short for a_a , its concentration in the aqueous humour, to remain low in comparison with a_c :

$$a_a = K_{ac} \frac{A}{M_a r'_{ca}} \int a_c dt$$

where K_{ac} is the endothelial permeability, M_a the mass of the aqueous, and r'_{ca} the value of the ratio a_c/a_a at equilibrium (Maurice, 1951).

From these two equations we have:

$$K_{ac} = \frac{a_a}{a_c} \frac{M_a}{A} r'_{ca} \frac{2}{t}$$

so that under comparable conditions the ratio a_a/a_c is a measure of K_{ac} .

Account must be taken of the exchanges between the aqueous humour and the blood. A small fraction of the ^{24}Na which enters the anterior chamber from the cornea is lost by the drainage of the aqueous humour. At the same time ^{24}Na passes into the general circulation from the test solution across the conjunctiva and enters the anterior chamber in the newly formed aqueous humour. When the two eyes of an animal are being experimented on in turn the second aqueous humour will gain a greater concentration of ^{24}Na from the blood and the comparison of the two values of a_a/a_c will be in error. From the experiments which were carried out with a bathing solution of tonicity 0.9 per cent. NaCl and of non-toxic pH, the ratio a_a/a_c for 29 first eyes was 5.25 per cent. ± 0.32 (S.E.M.) and for 25 second eyes 6.80 per cent. ± 0.54 . This difference agreed well with that which was calculated from the measurement of the gain of ^{24}Na by the plasma in some experiments and its known rate of exchange between blood and aqueous humour. The error from this cause can be reduced to negligible proportions by testing a solution on first and second eyes alternately.

The amount carried to the aqueous humour by the blood seems to be relatively of more importance in those eyes which have a low epithelial permeability. In the same 54 eyes those which had an epithelial permeability below the median value gave an average ratio a_a/a_c of 6.8 per cent. while those above gave 5.2 per cent. Caution needs to be exercised, therefore, in interpreting small rises in the ratio in eyes which have a low epithelial permeability.

Differing pH.—In Fig. 1(a) each point corresponds to a determination of a_c expressed as the number of counts/min./g.tissue. The experimental values are adjusted so as to make the activity of the test solution, a_e , equal to 100 counts/min./ml. It is seen that there is no significant change in epithelial permeability over the range pH 4–10. Beyond these limits it rises, the increase being particularly marked for alkaline solutions; at pH 11 the epithelial resistance is almost entirely destroyed. The values obtained appear to be independent of the buffer used.

In Fig. 1(b) each point corresponds to a determination of the ratio a_a/a_c . This ratio and therefore the endothelial permeability does not seem to show any significant trend over the whole range of pH. It is unlikely, however, that there are any large changes of pH within the cornea, because of the buffering power of the corneal proteins; certainly, changes in the buffering region of collagen, that is outside the range pH 5–9.5, are improbable (Bowes and Kenten, 1948).

Differing Tonicity.—In this series it was found that the weights of those corneae which had been in contact with the extremely hypertonic solutions

FIG. 1.—Influence of reaction on permeability of cornea.
 (a) Permeability of epithelium given by ^{24}Na activity in cornea; activity of bathing solution 100.
 (b) Permeability of endothelium given by ratio of activity in aqueous to that in cornea.
 Each point corresponds to one eye.
 Horizontal line and thick bars on right are median, quartiles, and range of 54 standard eyes.

were markedly low, presumably from the osmotic withdrawal of water (Fig. 2a). The loss of water from the aqueous humour does not appear to be significant under these conditions (Fig. 2b). On account of this variation and of marked trends of the ratio a_a/a_c with tonicity, the value of a_c cannot be used as a faithful indication of the epithelial permeability. Instead, this was measured by the total quantity of ^{24}Na which enters the eye, $a_c M_c + a_a M_a$. In order to make the values (plotted in Fig. 2c) directly comparable with those obtained in Fig. 1(a), they were divided by a representative value, 100 mg., of the conversion factor $M_c + M_a a_a/a_c$.

The epithelial permeability shows the expected increase when in contact with hypotonic solutions. The rise starts somewhere in the range 0.675–0.9 per cent. NaCl and continues to 0.30 per cent. NaCl, but increases no further for 0.18 per cent. NaCl. There is no evident difference between solutions of from 0.9 to 2.7 per cent. NaCl in their effect on the epithelium. The average epithelial permeability is apparently slightly reduced by contact with very hypertonic solutions.

The results of the experiments in which the test solution contained only 0.1 per cent. NaCl and was made isotonic with glucose all gave epithelial permeabilities within the normal range. Compared to the values in the control eyes, made isotonic with NaCl, the values of a_c in five animals were 1.2, 1.05, 2.2, 1.65, 0.79. It is possible that the high values in two cases were caused by incomplete washing away of the more viscous glucose solution since the relative values of a_a are more consistent, 1.0, 1.1, 1.0, 0.73, 1.75. It is evident that the large epithelial permeabilities in the main body of experiments are caused by the hypotonicity of the solution and not by an absence of salt. This conclusion together with that drawn from the results with hypertonic bathing solutions may be expressed in an alternative manner: that the flux of Na across the epithelium is proportional to its concentration, so that the concept of the permeability coefficient, K_{ec} , is valid, at least within the range 0.1–3 per cent. NaCl.

The value of the ratio a_a/a_c plotted against the tonicity of the bathing solution (Fig. 2d) shows a marked change in the region of 0.9 per cent. NaCl. Unfortunately, the low values corresponding to the hypotonic solutions do not unambiguously imply a lowered endothelial permeability. In this range the epithelial permeability is raised and it is possible that the increase takes place gradually throughout the experiment. This would give a low value to a_a/a_c with a normal endothelial permeability. The significance of the low ratios needs to be investigated by extending the measurements over a wider range of experimental times.

The values of the ratio corresponding to hypertonic bathing solutions are markedly raised above the level found for 0.9 per cent. NaCl both in this series and for the 27 eyes mentioned on p. 466 with epithelial permeabilities below the average. Since the epithelial permeabilities found with 1.35–2.7 per cent. NaCl test solutions are not significantly different from

FIG. 2.—Influence of tonicity on permeability of cornea.

(a) Mass of cornea.

(c) Permeability of epithelium.

(b) Mass of aqueous humour.

(d) Permeability of endothelium.

Each point corresponds to one eye.

Horizontal line and thick bars on right are median, quartiles, and range of 54 standard eyes.

those with 0.9 per cent. NaCl solutions, the increase in the ratio in this region must correspond to a true rise in endothelial permeability.

Fluorescein.—The examination of the cornea for staining was made with the naked eye in the direct light of a 100-watt bulb immediately after the eye was washed free of the test solution. This examination could only be cursory as it was necessary to collect the tissues as quickly as possible. The impression was gained, however, that the cornea was stained green only when a_e was more than 1 per cent. a_e , that is where the epithelial permeability was more than five times its average normal value.

In the more acid bathing solutions, the fluorescein stains the cornea readily regardless of the state of the epithelium, presumably because it is present in a non-dissociated form and enters by virtue of its fat solubility.

Discussion

Of the 54 standard epithelial permeabilities measured in these experiments, fifty are included in the range $3-9 \times 10^{-4}$ cm./hr. These values are less than one half of those which were thought to correspond to normal undamaged corneae in the previous investigation (Maurice, 1951). The reason for this difference has not been discovered, since attempts to reproduce the earlier experimental technique exactly have failed to give the higher permeabilities. Consistently lower values have been obtained recently, however, though small systematic variations still seem to occur.

The measurements of the endothelial permeability show a greater regularity. The value of the ratio a_a/a_e after 10 min. in 1951 was 5.6 compared to the average of 5.2 since found for 29 first eyes. The value then calculated for the endothelial permeability, 0.072 cm./hr does not, therefore, need to be revised.

The epithelial permeability to Na is resistant to large changes in pH and is independent of the total Na concentration, over a 30 : 1 range. This is in contrast to the frog skin where Na is known to be actively transported inwards and where the permeability is sensitive to changes of concentration and pH (Ussing, 1949), and it is difficult to believe that any active mechanism would be unaffected by these variations. The greater part of the movement of Na inwards across the epithelium, then, is probably determined by a passive resistance to diffusion: as previously suggested (Maurice, 1953) this may lie in the extracellular spaces of the wide flat cells of its outer layer.

There is a similarity in the behaviour of the limiting layers of the cornea when osmotic differences are produced across them. When the solution bathing the eye is made hypotonic to the stroma the permeability of the epithelium is increased, and when the aqueous humour is made hypotonic to the stroma by bathing the eye with a concentrated solution the permeability of the endothelium is increased. In both cases this increase does not go beyond a certain limit as the tonicity difference is made greater. This

behaviour and the magnitude of the raised permeabilities are very similar to those observed when the fluids bathing the layers are continuously stirred (Maurice, 1951). The tenfold increase in the permeability of the epithelium which results is almost certainly an indication of damage, and it is tempting to apply the same explanation to the endothelium. The mechanism which causes the damage might be an early stage in that which was shown to rupture whichever cellular layer was facing the hypotonic solution when the two sides of an excised cornea were bathed with solutions of differing tonicity (Cogan and Kinsey, 1942; Kinsey and Cogan, 1942). Before the epithelium was ruptured, it was seen to be lifted away from the stroma by an accumulation of fluid, which, it was believed, was formed by the layer acting as a semi-permeable membrane.

There is no firm ground for supposing that the raised permeability of the endothelium is a result of damage, however, nor for drawing any conclusions as to the tonicity of tears or of the most physiological test solution. An alternative explanation, for example, could be that the changes in this permeability are connected with a mechanism for maintaining the normal hydration of the cornea.

Nakamura (1923) and Potts (1953) have previously carried out systematic studies on the influence of tonicity on the permeability of the cornea to ions. They both produced evidence that it increased with solutions hypertonic as well as hypotonic to body fluids. Nakamura used a technique similar to that described here, but he measured the concentration of his test substances, fluorescein and iodide, in the aqueous humour only. He used only one eye at each tonicity so that the significance of many of the changes he observed is uncertain. In most of his experiments he measured the penetration of the test ion from a solution supposedly isotonic with tears (1.35 per cent. NaCl) after the cornea had previously been bathed for a period with a hypo- or hypertonic NaCl solution. The increases he observed in the latter case need not, as he believed, have taken place while the hypertonic solution was in contact with the cornea. The cornea itself would be hypertonic to the body fluids after this treatment, and the epithelial damage could occur during the second part of the experiment when the test solution would be relatively hypotonic to the corneal stroma. When, in experiments similar to those described here, he measured the penetration of fluorescein from hypertonic solutions, he obtained no significant changes in permeability if the hypertonicity was achieved with NaCl. If the solutions were made hypertonic with KI, on the other hand, the permeability to both fluorescein and iodide was increased; it is possible, however, that KI, in high concentrations damages the epithelium.

Potts brought the test solution into contact with the cornea in a glass applicator which was held on to it by suction. After 20 min. the eye was washed and the aqueous humour and a disk of cornea within the area covered by the applicator were collected and assayed for the test ion. Three

labelled ions were used, ^{24}Na , ^{134}Cs , and $^{32}\text{PO}_4$, and a range of tonicities of 0.6–2.25 per cent. NaCl. At neighbouring tonicities, the scatter of the values found for the activity of the test ion in the aqueous humour is much greater than that in the cornea—about 30 : 1 compared to 4:1. This large scatter perhaps results from damage to the epithelium at the lip of the applicator; comparisons between the values of the activity of the cornea, which were obtained from an area not in contact with the lip, should be the more meaningful.

Potts claimed that the permeability of the epithelium to ^{24}Na passed through a minimum at a tonicity the equivalent of 1.35 per cent. NaCl. He made only one measurement at each tonicity, however, and this conclusion is based on the concentration in two corneae with bathing solutions of 1.35 and 1.50 per cent. NaCl being about one half that found at neighbouring tonicities. The permeability to either $^{32}\text{PO}_4$ or ^{134}Cs showed no increase at higher tonicities. The concentration in the cornea of the former, for which there are in general two measurements at each tonicity, shows no significant change from 0.6–1.4 per cent. NaCl but is consistently lower from 1.6–2.25 per cent. NaCl. The amount of ^{134}Cs found in the cornea for the total of three measurements that were made at 0.75 and 0.9 per cent. NaCl were about four times the average of those at higher tonicities.

It will be seen that the results of these workers generally do not show any increase in permeability with hypertonic bathing solutions under the best conditions. Although their results concur with those described here in showing an increase with hypotonic solutions the evidence that the rise starts at a tonicity above 0.9 per cent. NaCl is based on very few experimental values.

The only investigation on the effect of the reaction of the bathing solution on the permeability to an ionized substance seems to be that of Rohbeck (1950) with sulphacetamide. In accordance with the findings with ^{24}Na , no change in its penetration into the aqueous humour was shown in the range pH 5–10. Unfortunately, the number of eyes used at any pH value was not disclosed.

The pharmaceutical application of these studies is that concentrated solutions brought into contact with the eye are unlikely to cause any damage by their osmotic force; in any case, if drops are instilled they are probably soon diluted by tear fluid. If it is desired to build up a large concentration of an ionized substance in the eye, the concentration that should be instilled is the maximum that will not cause discomfort, the equivalent of 1.5–2 per cent. NaCl (Krogh, Lund, and Pedersen-Bjergaard, 1945). Drops of higher concentrations may stimulate a flow of tears and wash the substance off the cornea. Similarly, if there is any benefit to be obtained in altering the reaction of the drops this can be done without harm to the cornea within the range pH 4–10, but discomfort and a flow of tears may result from exceeding the range pH 6.6–9 (Lipschütz, 1929).

Summary

(1) The permeability of the limiting cellular layers of the rabbit cornea to ^{24}Na has been investigated by bringing a solution of it in contact with the eye for 10 min., and analysing the amount that penetrates into the cornea and aqueous humour. Test solutions of NaCl concentrations from 0.18 to 10 per cent. and of pH from 2 to 11 were used.

(2) Solutions hypotonic to 0.9 per cent. NaCl cause a rise in epithelial permeability which reaches a maximum of about ten times normal at about 0.4 per cent. NaCl. Hypertonic solutions do not alter, or tend to decrease, the epithelial permeability.

(3) Increasing the tonicity of the solution bathing the cornea in the neighbourhood of 0.9 per cent. NaCl causes a rise in the permeability of the endothelium.

(4) The permeability of the epithelium to ^{24}Na is the same with a 0.9 per cent. NaCl solution as with an isotonic glucose solution.

(5) Solutions buffered from pH 4–10 do not affect the epithelium but outside this range increase its permeability.

(6) The bearing of these results on the nature of the cellular layers, and their application to the preparation of drugs for instillation in the eye is discussed.

REFERENCES

- BOWES, J. H., and KENTEN, R. H. (1948). *Biochem. J.*, **43**, 358.
 COGAN, D. G., and HIRSCH, E. O. (1944). *Arch. Ophthalm. (Chicago)*, **32**, 276.
 ———, and KINSEY, V. E. (1942). *Ibid.*, **27**, 466.
 KINSEY, V. E., and COGAN, D. G. (1942). *Ibid.*, **28**, 449.
 KROGH, A., LUND, C. G., and PEDERSEN-BJERGAARD, K. (1945). *Acta physiol. scand.*, **10**, 88.
 LIPSCHÜTZ, H. (1929). *Klin. Mbl. Augenheilk.*, **82**, 763.
 MAURICE, D. M. (1951). *J. Physiol. (Lond.)*, **112**, 367.
 ——— (1953). *Ophthalm. Lit. (Lond.)*, **7**, 3.
 NAKAMURA, B. (1923). *Klin. Mbl. Augenheilk.*, **70**, 195.
 POTTS, A. M. (1953). *Amer. J. Ophthalm.*, **36**, No. 6, pt. 2, 127.
 ROHBECK, M. (1950). *Klin. Mbl. Augenheilk.*, **117**, 405.
 SWAN, K. C., and WHITE, N. G. (1942). *Amer. J. Ophthalm.*, **25**, 1043.
 USSING, H. H. (1949). *Acta physiol. scand.*, **17**, 1.