ACIDIFICATION OF RECTAL FLUID IN THE LOCUST *SCHISTOCERCA GREGARIA*

by

JANET DOROTHY ISABELLA SPEIGHT
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Department of Zoology

The University of British Columbia
Vancouver 8, Canada

Date August 18, 1967

ABSTRACT

Acidification of rectal fluid in Schistocerca gregaria is due probably to active secretion of hydrogen ions, although bicarbonate absorption from H_2CO_3 leaving behind hydrogen ions cannot be excluded. This was concluded after the following mechanisms were eliminated: introduction of acid from some anterior region of the gut; bacterial fermentation; slow release of hydrogen ions from fecal material; preferential absorption of the basic form of the phosphate buffer pair; secretion of acid phosphate; release of hydrogen ions from the intima.

Any absorption of phosphate in the rectum must be as the monovalent ion since the cuticular intima was found to limit severely passage of the divalent form, as shown by studies of intimal permeability at various pH values.

The relationship of acidification of rectal contents to total acid-base regulation of the locust is not clear, although there is some evidence that excess acidity is lost via the excreta. Regulation of the hemolymph pH is rather slow, with a halfway return to normal in about one day when the tolerance level has not been exceeded.

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INTRODUCTION

In vertebrates, acid-base regulation is accomplished mostly by adjustment of the bicarbonate-carbonic acid buffer system. The concentration of carbonic acid is fixed by the link between the respiratory and circulatory systems, and vertebrate renal mechanisms regulate bicarbonate concentration. Intracorpuscular hemoglobin is also central to the efficiency of the system.

In contrast, there is very little known about the mechanism of acid-base regulation in insects. They do not show the same physiological association between respiratory and circulatory systems as do mammals. The insects have a true 'open' circulatory system rather than a closed system which is mediated by interstitial fluid. There is only one extracellular fluid which bathes the cells and circulates through the body. This is the hemolymph. Furthermore, the hemolymph of insects is not usually concerned with oxygen transport nor with the transport of carbon dioxide (Florkin and Jeuniaux, 1964).

The tracheal system of the insect spreads among all the internal organs and is in intimate contact with respiring tissues which it supplies with gaseous oxygen. Transport in the liquid phase is minimized. Tracheae end in very slender tubes called tracheoles, and the final transport of oxygen takes place by diffusion through tissue fluids and cytoplasm to the mitochondria (Miller, 1964). Carbon dioxide is about thirty-five times as permeable as oxygen in animal tissues, so that if

tracheal arrangement is adequate for oxygen transport, these same conditions should be more than sufficient for removal of carbon dioxide (Miller, 1964).

There is no cellular element comparable to the erythrocyte in the hemolymph (Jones, 1964) which could serve in an analogous manner to increase the buffering efficiency of a bicarbonate-carbonic acid system. There are no respiratory pigments found in insects, except for the occurrence of hemoglobin in the hemolymph of some chironomids (Florkin and Jeuniaux, 1964). The green color of the hemolymph of the solitary phase of Schistocerca gregaria and Locusta migratoria is due to the presence of a yellow chromoprotein, the prosthetic groups of which are β-carotene and lutein, and of a blue chromoprotein, the prosthetic group of which seems to be mesobiliverdin (Goodwin and Srisukh, 1951). Mesobiliverdin is absent from the gregarious hoppers which have yellow hemolymph.

While there is no analog in the insects of acid-base regulation via a link between the respiratory and circulatory systems, it has been suggested by Phillips (1961) that the rectum of the desert locust, Schistocerca gregaria Forskål is functionally analogous to the distal and collecting tubes of the mammalian kidney. They are both able to absorb NaCl against large gradients and can also absorb water so as to produce a hypertonic urine. In the vertebrates, the exchange of H^+ for Na^+ is central to the regulation of pH; perhaps a similar mechanism is involved in this insect. Before considering

preliminary observations which support this suggestion, a review of the excretory system in insects and particularly the desert locust is in order.

The excretory system of Schistocerca gregaria is primitive and typical anatomically. It includes the Malpighian tubules which lie free in the hemocoel and open into the digestive tract between the midgut and hindgut in the third abdominal segment. There are about 250 tubules in all (Savage, 1956; Phillips, 1965), and they extend forward to the mesothorax and back to the penultimate abdominal segment. They are blind-ended and the walls are one cell layer thick. The Malpighian tubules secrete a fluid into the gut and, while this fluid does not differ osmotically from the hemolymph, the relative concentration of ions is very different (Stobbart and Shaw, 1964). The concentration of K^+ is usually at least six times that of the hemolymph and as much as thirty times in some aquatic forms. The concentration of Na^+ and Cl^- are lower than in the hemolymph, as found in Dixippus morosus (Ramsay, 1955b, 1956) and Schistocerca (Phillips, 1964c). The pH of Malpighian tubule fluid has been shown by Ramsay (1956) in Dixippus to be higher than the hemolymph pH value. Ramsay also found that when he varied the pH of the fluid bathing the tubules, the contents were always slightly alkaline, so it has been concluded that the Malpighian tubules do not function in pH regulation in this insect at least. Basically the Malpighian tubules appear to be secretory; their product is further modified by the epithelium of the hindgut and rectum, especially

the latter. Knowledge of the physiological role of the hindgut is rather limited (Stobbart and Shaw, 1964), but it does not seem normally to be concerned in ionic regulation.

The rectum of the insect functions principally in selective reabsorption of ions secreted by the Malpighian tubules. Wigglesworth (1932) suggested that the rectum reabsorbed water and salts, and this was confirmed by the work of Ramsay (1950, 1953a, 1953b) on mosquito larvae, and of Phillips (1961, 1964a, 1964b, 1964c, 1965) on the desert locust. Their work made untenable the older hypothesis that the Malpighian tubules alone are responsible for ionic regulation. Ramsay (1956) stated: 'There is nothing in the response of the tubules ... which might suggest that they are responsible for the maintenance of the normal composition of the hemolymph; rather it is that the activity of the tubules alone would radically alter the composition of the hemolymph were it not for the participation of the other important organs of the excretory system, the rectal glands'. The locust rectum has been shown by Phillips to transport actively sodium, potassium and chloride from the lumen against electrochemical gradients, independent of solvent flow. It modifies the primary excretory fluid generated by the Malpighian tubules and is responsible for regulating the normal composition of the hemolymph.

The six long rectal pads, or rectal glands, consist of a single layer of very large columnar epithelial cells, while between the pads the epithelium is considerably reduced with

cells cuboidal to squamous in shape (Phillips, 1964a, 1965). The whole lumen of the rectum is lined with a rather thick chitinous cuticle or intima unattached over the pads. On the basis of cytological studies, Phillips (1965) concluded that only the large epithelial cells of the rectal pads are involved to any extent in ion transport across the rectum.

To return to the analogy suggested by Phillips (1961), he found that the contents of the rectum were acid (pH 5-6) even after persistent rinsings with alkaline buffer. Ramsay (1956) had found previously in Dixippus morosus that while the urine is always alkaline to the serum, it becomes acid in the rectum. In Rhodnius the Malpighian tubules have a somewhat different structure than in either Schistocerca or Dixippus, there being two functionally distinct regions, i.e. the distal portion of the tubule is secretory and resorption occurs in the proximal portion (Wigglesworth, 1931b). It was shown that the contents of the secretory portion are weakly alkaline and then become distinctly acid in the lower portion of the tubule. The acidification, according to the theory of Wigglesworth, is important in nitrogen excretion causing precipitation of uric acid as uratic spheres which are then eliminated in the feces.

The pH of Schistocerca hemolymph is about 7.13, and the pH of gut contents from all regions is lower than this: midgut fluid is 0.7 pH units lower, hindgut 1.4 and rectal 2.4 pH units lower than the hemolymph (Phillips, 1961). The acidification does not appear to be due to the introduction of

acidic material from the Malpighian tubules (Ramsay, 1956). Two main possibilities were suggested at this time by Phillips to account for acidification of the rectal fluid: (1) The gradient in pH arises because the rectal wall is impermeable to hydrogen ions, which enter the rectum from the hindgut or arise from bacterial fermentation. (2) The epithelium actively maintains the pH gradient.

The experiments done by Phillips (1961) investigating the acidification of rectal fluid have been repeated here, since they were preliminary in nature and since it was thought that the process might be associated with acid-base regulation. His conclusion was that the epithelium actively maintains the gradient. Three mechanisms were suggested to account for this: (1) The cells of the rectal wall may themselves have a low pH due to the presence of H_2CO_3 derived from metabolic activity. The rectal lumen could be in equilibrium with the low pH of these cells, and the gradient would be instead between the rectal epithelium and the hemolymph. (2) The basic form of a buffer pair is taken up by the rectal cells e.g. $HPO_4^{=}$ rather than $H_2PO_4^{-}$, or HCO_3^{-} rather than H_2CO_3. It is also possible that carbon dioxide could diffuse into the lumen from the epithelium, hydrate and dissociate. Then HCO_3^{-} could be preferentially absorbed to leave H^{+} behind. This last scheme might require the presence of carbonic anhydrase in the rectal lumen since hydration of carbon dioxide is slow when not enzymatically catalyzed. Or, it could be a slow reaction, as

in the system described by Pitts (1963) in the lumen of the proximal tubules of the vertebrate kidney. (3) There is active secretion of hydrogen ions by the rectal epithelium, either as free ions or as an acid which dissociates.

The purposes of this study have been to test the above hypotheses as to the source and mechanism of acidification, and to determine whether the acidification process plays some role in pH regulation of the locust by following the pH of excreta in acid loaded animals.

MATERIALS AND METHODS

Animals

The colony of Schistocerca gregaria Forskål was reared in a controlled environment of 28°C. and 50% relative humidity under continuous light, and fed on a diet of bran, fresh lettuce, grass and tap water. Immature adult male animals that were three to four weeks past the final moult and still a pinkish color were taken from the colony as required. Animals used for a given experiment were taken from the same egg batch reared in a single cage so as to reduce variation due to age, environmental history and other variables. Unstarved animals were used in all experiments in case starvation had any effect on the normal processes of ionic and osmotic regulation. Young adults were used as more body fluids could frequently be obtained from these animals than from older ones.

Removal of gut contents for pH determination

Locusts were anaesthetized with a mixture of ether and carbon dioxide and a longitudinal cut made along the dorsal surface. Hoskins and Harrison (1934) showed that ether has no effect on the pH of gut contents of the honeybee. Gut contents were removed by slitting the gut anteriorly to posteriorly, care being taken to avoid contact with the hemolymph. In most cases the contents were sufficiently moist to measure pH. However, sometimes rectal contents were dry due to water absorption, and in these cases distilled water was added for moisture. Addition of a small amount of distilled water to already moist material did not significantly change the pH.

Preparation of the ligated locust rectum

The method used in preparation of the ligated locust rectum was developed and described by Phillips (1961, 1964a) with the following slight modifications. The animal was placed on its right side in a short length of glass tubing, diameter 1.5 cm., with cotton wool loosely packed at one end and the last five or six abdominal segments protruding from the end of the tube. To anaesthetize the animal, a mixture of carbon dioxide and ethyl ether was blown gently through the cotton wool. The stream was continued for about 15 to 20 min. while the ligation was being performed; this is a longer time than mentioned by Phillips who used 3-5 min. of ether vapour after several minutes of carbon dioxide. Also, in making the incision, it was found convenient to prick first a small hole in the cuticle, and to use this hole as a starting point for the scissors. Originally human hair was used to tie off the gut, but in later experiments cotton thread was used with equal success.

According to Phillips (1961), the blood osmotic pressure after the operation is similar to that reported for normal locusts by Treherne (1959). Animals were invariably active until the end of an experiment, and for several days thereafter. Three to seven days was reported by Phillips (1964a). In general, experiments were run immediately after the operation, and an experiment would take no more than three to four hours. Measurements of Phillips (1961) indicated that the rectal epithelium was able to move water and salt actively for 24 to 48 hr. after ligation.

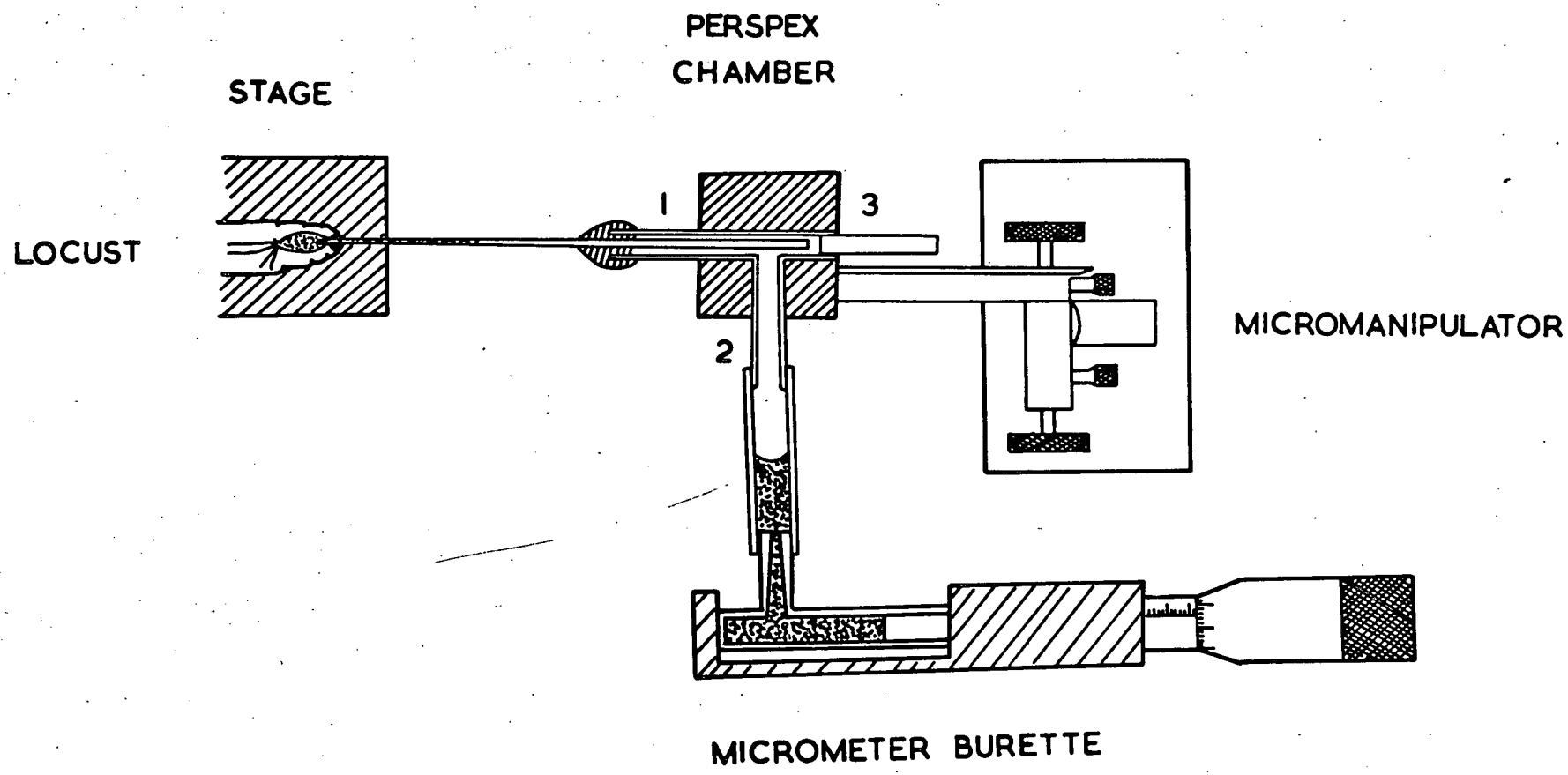
Injection and removal of locust rectal fluid

The ligated rectum was rinsed thoroughly using 1.2M NaCl injected through the anus with a syringe and fine polyethyelene tubing (PE 10), so that all fecal material was removed before injection of experimental buffer. Salt solution was used, although alkaline buffer made up with salt solution was used by Phillips (1961) for rinsing with no differences in subsequent experimental results. After completion of an experiment, the animal was disected and results discarded if any visible material was found in the rectum, or if the ligature had loosened.

The terminal segment of the locust was waxed around all the protruberances to prevent leaking, and the animal placed in a wire cage padded with cotton wool on the stage of a binocular microscope. The abdomen of the animal extended from the cage and was clamped to a piece of plasticene. The injection apparatus (Fig. 1) used was similar to that designed by Phillips (1964a). It consists of an injection needle approximately 2 mm.in diameter with the tip pulled to about 0.5 mm. and the edges well annealed by heating in a Bunsen burner to prevent puncture of the cuticular lining of the rectum, the intima. The needle was sealed in one arm (1) of a small perspex chamber with three outlets and attached to a micromanipulator. The second sidearm of the chamber (2) lead by surgical rubber tubing to a syringe filled with water. The remaining opening (3) was fitted with an O-ring through which

FIGURE 1

Diagram of apparatus arrangement for injection of experimental buffer into the ligated locust rectum. Heavy stippling indicates water; light stippling, experimental solution. Solutions are introduced into the rectum through the anus by means of a microcapillary tube attached to part 1 of the perspex chamber. Fluid is introduced into the rectum by adjusting the micrometer burette with the metal rod (3) in place. Samples of rectal fluid can be taken periodically by removing the rod and inserting a very fine glass capillary through parts 3 and 1 into the injection capillary.


PERSPEX
CHAMBER
STAGE
LOCUST
1
3
2
MICROMANIPULATOR
MICROMETER BURETTE

was pushed a removable metal rod. With the rod in place, the desired amount of experimental solution was drawn up into the injection needle. The needle was inserted by means of the micromanipulator through the anus of the animal into the rectal lumen. To stop leakage, a beeswax-resin mixture was used to seal the needle in place. The experimental fluid was then driven into the rectum using the syringe to force water down the tube and the resulting air pressure to push the other fluid out of the injection needle into the rectum. Mixing was assured by the ventilatory movements of the locust. Samples could be withdrawn at intervals by pulling out the metal rod (3) and inserting a long thin glass capillary into the injection pipette.

In general, 65 µl. of fluid were injected. One sample of 15 to 20 µl. was removed at various time intervals and the remainder constituted the final sample. When only a final sample was required, then 50 µl. were injected initially. Thus volume changes due to sampling were standardized. Values for a given time might consist of a mixture of samples taken in both these ways.

Solutions used in injection experiments

Two buffers were used in the injection experiments. These were McIlvaine's phosphate-citrate buffer and Tris (hydroxymethyl) aminomethane with HCl. Both of these were made up with 1.2M NaCl to minimize water movement in the rectum (Phillips, 1961). Salt was used instead of a saccharide solution to discourage bacterial activity.

To prepare 0.02M McIlvaine's buffer, 0.02M Na_2HPO_4 was titrated with 0.02M citric acid to pH 7.2-7.4. A weaker buffer was prepared by titrating 0.002M Na_2HPO_4 with 0.002M citric acid to pH 7.2-7.4. This value of pH was chosen since pH 7.4 is the pH predicted from the Ussing equation for rectal fluid at equilibrium if hydrogen ions were distributed passively across the rectal wall (Phillips, 1961). An acid buffer pH 3.8-3.95 was also prepared using 0.002M citric acid and 0.002M Na_2HPO_4 . To prepare the Tris-HCl buffer, 0.002M Tris was titrated with 0.1N HCl to pH 7.4. Because the acid was relatively concentrated and the amount added very small, its addition was assumed not to change the molarity of the original NaCl solution significantly.

Detection of leakage from the rectum

Unless otherwise stated, the dye amaranth was included in all experimental buffers injected into the rectum of Schistocerca. Two milligrams were added to 10 ml. of buffer prior to use.

Amaranth is not absorbed from any part of the locust gut (Treherne, 1957, 1958). Phillips (1961, 1964a) found that amaranth behaves like phenol red and many other dyes and is actively secreted by the Malpighian tubules against a 100- to 1000-fold concentration gradient. When injected into the hemolymph, amaranth was almost completely removed from the blood within 1-2 hr. and was found concentrated in the hindgut anterior to the ligature. Hence the leakage of a small amount

of dye could be detected by a post-experimental examination of the hindgut. Leakage did not occur often, and any large punctures could be immediately detected since there was poor recovery of injected fluid during sampling.

Permeability of the intima to inorganic phosphate

(a) Preparation of the intima

The intimal preparation used in studies of permeability to phosphate ions was set up as described by Phillips (1961, 1965) with minor modifications. The locust was sacrificed by cutting off the head, and the animal held on the stage of a binocular microscope with a slab of plasticene. Polyethylene tubing (PE 90) was inserted a short distance (0.2-0.4 cm.) into the rectum through the anus. While held by a micromanipulator, the tubing was waxed securely in place to the last segment. The whole of the final abdominal segment and several millimeters of the cannula were waxed well to prevent leaks. Next, the intersegmental membrane between the penultimate and final abdominal segment was cut, and the gut, still waxed to the cannula, was pulled out slightly using the micromanipulator. It was cut through in the hindgut region several millimeters anterior to the rectum. A concentrated solution of amaranth dye (0.05M) was flushed through the rectum via the polyethylene tubing to rinse out any solid material in the rectum. A ligature of cotton thread was then tied firmly at the anterior end of the rectum. The preparation was filled with the amaranth solution so that leaks could be detected.

To loosen the rectal pads and other external tissues from the chitinous intima, the preparation was soaked overnight in distilled water. Any leaks were detected by the appearance of the red amaranth dye in the surrounding water. After soaking, the external tissue could be pulled away from the cuticle in strips with fine forceps.

A small hole was cut in the PE 90 tubing about 1 cm. above the waxed joint with the intima. A length of about 5-10 cm. of smaller polyethylene tubing (PE 10) was inserted through this hole down into the lumen of the sac so that the end was visible within but not rubbing against the side. The joint of the two tubes was sealed with wax. The smaller tube was used for running the perfusate into the sac, and the larger for collection of the solution which was forced out. The chitinous intima was left then as a cannulated sac whose permeability to phosphate could be studied in the absence of the cellular material.

(b) Perfusion of the intimal sac

Non-radioactive solutions were contained in glass reservoirs held by clamps to a horizontal bar. Tubing from the reservoir led by a needle into the PE 10 tubing. Hydrosatatic pressure (range 35-40 cm. H_2O) was used to perfuse the solution. The rate of perfusion could thus be controlled roughly by adjusting the height of the reservoir.

Radioactive solution of identical chemical composition to the non-radioactive solution was placed in small plastic

vials, and each rectal sac was put in one of these. Radioactive molecules diffusing into the sac were washed out in the perfusate which was collected for determination of radioactivity. The plastic vials were kept in a constant temperature bath at 28°C. A total of 14 preparations was run, and five successive replicate collections were made for each prepration for each of the four solutions run.

(c) Solutions used in study of intimal permeability to phosphate

The premeability of the cuticular intima to the various ionic species of inorganic phosphate was studied by observing the diffusion of labelled phosphate at three different pH values. The dissociation of phosphoric acid occurs as follows (Giese, 1962):

$$H_3PO_4 \underset{pKa\ 2.12}{\rightleftharpoons} H^+ + H_2PO_4^- \underset{7.20}{\rightleftharpoons} H^+ + HPO_4^{=} \underset{12.66}{\rightleftharpoons} H^+ + PO_4^{\equiv}$$

Using the Henderson-Hasselbalch equation, it was calculated that at pH 4 the phosphate is found chiefly as the monovalent ion, and at pH 8 primarily as the divalent form. A value of pH 6 was chosed as an intermediate value and since it approximates the normal rectal pH value. Any molecule must penetrate the intima before contacting the rectal epithelium, and so for absorption to occur it is essential that the molecule be able to pass this first membrane relatively unrestricted. If the intima were found impermeable to phosphate at pH 6 as some preliminary observations suggested, involvement of phosphate in acidification could then be eliminated at the outset.

The buffer solutions used consisted of 20mM PO_4 made up with the sodium and potassium salts, and 5mM/l. each of NaCl, KCl and $MgCl_2$, adjusted to pH values of 4, 6, and 8. Labelled phosphate as $H_3P^{32}O_4$ was added to the external solution, in such small amount as to have no effect on the buffer concentration of phosphate or the pH. Enough P^{32} was added to give a count of about 10^5 cpm/ml. in the outside solution. To test whether presence of Ca^{++} had a significant effect on the phosphate permeability of the membrane, a control experiment was run at pH 6 in which the solution contained 5mM/l. $CaCl_2$ in addition to the other ions mentioned above. Calcium could not be added at extreme pH values because of precipitation of calcium phosphate. Solutions were then all 35mM except for the calcium solution which had a total solute concentration of 40mM. The wide spectrum antibiotic tetracycline (5 mg./l.) was included to prevent bacterial incorporation of phosphate.

(d) Collection and counting of radioactive samples

To collect the radioactive perfusate, a planchet was left under the open end of the PE 90 tubing for a known length of time, 15 min. for the first three preparations and thereafter for 30 min. After a change of perfusing solution, the new solution was allowed to run ½ hr. before making the first collection so as to rinse out any vestiges of the previous solution. Samples were dried before counting. If the volume collected was insufficient to wet the surface of the planchet evenly, a few drops of 'Tween 80', a detergent,

were used. To measure the activity of the external solutions for later calculations, 10 µl. samples of each were counted. Both initial and final activity of the external solutions were checked each day. Significant changes never occurred in activity of the external solutions over the course of a day.

All counting was done on a 'Phillips' Electronic Counter PW4035 and Geiger-Mueller detector. The counting of samples from a single preparation was done on the same day to reduce variations due to the efficiency of the instrument and amount of radioactive decay. The total number of counts per minute was about four times background count for pH 4 where the greatest amount of diffusion occurred. Background counts were run frequently between counts of unknowns, and the day's average was subtracted from individual samples. The number of counts per minute was determined from a total of 1000 counts. The range for the five replicates was about 10% of the mean.

Since the molarity of the radioactive solution was known, the amount of phosphate in 10 ul. could be calculated and related to the count obtained. From these standards, the amount of phosphate which had diffused into the sac could be calculated. The calculation was done for each sample collected and the five values for a given preparation at each of the three pH values then averaged.

(e) Inorganic phosphate precipitation

Antibiotic was included in the experimental solutions to prevent bacterial incorporation of the inorganic phosphate. MacKay and Butler's modification of Mathison's method (Peters and Van Slyke, 1932) was used as a check for this. The technique results in the precipitation of inorganic phosphate. If the labelled phosphate solution is treated according to this method, all labelled material should be contained in the precipitate if all phosphate has in fact remained as inorganic phosphate. However if a significant count is obtained from the supernatant, then some phosphate must be present in dissolved organic form or incorporated into bacteria.

The solutions were made up as given by Peters and Van Slyke (see Appendix A). The quantities for the assay were scaled down but the relative proportions were the same. One milliliter of the external solution was pipetted into a small centrifuge tube. Then 0.2 ml. of the magnesium citrate mixture of Fiske was added, plus 0.4 ml. of strong ammonia water. The tube was shaken for a few minutes, corked and left in the refrigerator overnight. Ten microliters of the supernatant obtained after centrifugation was placed on a planchet for counting. This assay was done for each radioactive solution at the start and at the finish of each day's experimental runs. It was found that P^{32} remained 100% in the inorganic form, as the activity of the supernatant never differed significantly from background counts.

Collection of body fluids of Schistocerca gregaria

(a) Hemolymph

Samples of hemolymph were easily obtained by cutting off one of the large jumping legs of the locust. Amounts of 50-60 µl. were collected in a capillary tube as the ventilatory movements of the animal forced out drops of fluid. Samples were used for measurements of pH and inorganic phosphate. In cases when determinations were not made immediately the hemolymph was stored under paraffin oil.

(b) Malpighian tubule fluid

Malpighian tubule fluid could be collected by inserting a fine glass cannula with the aid of a micromanipulator, through the anus up to the region of entry of the tubules into the gut. Amounts of fluid from 3-5 µl. could sometimes be collected in this way, though difficulty was often encountered due to material in the gut. Phillips (1961, 1964c) had used the method for collection from starved animals. The fluid collected in the hindgut and posterior midgut was assumed to be derived almost totally from the Malpighian tubules as it was clear and watery with numerous small red crystals in some cases. It could be easily differentiated from the dark brown fluid found in the midgut and foregut, which resembles molasses in consistency.

Consequently an alternate method was more frequently used, taken from Ramsay (1955a) who used the method for collection of urine in Dixippus morosus. The locust was anaesthetized with ether and carbon dioxide and the abdomen opened along the mid-ventral line at about the level of entry

of the Malpighian tubules into the gut. A ligature of cotton thread was tied around the intestine just posterior to the junction of the tubules, taking care not to tie off the tubules as well. Anterior to this, a cut was made into the wall of the midgut, and any material in the area was removed using fine forceps. Frequently, fluid could be squeezed from this material and recovered with a capillary tube. After removal of gut contents, a fine glass cannula was inserted, and either tied into place or merely held in position. Fluid would accumulate in the length of gut between the ligatures, and this fluid had been presumably elaborated by the Malpighian tubules. Samples usually had to be pooled using this method: 2.5-4 µl. were used in the phosphate analysis. The rate of collection was not as good as that given by Ramsay of 4.0-6.5 mm^3/hr. When necessary, samples were stored under paraffin oil.

(c) Rectal fluid

Rectal fluid was collected by squeezing gently the terminal abdominal segment of the locust and collecting any resulting fluid in a capillary tube. In some animals a drop of fluid could be obtained, while in others all fluid had already been absorbed from the rectum (Phillips, 1964c). Cannuli were not used since the animals were unstarved and the pipette became clogged with solid material. Samples usually had to be pooled, as volumes used in the phosphate determination ranged from 2-4 µl. The fluid was stored under paraffin oil unless measurements were made on it immediately.

Measurement of inorganic phosphate by colorimetric method

(a) Body fluids

The method used to measure inorganic phosphate concentrations in the body fluids of Schistocerca gregaria was that of Gomori (1942) with minor changes used to deal with the small sample volumes. The solutions are listed in Appendix B.

Hemolymph was allowed to clot for 20 to 30 min., and the serum protein was then precipitated with 1 ml. 5% trichloroacetic acid (TCA) for 1 hr. in a polyethylene micro-centrifuge tube. Hemolymph sample sizes ranged from 25 to 60 µl. and sometimes samples were pooled. If untreated with 5% TCA, the sample formed a precipitate when the molybdate-sulfuric reagent was added, and a filtered sample had no blue color although the precipitate was blue. Fiske and Subbarow (1925) state that precipitate formation means that protein is present. They found that 1 mg. of protein will precipitate 0.01 mg. phosphorus and diminish the color of the supernatant by an equivalent amount. The TCA precipitate was centrifuged down for 15 min. at the maximum speed in an Electrifuge Model 50 centrifuge. Five percent TCA was also added to samples of rectal fluid and Malpighian tubule fluid although no precipitate resulted and centrifugation could be omitted.

The amount of reagents was modified from Gomori to deal with the small quantities of the unknowns but the proportions are essentially the same. The procedure was, in order of addition, as follows:

(1) Blank: 0.5 ml. molybdate-sulfuric reagent
1 ml. 5% TCA
0.2 ml. 'elon'
water to 3 ml.

(2) Standard: 0.5 ml. molybdate-sulfuric reagent
0.2 (0.4, 0.6) ml. standard 0.5mM KH_2PO_4
1 ml. 5% TCA
water to 3 ml.

(3) Unknown: 0.5 ml. molybdate-sulfuric reagent
supernatant from TCA precipitation
0.2 ml. 'elon'
water to 3 ml.

The samples were incubated at room temperature for 45 to 90 min. and read at 675 mμ on a Bausch and Lomb Spectronic 20. A sample was sometimes checked by the addition of a known amount of standard if the concentration was at the lower limit of the curve, or by using a larger sample size whenever possible. A calibration curve was prepared for each group of unknowns. Sample size was adjusted so that percent transmission was not greater than 92% and not less than 32% on the calibration curve. The experimental error is estimated at $\pm$ 1mM/1., taken from hemolymph determinations since error due to transfer and centrifugation was at a maximum here.

(b) Phosphate secretion

The same method of Gomori (1942) was used in experiments investigating possible phosphate secretion by the rectum. In these, amaranth dye was omitted from the injected buffer so as

to avoid complications with the colorimetric determination of phosphate. The absence of any protein precipitate with the addition of 5% TCA was used as an alternate method of detecting leaks into the lumen, as the presence of any hemolymph would have caused precipitation of serum protein. The experimental error was of the order of ± 0.3 mM/l.

pH measurements

All pH measurements were made using a Radiometer pH Meter 27. For routine adjustment of buffer solution, Radiometer Type G202B glass electrode with reference electrode K401 was used. The 'B' type of electrode is recommended by Radiometer for measurements on poorly buffered neutral solutions of strong electrolytes, and hence was suited for the weak buffers made up with 1.2M NaCl. Buffer solutions used in standardizing these electrodes were accurate to ± 0.01 pH unit.

Two types of glass electrode were used in measuring the pH of micro-quantities:

(1) The Radiometer Type G252C with reference electrode K150 requires at least 2 µl. for one determination. In practice, 5 µl. was used as the larger sample gave better accuracy (± 0.1 pH unit). The 'C' type of electrode is designed with a special view to mechanical strength. It was used for pH measurements of injected buffer, hemolymph, gut contents, and fecal material. Salt errors do not occur with this electrode until pH 9, and this is out of the range encountered experimentally. Whenever sample size permitted, a number of

determinations (4-5) were made using electrode G252C. The first sample of unknown was considered as a rinse and readings discarded. Before the next group of determinations were made on another unknown, a fresh drop of KCl was released from the calomel electrode to get rid of any material which might have diffused into the KCl. This was not done between replicates. All measurements were carried out at room temperature.

It was found necessary to calibrate the microelectrode regularly because of variations due to deviations of microelectrodes from the ideal and the relative weakness of the experimental buffer as compared to buffers used to standardize the electrode. (This exhibits itself by a drift of the meter needle in the case of the weak buffer with a more rapid stabilization for stronger buffer.) As an electrode aged, it also sometimes required calibration. To do this, 0.002M buffer identical to that used in experiments was adjusted to various pH values e.g. 5.5, 6.0, 6.5, 7.0, as measured by Type G202B glass electrode. These same solutions were then measured using the microelectrode G252C, and a calibration curve drawn. Mean values for unknowns could then be read off the curve to obtain a corrected value. Calibrations were done only in those cases where an absolute value was desired, and not for those where only relative values were required. Values for hemolymph, gut contents, and fecal material were not corrected since they have a high enough buffer capacity that excessive drift of the meter does not occur. The time allowed for the needle of the meter

to equilibrate was standardized at 2 min. The buffer standards were accurate to ± 0.01 pH units.

(2) The Radiometer Type G297 electrode with reference K497 calomel electrode required a minimum volume of 20 µl. In the measurements done, 50 µl. was used. The electrode is mounted in a water jacket of glass from which only the plastic tips of the fine capillary tubing protrude at either end. Loss of carbon dioxide is kept at a minimum by this design, and the electrode will henceforth be considered 'anaerobic'. The microelectrode unit is intended primarily for measuring the actual pH of mammalian blood in small quantities at a specific temperature. There is no reason to suppose the system is less accurate for measuring the pH of locust hemolymph, e.g. there are not large amounts of carbonic anhydrase in the hemolymph which would cause greater loss of carbon dioxide. Heated water from the circulation thermostat (VTS13) flows through the jacket keeping the electrode at an even temperature (25°C). The electrodes were standardized with precision buffer solution, Radiometer Types S1500 and S1510, accurate to ± 0.005 pH units. Any gas exchange with the environment prior to measurement was kept at a minimum by storing samples under paraffin oil before drawing them into the capillary of the electrode. The system was used for measuring pH values of locust hemolymph and experimental buffer, to check effects due to loss of carbon dioxide. Equilibrium time was set at 2 min.

RESULTS

pH of different regions of the gut

(a) Normal values

To determine if the rectal acidity is due to introduction of acid from any anterior region of the gut, the pH of its contents was measured in the different regions (Fig. 2). Ten animals were used and values for each region were averaged. The results are given in Table I.

The midgut fluid was found to be acid with a pH of 5.46± 0.39, and this is significantly lower than all posterior values. In the region of entry of the Malpighian tubules between midgut and hindgut, the contents became alkaline with values of 7.59±0.72, 7.94±0.49, and 7.13±0.47 anterior to posterior. The final value of 7.13±0.47 occurred in the ileum and so this part of the gut, while it does not modify the pH to the acid level as found in the rectum (5.91±0.44), does lower it somewhat. The value 7.13 differs significantly from 7.94 but not from 7.59. Material was seldom found in the colon, which appears to act as a sphincter, although the value of 6.17±0.14 for the posterior ileum may indicate some forward movement of acid. The anterior and posterior rectal contents were found to have a pH of 6.19± 0.45 and 5.91±0.44 respectively, which is significantly more acid than all anterior values except those for posterior ileum and midgut. Two values are presented since frequently the fecal pellet could be divided and two readings taken. The difference, while not significant at the 0.05 level of probability, may be a

FIGURE 2

Regions of the gut of Schistocerca gregaria: a, pharynx; b, oesophagus; c, crop; d, caeca; e, ventriculus; f, Malpighian tubule; g, proctodaeal valve; h, ileum; i, colon; j, rectal sac (after Phillips, 1964a).

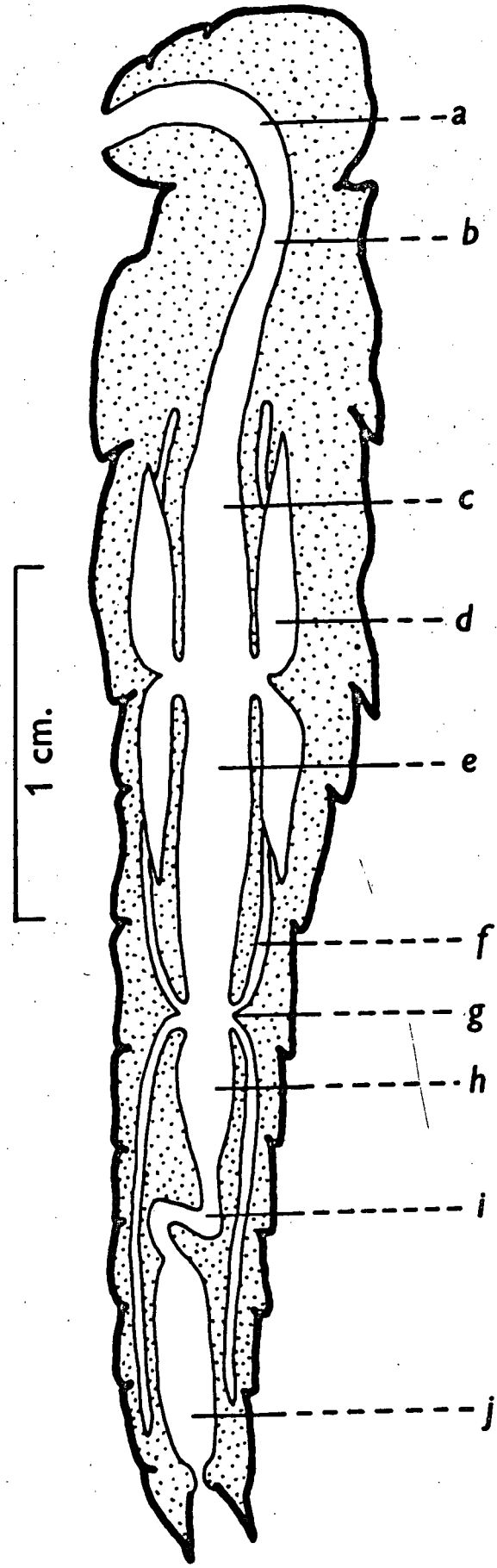

a
b
c
d
e
f
g
h
i
j
1 cm.

TABLE I pH of gut contents in normal and tetracycline-fed *Schistocerca gregaria*.

	Region of gut	pH	
		Normal Mean ± S.D.	Tetracycline fed Mean ± S.D.
Midgut	Mid ventriculus	5.46 ± 0.39	6.12 ± 0.43
Midgut	Posterior ventriculus	7.59 ± 0.72	7.26 ± 0.75
	Proctodaeal valve	7.94 ± 0.49	8.07 ± 0.54
Hindgut	Anterior ileum	7.13 ± 0.47	7.48 ± 0.58
Hindgut	Posterior ileum	6.17 ± 0.14	--
Hindgut	Colon	--	--
Hindgut	Anterior rectum	6.19 ± 0.45	6.32 ± 0.47
Hindgut	Posterior rectum	5.91 ± 0.44	5.80 ± 0.60

reflection of the fact that the more posteriorly occurring material has been in the rectum for a longer period, and presumably has reached a terminal value. The pH of excreted fecal pellets also had a mean of 5.95±0.55. This agrees with the value for the most posterior material found in the rectum. Some of the variation in values for a given region was due probably to the handling of the animal which often caused the locust to defecate and probably shifted gut contents posteriorly generally.

(b) Values for animals fed antibiotic

So that the role of bacterial fermentation in acidification could be evaluated, the gut flora was killed off in ten animals. They were fed the normal bran and water except that a large dose of the wide spectrum antibiotic tetracycline was included in the diet for four days.

Values for tetracycline treated animals are included in Table I. There was no significant difference between the pH of gut contents of control animals and those treated with tetracycline ($p=0.05$). It can thus be concluded that acidification occurs in the rectum, and is not due either to the introduction of acid from any anterior region or to bacterial fermentation.

Demonstration of a state of dynamic equilibrium

(a) Permeability of the rectal epithelium to hydrogen ions

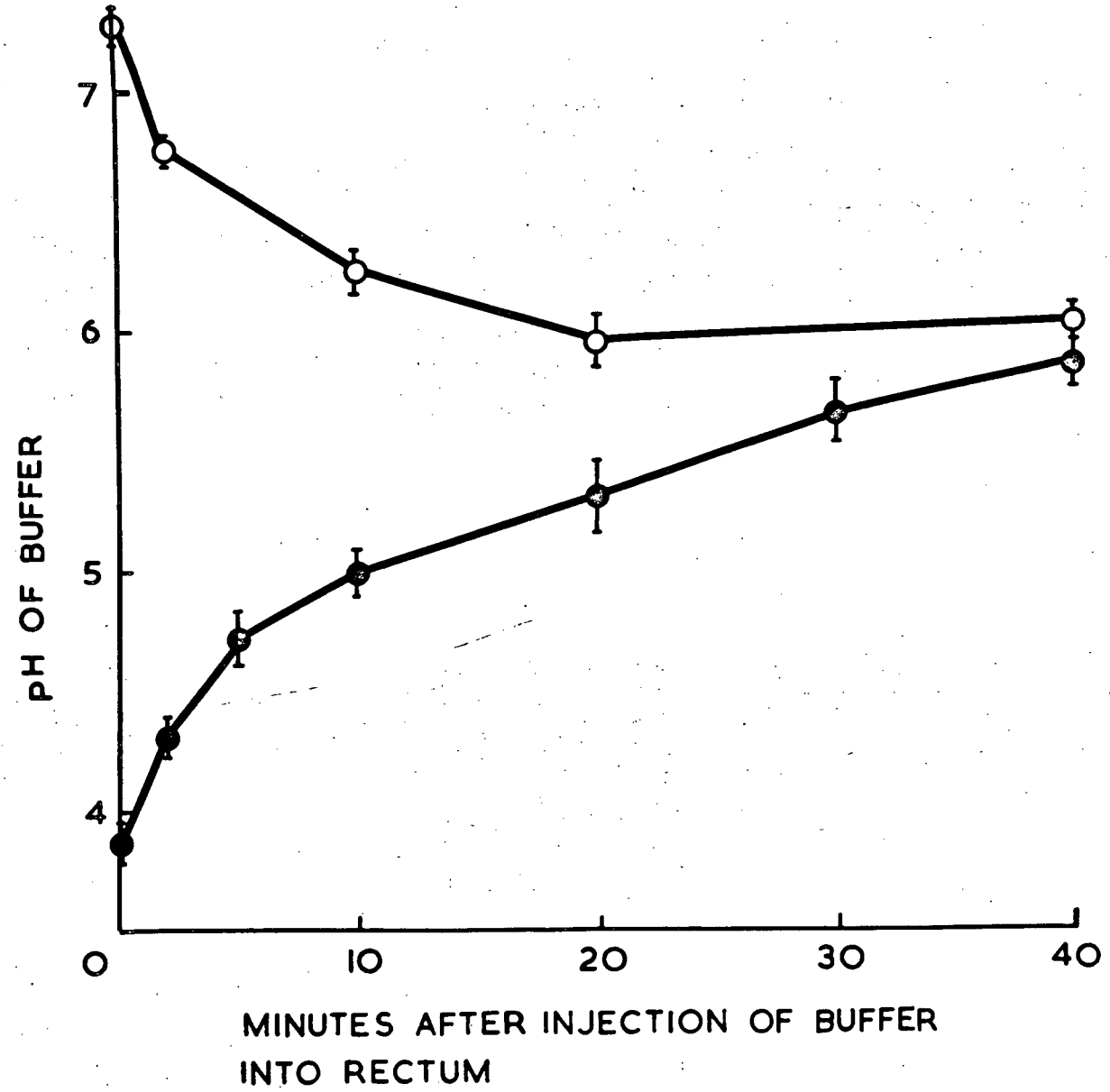
Experiments were done to demonstrate that the pH gradient between the rectal lumen and hemolymph represents a dynamic

equilibrium maintained by the rectal wall. The experiments were designed to show that the same acid pH is attained whether a more alkaline or a more acid buffer is introduced into the ligated rectum. If so, this would suggest that the epithelium is permeable to hydrogen ions, and would require rejection of the hypothesis that the acidity arises anteriorly and that the pH of the lumen contents does not equilibrate with the hemolymph because the rectal wall is impermeable to hydrogen ions.

The buffers used in these experiments were 0.002M McIlvaine's buffer at pH values 7.2-7.4 and 3.8-3.95. Ten animals with the recta ligated and rinsed out were injected with alkaline buffer and twelve with the acid buffer. Results are presented graphically in Fig. 3. When alkaline buffer was injected, the pH fell 0.5 units within 1 to 2 min. and a steady pH was reached between pH 5.95 and pH 6.02 within 20 min. When acidic buffer was injected into the locust rectum, there was an increase in pH most rapidly in the first 10 min. and then more slowly until a steady value was approached similar to that observed following injection of alkaline buffer solution. Clearly the system approaches the same acid pH whether an acid or alkaline buffer is injected. There was no significant difference ($p=0.05$) between the level of acidity reached at 40 min. after injection of alkaline buffer as compared to that for acid buffer injection at the same time. Time taken to reach an equilibrium value is a reflection of the buffering capacity of the experimental solution. The pH values of rectal fluid

FIGURE 3

The change in pH of rectal fluid with time after injecting 0.002M McIlvaine's buffer with original pH values 7.2-7.4 and 3.8-3.95. Horizontal bars, mean values; vertical bars, standard error of the mean.


7
6
5
4
pH OF BUFFER
0
10
20
30
40
MINUTES AFTER INJECTION OF BUFFER
INTO RECTUM

reached at equilibrium in these experiments are not significantly different from normal rectal values (Table I).

(b) Role of bacterial fermentation

To demonstrate that acidification of fluid injected into ligated recta was due solely to the activity of the rectal wall and not to bacterial fermentation, the following experiment was performed in which an antibiotic was included in the buffer. Ten animals were injected with 0.002M McIlvaine's buffer pH 7.2-7.4 to which 4 mg./l. of tetracycline had been added. Acidification occurred in the presence of the antibiotic (Fig. 4) indicating that bacterial fermentation is not involved in the phenomenon. The pH reached at 40 min. was 6.06 ± 0.28 as compared to pH 6.02 ± 0.19 for the same system without tetracycline. The difference was tested and found not to be significant at the 0.05 level of probability. It should also be noted that the rectal contents had been rinsed out, thus drastically reducing the indigenous bacterial population.

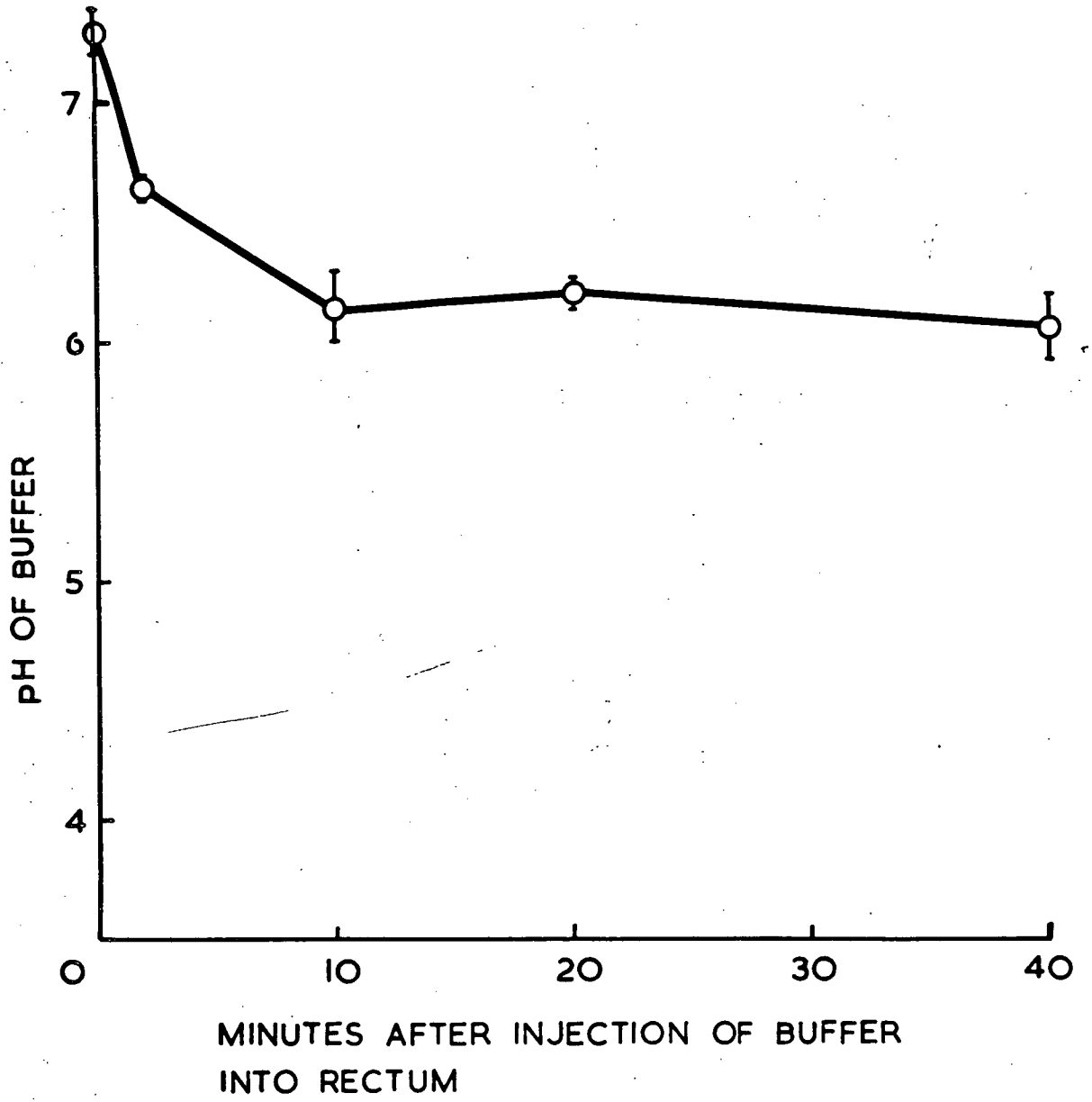
It is concluded from the results of Fig. 3 and Fig. 4 that the rectal wall is permeable to hydrogen ions and that the pH gradient across the rectal wall represents a dynamic equilibrium maintained by the epithelial cells.

(c) Possible contribution of fecal material

Although care was taken always to rinse the rectum thoroughly before injection of buffer, and the rectum was checked by disection after the experiment, small amounts of fecal material could possibly be contributing to the acidity since

FIGURE 4

The change in pH of rectal fluid with time after injecting 0.002M McIlvaine's buffer with original pH value 7.2-7.4 containing 4 mg./l. tetracycline. Horizontal bars, mean values: vertical bars, standard error of the mean.


7
6
5
4
pH OF BUFFER
0
10
20
30
40
MINUTES AFTER INJECTION OF BUFFER
INTO RECTUM

they have been shown to be acid themselves (pH 5.91). An experiment was therefore undertaken to check whether there is significant or sufficient slow release of hydrogen ions by fecal material to account for the acidification. Small pieces of fecal material just obtained from a locust were placed in 50 µl. of 0.002M alkaline buffer. The suspension was placed in a capillary tube to reduce loss by evaporation, and the pH was measured against time to see if in fact there was a slow release of hydrogen ions occurring.

The results are shown in Fig. 5. By inspection, there is no significant lowering of the pH after the initial change caused by addition of fecal material so that no slow release of hydrogen ions took place within a three hour period. This time is greater than the period of experimentation; also it is most unlikely that visible fragments such as were added here would have been consistently undetected during the injection experiments because of the precautions already mentioned.

It is concluded that there is not a slow release of hydrogen ions by fecal material which would account for the observed level of acidification in the ligated rectum. This is a further confirmation of the fact that the acidity is produced by the rectal wall itself.

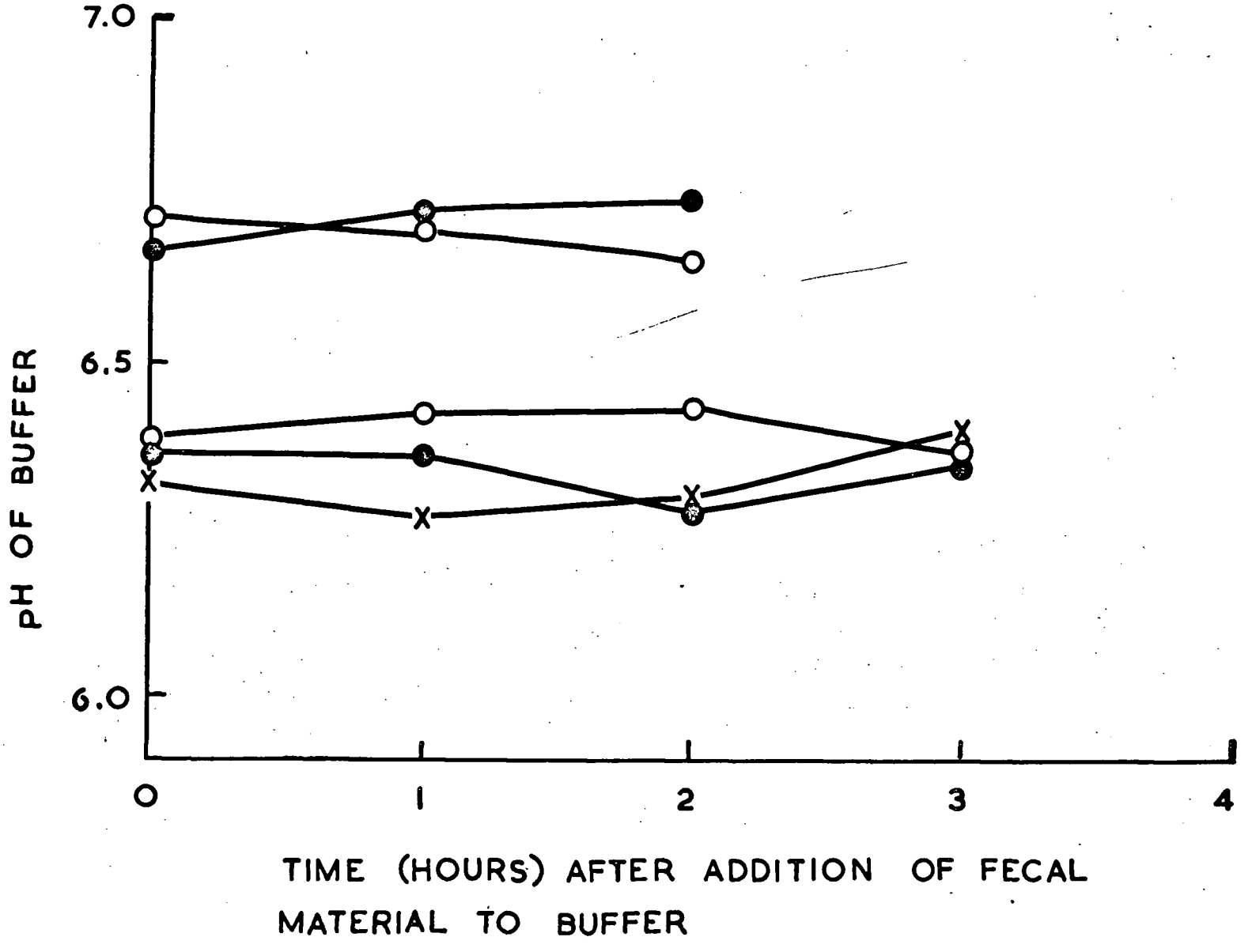
Role of phosphate in acidification

(a) Permeability of the intima to phosphate

A possible mechanism of acidification was visualized as the absorption of the basic form of a buffer pair, e.g. $HPO_4^{=}$

FIGURE 5

The pH of 50 µl. aliquots of alkaline 0.002M buffer with added fecal material against time since addition. Points are the mean of at least two values. Standard deviation, ±0.04 pH units.


7.0
6.5
6.0
pH OF BUFFER
0
1
2
3
4
TIME (HOURS) AFTER ADDITION OF FECAL MATERIAL TO BUFFER

rather than $H_2PO_4^-$, or HCO_3^- rather than H_2CO_3, these being the two most prevalent buffer systems found in biological material (Phillips, 1961). The concentration of phosphate is high in the rectal lumen (section c). It was decided to investigate the role of phosphate in the acidification process largely because some preliminary results had suggested that the intima was impermeable to inorganic phosphate. If this could be confirmed, then any preferential absorption of the basic form of the phosphate buffer pair could be eliminated, i.e. the phosphate must be able to penetrate the intima lining the rectal lumen if absorption is to occur.

The permeability of the intima to phosphate was determined at three different pH values; one at the normal rectal pH of 6 and the other two values chosen so that the phosphate existed either largely as monovalent ion (pH 4) or divalent ion (pH 8). A summary of results is given in Table II. While the intima is almost impermeable to phosphate at pH 8, this is not true at the other pH values tested. The intima is about thirteen times more permeable at pH 4 than at pH 8, and about twice as permeable as at pH 6. Differences between all possible pairs are significant at the 0.05 level of probability.

Using the Henderson-Hasselbalch equation and the following pK_a values for 25 C. (Giese, 1962):

$$H_3PO_4 \underset{pK_a = 2.12}{\rightleftharpoons} H^+ + H_2PO_4^- \underset{7.20}{\rightleftharpoons} H^+ + HPO_4^- \underset{12.66}{\rightleftharpoons} H^+ + PO_4^{\equiv}$$

it was calculated that at pH 4, phosphate exists as 76

TABLE II Permeability of the intima to phosphate at three pH values.

	$\mu MPO_4/hr/\Delta M$			
Preparation	pH 8	pH 6	pH 6 with Ca^{++} added	pH 4
1	0.225	1.73	2.07	3.77
2	0.499	2.08	2.39	6.07
3	-	2.88	4.29	7.68
4	0.413	1.36	2.69	5.18
5	0.600	3.08	2.71	5.94
6	0.275	1.92	2.36	4.85
7	0.238	2.18	3.77	5.22
8	0.306	1.89	2.74	6.83
9	0.462	3.90	4.77	9.20
10	0.524	2.88	3.39	7.19
11	0.913	4.82	6.06	7.95
12	0.403	1.56	2.30	4.20
13	0.568	4.38	6.08	8.69
14	0.625	3.02	2.55	4.89
Mean	0.465	2.69	3.44	6.26
S.D.	0.093	1.08	1.37	1.70
S.E.	0.026	0.287	0.365	0.453

$H_2PO_4^-$: 1 H_3PO_4 and at pH 8 as 6.3 $HPO_4^=$: 1 $H_2PO_4^-$. At pH 6, $H_2PO_4^-$ predominates over the divalent form in a ratio of about 16 : 1. Thus the change in phosphate permeability with pH may be due to the ionic form in which the phosphate exists at that pH. The difference in size between monobasic orthophosphate and dibasic orthophosphate is a factor of 25% (Brindley, 1962). The intima appears to act as a molecular sieve limiting the passage of the larger $HPO_4^=$ ion, as described by Phillips (1965) for other molecules. Absorption from the rectum requires that molecules first pass through the cuticular intima before contacting the large epithelial cells where mechanisms of active reabsorption and acidification probably reside. Hence the intima probably limits absorption of dibasic orthophosphate in favor of the monobasic form. Any preferential absorption of the basic form of the phosphate buffer pair would be severely limited by the low permeability of the intima to the divalent form.

The presence of calcium ion in the medium does influence the permeability of the intima to phosphate although the effect is small. This may be a charge effect, just as it is possible that the changes in pH inflicted experimentally on the intima are effecting the charge on the membrane, and hence its permeability to ions. An experiment to test this has been carried out by Chabun (1967). He studied the movement of chloride ions at various pH values, and showed that penetration was at the same rate at all pH values. It can therefore be concluded

that variations in pH do not effect the charge on the membrane, and therefore the variation in penetration of the various phosphate forms must be due to differences in size.

It can be concluded that the intima is slightly permeable to phosphate at its normal pH value, but that the pore size of the membrane severely limits entry of the basic form of the buffer pair, and hence casts doubt on the theory of selective reabsorption of the basic form of phosphate as a mechanism of acidification.

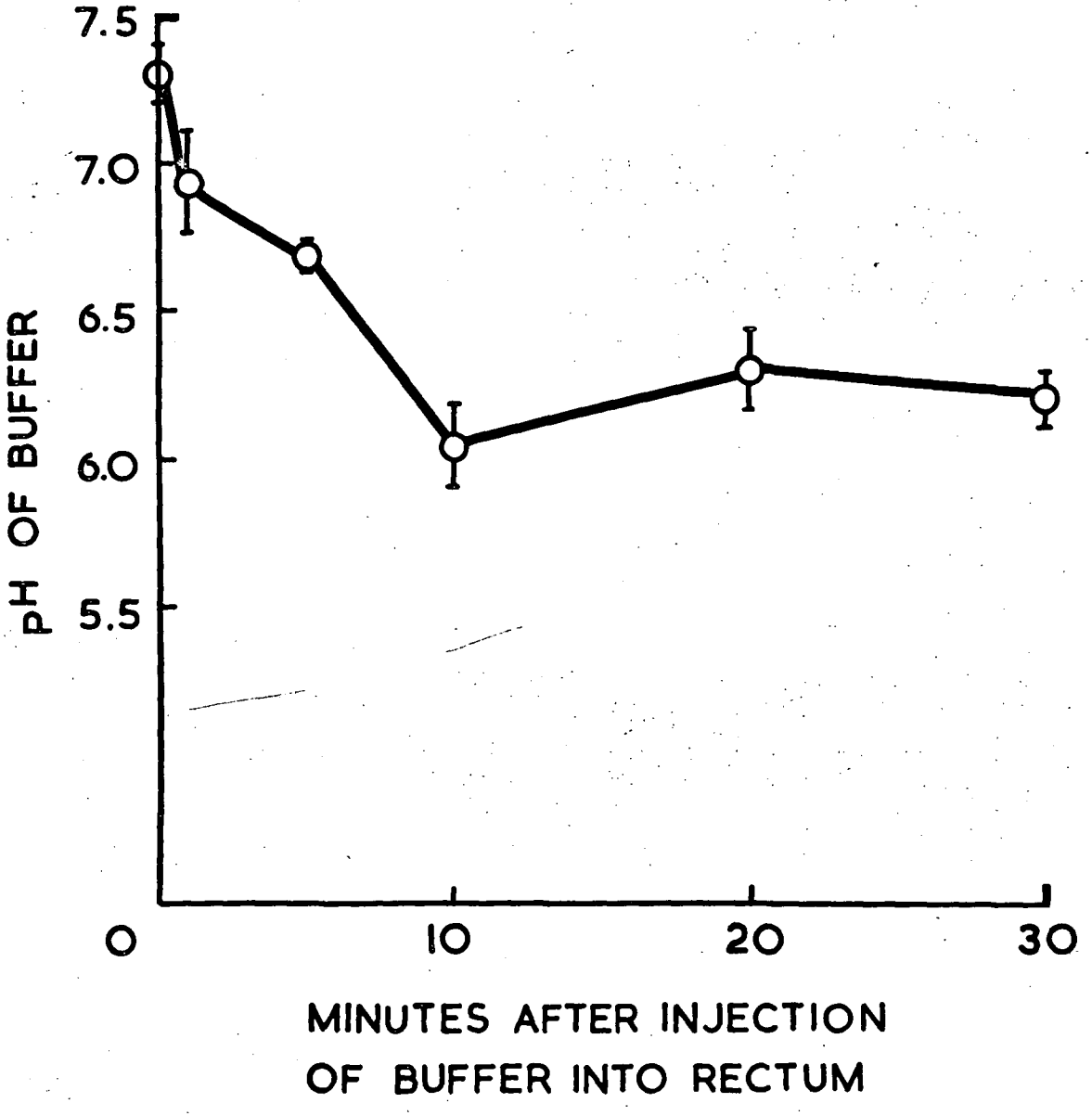
(b) Acidification of Tris-HCl buffer

To investigate further whether phosphate buffer is necessary for acidification, injection of Tris-HCl buffer into the ligated and rinsed rectum was performed. If no acidification occurred with this buffer, it would suggest that the phosphate of the previously injected buffer solution might be necessary for acidification. If acidification persisted, it would seem unlikely that there exists a particular mechanism for the uptake of the Tris molecule, and that more probably there had been an active secretion of hydrogen ions or some acid (e.g. carbonic) into the lumen.

Fig. 6 shows the results of injection experiments on five animals. The level of acidity reached at equilibrium was comparable to that obtained with McIlvaine's phosphate-citrate buffer (i.e. 6.04 - 6.18 vs. 6.02). Equilibrium was reached with both buffers in 10 to 20 min. Five other animals treated identically and judged to have no tracheal damage or leaks

FIGURE 6

Acidification of 0.002M Tris-HCl buffer, pH 7.2-7.4. The graph represents results from five animals. Horizontal lines, mean values; vertical bars, standard error of the mean.


7.5
7.0
6.5
6.0
5.5
pH OF BUFFER
0
10
20
30
MINUTES AFTER INJECTION
OF BUFFER INTO RECTUM

failed to acidify the buffer to a pH value of at least 6.5 on the first run. This is possibly the result of varying cuticular permeability to Tris: a more permeable intima might have allowed its penetration to the cell layer where it would be expected to cause damage of transport activity. The cuticular intima of individual locusts is known to exhibit widely varying permeability properties (Phillips, 1967).

The results show that acidification occurs in the absence of phosphate buffer. They substantiate the conclusion drawn from intimal permeability studies that absorption of the basic form of the phosphate buffer pair is not the only mechanism of acidification, if this buffer pair plays any role at all.

(c) Possible involvement of acid phosphate

While the basic form of the phosphate buffer pair is not selectively taken up, the possibility still remains that the acid form of the buffer pair is being secreted into the lumen. Before investigating this hypothesis directly, it was necessary to establish where the gradients in phosphate concentration occurrred in the animal, and so the phosphate concentrations of the body fluids were measured.

Results are given in Table III. The concentration of phosphate in the Malpighian tubule fluid is about twice that of the hemolymph. The concentration of phosphate in rectal fluid is considerably higher than in either hemolymph or Malpighian tubule fluid, these differences being highly significant ($p < 0.001$). From a consideration of these figures alone, it

TABLE III Concentrations of inorganic phosphate found in the body fluids of the locust.

Fluid	mM PO_4	S.D.	No. Obs.	Range
Hemolymph-serum	6.24	1.273	10	4.69-8.24
Malpighian tubule	14.6	5.65	5	9.5-23.9
Rectal fluid	47.9	5.89	9	14.0-81.4

could be supposed that the high concentration of phosphate in the rectal fluid is due either to secretion of phosphate or to a concentrating of phosphate resulting from absorption of water in the rectum.

First, the passive movement of acid phosphate was investigated. In previous experiments involving injection of 0.002M buffer, the gradient for diffusion of the acid form of phosphate was from the hemolymph into the rectal lumen. However, if a stronger buffer (0.02M) were used, then the gradient would favour passive movement of $H_2PO_4^-$ out of the rectum rather than inward. This follows from the following considerations: The phosphate concentration of the hemolymph was found to be 6.24 mM/l. (Table III). At the normal pH of the hemolymph, 7.13 (Table X), it can be calculated from the Henderson-Hasselbalch equation that the ratio of divalent to monovalent phosphate ions is 1 : 1.18. Doing the same calculation for McIlvaine's buffer at pH 7, the ratio of divalent to monovalent ions is 1 : 1.6. Then since the total amount of phosphate is known, the amounts of monovalent and divalent phosphate can be calculated. A summary of these calculations is given in Table IV. Total phosphate concentration for the buffer was equated to molarity, since only a few drops of citrate had been added to reach this pH. The electropotential gradient of 20 mv. (Phillips, 1965) with the rectal lumen positive to the hemocoel would support a three-fold concentration gradient, e.g. 10mM $H_2PO_4^-$ in the lumen would equilibrate with 3.38mM $H_2PO_4^-$ in the

TABLE IV Comparison of amounts of monovalent and divalent inorganic phosphate in McIlvaine's buffer and locust hemolymph.

	m M PO_4	
	$HPO_4^=$	$H_2PO_4^-$
Hemolymph (pH 7.13)	2.86	3.38
0.002 M McIlvaine's buffer (pH 7.0)	0.77	1.23
0.02 M McIlvaine's buffer (pH 7.0)	7.7	12.3

hemolymph; this value is exceeded by 12.3mM $H_2PO_4^-$ in the 0.02M buffer.

Seven locusts with recta ligated were injected with 0.02M McIlvaine's buffer. The pH of the injected buffer dropped 0.5 units in the first 10 min. and thereafter more slowly (Fig. 7). At 90 min. the buffer had a pH value of 6.39 ± 0.14. The rate of acidification for the stronger buffer was slow as compared to that for 0.002M buffer (Fig. 3), as expected. Equilibrium was only approached in about 90 min. as compared to 15-20 min. for 0.002M buffer. The results suggest that acidification is not due to passive diffusion of the acidic form of the phosphate buffer pair into the lumen.

While the possibility that the acid form of the phosphate buffer pair is moving into the rectal lumen passively was tested by experiments with 0.02M buffer, and it was found that acidification occurred even when the gradient favoured $H_2PO_4^-$ movement out of the rectum, there is still the possibility of active secretion of acid phosphate into the lumen. This was investigated by the following experimental procedure: 65 µl. of 0.002M McIlvaine's buffer (0.0045 for two other cases) at pH 7.2-7.4 were injected into the ligated locust rectum and left for 20 min. The pH of the acidified buffer was measured as well as the concentration of inorganic phosphate (Gomori, 1942). Table V shows the results. Five animals were used, and three experiments were carried out on each. The volume of fluid in the rectum was not rigorously controlled but

FIGURE 7

Acidification of 0.02M McIlvaine's buffer, pH 7.2-7.4. The graph represents results from seven animals. Horizontal bars, mean values; vertical bars, standard error of the mean.

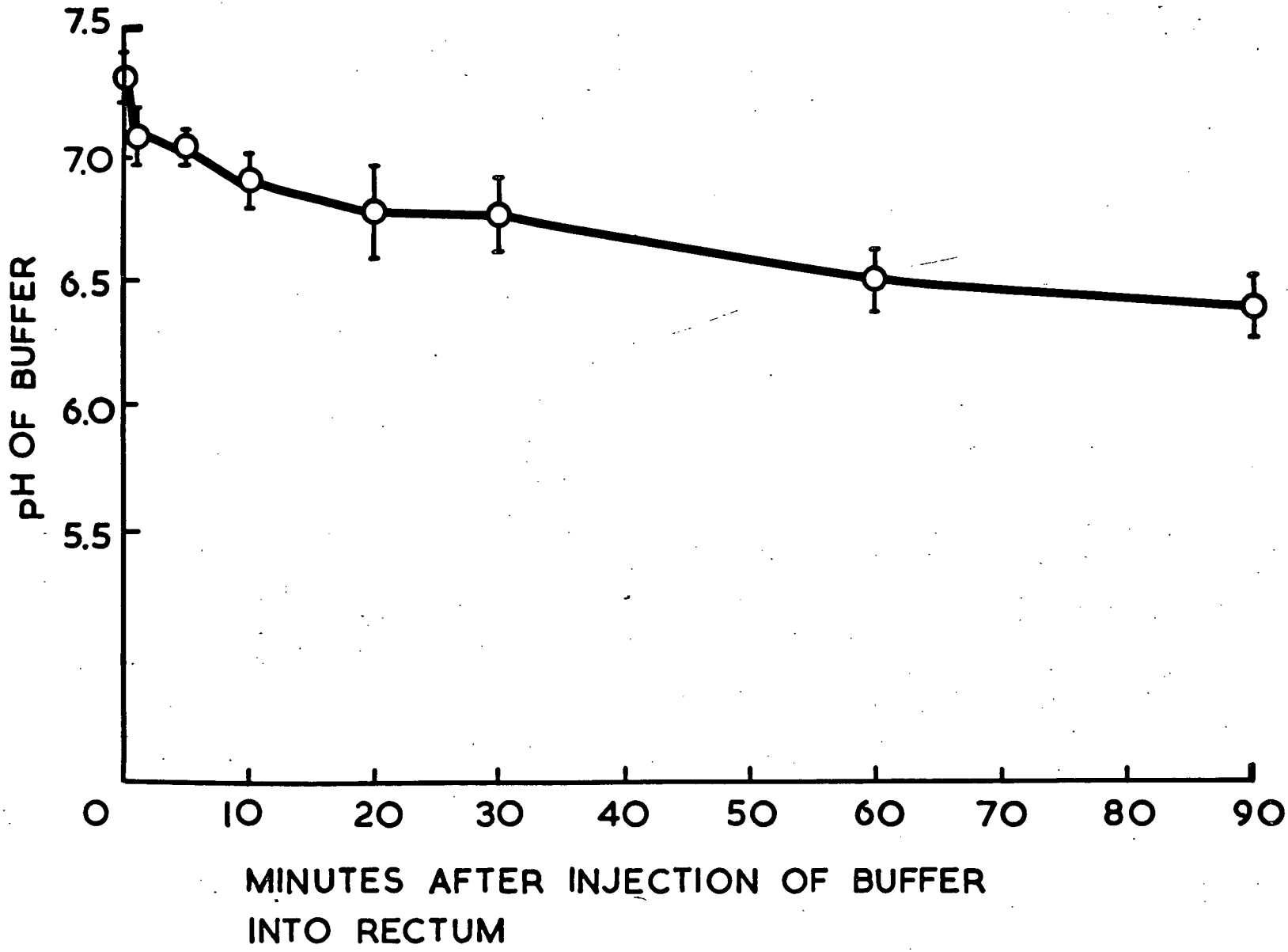

7.5
7.0
6.5
6.0
5.5
pH OF BUFFER
0
10
20
30
40
50
60
70
80
90
MINUTES AFTER INJECTION OF BUFFER
INTO RECTUM

TABLE V Phosphate secretion experiments. The pH and phosphate concentration of rectal fluid 20 min. following the injection of 65 µl. of 0.002 or 0.004 M McIlvaine's buffer (pH 7.2-7.4) into ligated rectum.

Locust	Experiment	pH	mM PO_4 Initial	Final
1	1	6.94		1.90
	2	6.86	2.00	1.70
	3	6.85		1.70
2	1	6.53		1.83
	2	6.75	2.00	1.67
	3	7.05		1.83
3	1	6.64		2.00
	2	7.02	2.00	2.00
	3	7.04		1.86
4	1	6.18		4.83
	2	6.32	4.63	4.50
	3	6.60		4.67
5	1	6.60		4.33
	2	6.81	4.36	4.33
	3	6.79		4.80

the 1.2M NaCl content of the buffer prevented any sizeable water absorption over the time period involved and consequent increase of phosphate concentration from this effect. However, some of the variation in results can be attributed to slight volume changes caused by water movement.

It was calculated from the Henderson-Hasselbalch equation that if the pH was lowered from 7.2 to 6.5 only by secretion of acid phosphate then the original concentration of 2mM would have to rise to 6mM. The concentration of phosphate never rose to the extent that was calculated as necessary to reach a given level of acidification during any of the five experiments. For example, animal 2 lowered the buffer pH to 6.53 with no increase in buffer phosphate concentration. Furthermore, the same phosphate concentration of 1.83 mM was found even when the buffer pH had a value of only 7.05 pH units. For an initial phosphate concentration of 4.5 mM, the value would be expected to rise to 13.5 mM in order to reach pH 6.5, whereas actually the pH went down to 6.18 and 6.32 with no significant rise in phosphate concentration. Thus there does not appear to be any secretion of phosphate which correlates with the hydrogen ion concentration.

The carbon dioxide tension of acidified rectal fluid

It is possible that the metabolic activity of the rectal epithelium produces a high partial pressure of carbon dioxide, and hence carbonic acid, in the epithelium and lumen of the rectum. The lumen might then be in equilibrium with a low pH in the epithelium (Phillips, 1961).

This hypothesis was tested by injecting alkaline McIlvaine's buffer (.002M, pH 7.4) into the ligated locust rectum, and removing the acidified fluid 20-30 min. later. The pH was measured at 0.5-1 min. and the rest of the fluid kept in capillary tubing for 3 hr. to allow the carbon dioxide in the acidified fluid to equilibrate with that in the air. Results are shown in Table VI. Small pH changes occurred in both a positive and negative direction. The average increase was 0.19 pH units and the average decrease was 0.16 pH units. On this basis, little of the acidity of rectal fluid can be attributed to a high carbon dioxide tension in the epithelium or lumen; the pH would have increased with time if carbon dioxide were being given off from the buffer.

Readings of pH were taken in all experiments up to this point using the microelectrode G252C, its chief advantage being the small sample size required (5 μl.). The anaerobic electrode G297 required too large a volume to be used routinely for all measurements (50 μl.) but it was used at this stage as an additional way of checking whether acidified buffer had a high partial pressure of carbon dioxide. If the readings obtained with the anaerobic electrode were significantly more acid as compared to those with the microelectrode, then this would indicate that significant amounts of carbon dioxide were being lost to the atmosphere in using the open system.

McIlvaine's buffer (pH 7.4, 0.002M) was injected and left for 20 min. in the ligated rectum. On removal from the animal,

TABLE VI Change in pH of acidified buffer on exposure to air for 3 hr.

Experiment	pH of buffer		Difference
	1 min.	3 hr.	
1	5.98	6.21	+ 0.23
2	5.81	5.65	- 0.16
3	6.29	6.51	+ 0.22
4	6.12	5.94	- 0.18
5	6.00	5.87	- 0.13
6	6.22	6.33	+ 0.11
Mean			+ 0.02

the sample was kept under paraffin oil until pH was measured. The sample could be drawn directly into the capillary of the electrode from beneath the paraffin oil in many cases; in others, maximum exposure to the air was 0.5 - 1 min. Within this time, the change in pH, if carbon dioxide were in fact being given off, would be of the order of 0.05 pH units. (In 5 min., the experimental buffer bubbled with carbon dioxide changed from pH 6.10 to pH 6.30).

Table VII gives the results obtained with the anaerobic system. The pH values are not significantly lower ($p=0.05$) than values obtained previously in experiments in which no special precautions were taken to prevent escape of carbon dioxide (the reading for 20 min. in Fig. 3, pH 5.95 ± 0.27).

Error due to carbon dioxide loss is not significant then, and once again a high partial pressure of carbon dioxide does not appear to be the immediate cause of the acidification of the injected buffer.

Changes in the preparation's capacity to acidify buffer

(a) Adjustment to hemolymph pH

In the experiments in which McIlvaine's buffer and Tris-HCl were injected into the ligated locust rectum, no more than three runs were performed on the same animal since a progressive change was noted in the preparation. For example, pH values obtained would frequently follow this pattern, given here for 0.002M alkaline McIlvaine's buffer:

TABLE VII The pH of rectal fluid as measured with an anaerobic electrode system 20 min. after injecting 60 µl. of 0.002 M McIlvaine's buffer (pH 7.4) into the ligated rectum.

Preparation	Experiment	pH
1	1	6.29
	2	6.48
2	1	6.09
3	1	5.91
	2	6.34
	3	6.40
4	1	6.30
5	1	6.08
	2	6.23
	3	6.31
Mean		6.24
St. deviation		0.17
St. error		0.05

Run	1	2	3
2 min.	6.60	6.84	7.00
20 min.	6.11	6.40	6.52

Failure to acidify buffer after three to four runs in a given preparation might conceivably show some relationship to hemolymph pH, i.e. to a change in the concentration gradient due to altered hemolymph pH. Injection of acid buffer into the rectum might cause the hemolymph to become more acid, and injection of alkaline buffer might cause the hemolymph to become more alkaline. In the case of the latter, the reduced pH gradient observed during the experiments might then represent an adjustment to the higher pH of the hemolymph resulting in reduction of the secretion of hydrogen ions into the lumen.

In the experiments, 65 µl. of either acid or alkaline McIlvaine's buffer (0.002M) were injected into the ligated rectum (the pH values for these same experiments are given in Fig. 3). At the end of 3-4 experiments on a given animal, the pH of the hemolymph was measured. Values are given below for 15 animals:

pH of injected buffer	Final pH of Hemolymph Mean±S.D.	Range
7.2-7.4	7.07±0.15	6.84-7.26
3.8-3.95	7.03±0.13	6.83-7.17

There is no statistical difference between the hemolymph pH of those animals injected with acid buffer compared to those injected with alkaline buffer, though pH values of both are significantly lower than control animals (unoperated), as discussed in the next section. While the data do not demonstrate

that the animals are adjusting acid secretion in response to an altered hemolymph pH, it cannot be concluded that the animals do not adjust since it is always possible that the change of pH required to initiate regulation in any individual may be smaller than the variation in hemolymph pH of the whole population.

The same change after three runs was noted in experiments in which Tris-HCl buffer was injected into the ligated rectum as had been observed with McIlvaine's buffer, although failure in the case of the Tris buffer cannot be entirely separated from probable cellular damage if the Tris molecule penetrated to the epithelium (see earlier section).

(b) Alkaline pH values

When failure of the ligated rectum to acidify injected fluid was first encountered, it was not clear whether this was due to damage to the preparation or whether the rectum could also alter the pH to high values as well as acid values. The question was asked whether, with successive injections, the pH of the buffer ever fluctuated significantly in the alkaline direction to a value higher than the pH expected if hydrogen ions were distributed passively across the rectal wall. That is, pH 7.4 is the pH predicted from the Ussing equation for rectal fluid at equilibrium if hydrogen ions were distributed passively across the rectal wall (Phillips, 1961). The lumen of the rectum is 20 mv. positive to the hemocoel so that the pH of rectal fluid should be 0.3 pH units above that of the

hemolymph (7.13) at equilibrium, provided that hydrogen ions move across the rectal wall by diffusion only. To answer this question, pH values taken 30 min. after injection of 0.002M Tris-HCl buffer (pH 6.65-7.4) during the second or third experiment on the same animal were compared to the original pH of the buffer injected into the rectum. These values are presented in Table VIII. It can be seen that while the buffer may not have been acidified by the rectum to the usual extent (pH 5.19-6.1), it has in no case become significantly more alkaline than pH 7.4. Thus any variation in pH is in the acid direction, although for animals 6 and 7 the pH went up to that value expected passively. Failure to acidify in these cases appears to be caused by damage to the preparation.

(c) Deterioration of preparation

The operation and repeated buffer injections into the ligated rectum may cause injury to the locust and result in a failure to regulate hemolymph pH within its usual limits. One way this could be tested would be to compare the pH of hemolymph of animals at the end of experiments to the pH in animals that had not been subjected to the operation. The pH values for hemolymph for animals which had notundergone the operation were drawn from Tables X and XII. These values were compared to the pH values for locusts in which the ligated rectum was injected with either acid or alkaline McIlvaine's buffer (section a). Animals subjected to the ligation and injection procedures did in fact have a hemolymph pH significantly lower

TABLE VIII The pH of 0.002M Tris-HCl buffer 30 min. after injection of 65 µl. into the ligated rectum for the second or third replicate experiment on the same locust.

Animal	Buffer pH	
	Original	Final (30 min.)
1	7.28	7.25
2	7.43	7.51
3	6.90	7.07
4	7.08	7.21
5	6.98	6.92
6	6.65	7.38
7	6.65	7.67

TABLE IX Acidification of 0.002 M McIlvaine's buffer with time after injection of 50 µl. into ligated rectum.

Animal	Time buffer left in rectum	Buffer pH Original	Final
1	70 min.	7.2	5.76
2	70 min.	7.2	6.38
3	2 hr.	7.10-7.15	6.36
4	2 hr.	7.10-7.15	5.91
5	2.5 hr.	7.25	5.91
6	2.5 hr.	7.25	6.83*
7	2.5 hr.	7.21	6.19
Mean			6.09
S.D.			0.261
S.E.			0.107

than the control animals ($p=0.05$). This may reflect a general deterioration of the preparation as a whole, and a failure to regulate hemolymph pH within its normal limits. Also the animal may have been unable to excrete acid due to ligation.

Possible deterioration of the preparation was checked by another method, i.e. by testing how long the ligated rectum could maintain the pH gradient. To check whether failure to acidify after three runs is due to the death of the preparation within the time of the experiment, the first injection of buffer was left in the rectum for a length of time equal to or greater than the time normally taken to perform three injections. If the preparation were in fact dying, then the pH gradient across the membrane would no longer be maintained. This assumes that the membrane is freely permeable to the acidifying entity in both directions, and that there is not secretion of acid which cannot back-diffuse. The assumption appears justified since it was shown previously that the system approaches the same acid pH whether an acid or an alkaline buffer is injected (Fig. 3), and hence is freely permeable to hydrogen ions.

Seven animals were studied; 50 µl. of 0.002 McIlvaine's buffer, pH 7.10-7.25, were injected into the ligated rectum for times varying from 70 min. to 2.5 hr. Results are given in Table IX. Only one out of the seven animals tested failed to acidify the experimental buffer. The equilibrium pH value was 6.09 ± 0.26; that for original experiments was 6.02 ± 0.19 (Fig. 3).

The preparation is able to maintain the pH gradient across the rectum for at least 2.5 hr. so that failure to acidify after three runs is not due to the death of the epithelium even though the pH of the hemolymph of treated animals does tend to drop within this length of time.

Amount and rate of acid secretion

In order to estimate the quantity of acid secreted by the rectal epithelium, both 0.02M and 0.002M McIlvaine's buffer were titrated with 0.1N HCl. Amaranth dye (2mg./10 ml.), which was normally added to injected buffers, was included. The pH was measured with a Radiometer Type G202B glass electrode. The values in Fig. 8 and Fig. 9 represent the average from three titrations. From the titration curves, it was calculated that 3.9 µl. of 0.1N HCl are needed to acidify 65 µl. of 0.02M buffer from pH 7.2-7.4 to pH 6.39; and 0.68 µl. of 0.1N HCl in the case of 0.002M buffer to reach a level of 6.02. Over the pH range 7.4 to 6.0, the buffering capacity is approximately constant, so that 0.007 µequiv. H^+ were needed to lower 1 µl. of 0.02M buffer 1 pH unit; and 0.0007 µequiv. for 0.002M buffer. The calculation for 0.02M buffer gives identical results to Phillips (1961). Then, assuming that there was no significant absorption of buffer from the rectum within 5 min., there must have been a total net influx of at least 0.11 µequiv. H^+, or 1.4 µequiv. H^+/hr. to account for the observed acidification of 0.02M buffer. Phillips gave a minimal estimate of 0.4 µequiv. H^+/hr. Both these estimates could be much lower than

FIGURE 8

Titration of 10 ml. of 0.002M McIlvaine's buffer with 0.1N HCl. Each point is the average from three titrations. Amaranth dye was included.

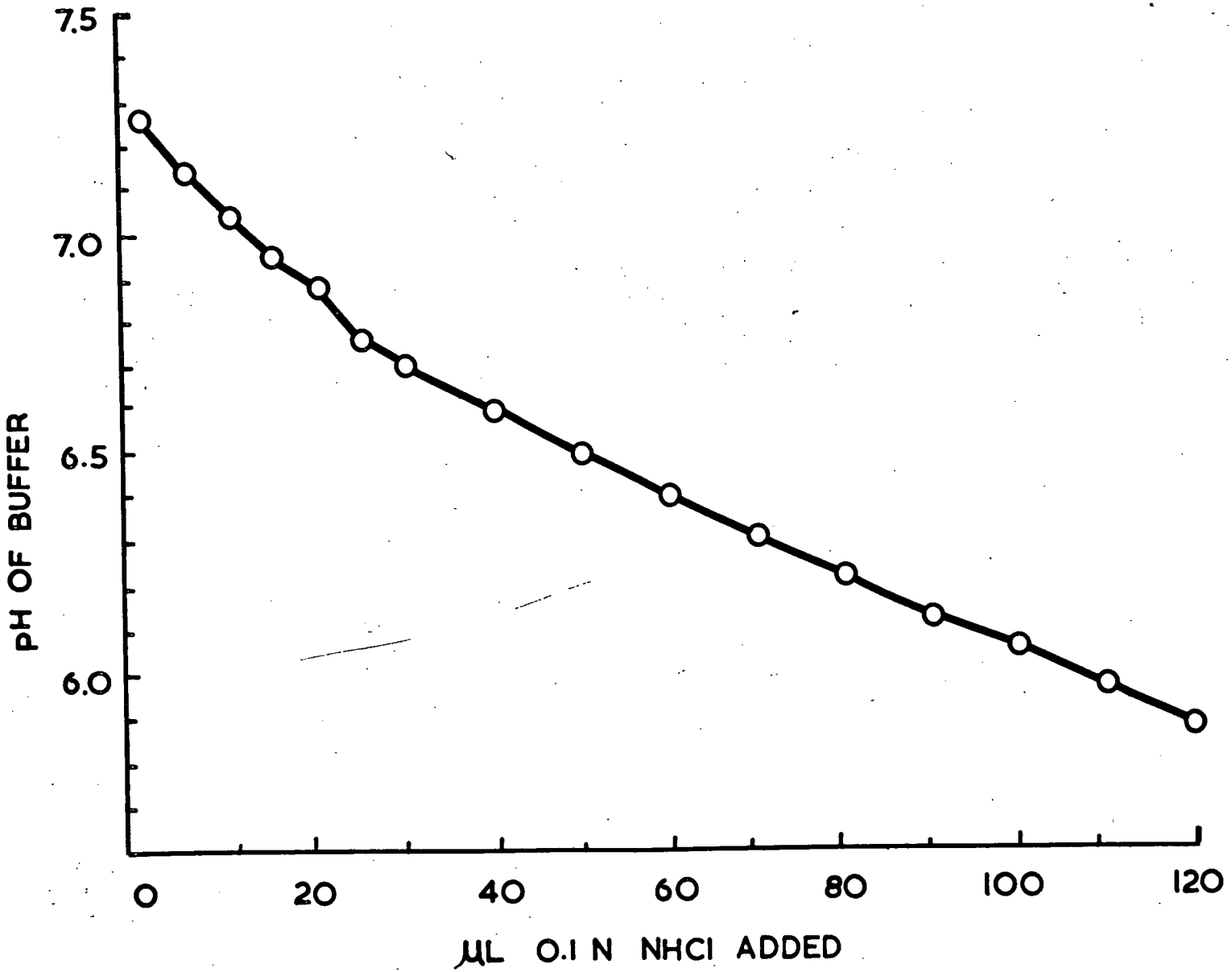
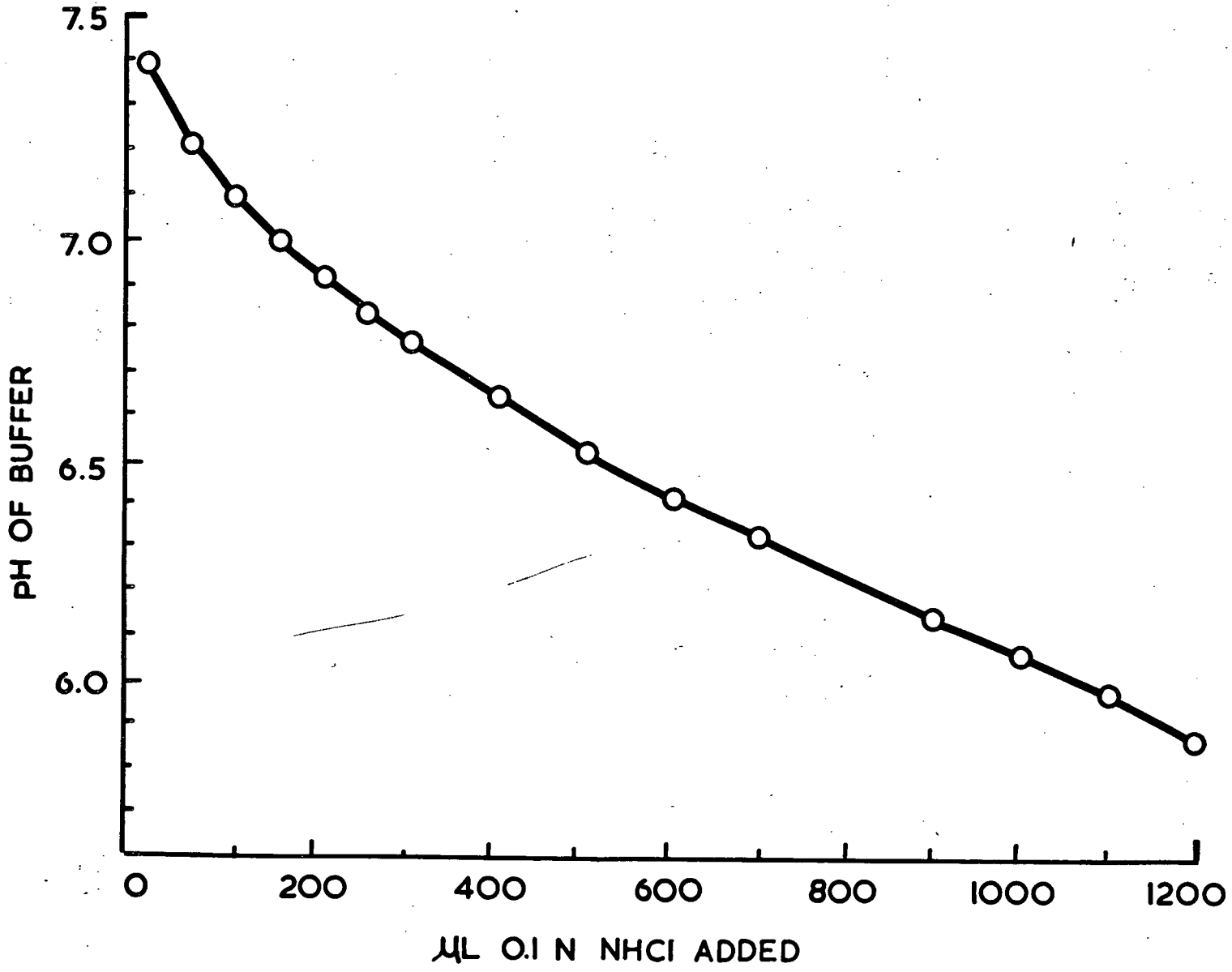

7.5
7.0
6.5
6.0
pH OF BUFFER
0
20
40
60
80
100
120
µL 0.1 N NHCl ADDED

FIGURE 9

Titration of 10 ml. of 0.02M McIlvaine's buffer with 0.1N HCl. Each point is the average from three titrations. Amaranth dye was included.


7.5
7.0
6.5
6.0
PH OF BUFFER
0
200
400
600
800
1000
1200
μL 0.1 N NHCl ADDED

the actual secretion rate since the value calculated depends on determining accurately the initial slope of the curve. Moreover, the rate of back diffusion, which increases as acidification proceeds, is not known. The calculation was done for the first 5 min. of the stronger buffer for two reasons: the concentration gradient is lowest at the beginning of the curve and hence gives the closest estimation of rate in the absence of a concentration gradient; times were considered more accurate for the stronger buffer.

Possible role of the excretory system in pH regulation

(a) Tolerance level

In the course of this investigation, some preliminary observations were made concerning pH regulation and the possible role of the excretory system, and more particularly rectal acidification, in this process. Unstarved locusts were injected with various amounts of 0.1N HCl to determine the amount of acid that Schistocerca can tolerate and whether it can regulate hemolymph pH when it has been loaded with acid. The animals were anaesthetized and injected beneath the cuticle of the third abdominal segment. One hour was allowed for distribution of the acid throughout the animal. Hemolymph samples were taken from the joint of a jumping leg over a 72 hr. period. Animals were not fed during the experimental period.

All measurements of hemolymph pH made during these experiments were done with the microelectrode, since only

10-15 µl. could be taken at each time of sampling. To check the error involved due to loss of carbonic acid due to sampling and time of measurement (Buck, 1953; Phillips, 1961), the pH values of locust hemolymph were compared using the two types of glass electrodes: Radiometer Type G252C, exposed to air, and Radiometer Type G297, which is anaerobic.

About 60 µl. of hemolymph could be taken from the leg joint of the locust into a capillary tube. This was done as quickly as possible, and the sample then kept under paraffin oil until measured, for the anaerobic system. For the other type, hemolymph was kept in 5 µl. capillary tubes and measured within 2-5 min.

The results for eight animals are presented in Table X. The error was found to be about 0.03 pH units. This agrees with Phillips (1961) who estimated that the error in measured pH value for locust hemolymph is less than 0.1 pH unit.

The tolerance level was judged to be about 100 µl. of 0.1N HCl, since there was some evidence at least of recovery to normal pH values in this group, i.e. animals 2 and 3 in Table XI at 72 hr. had hemolymph pH values of 7.40 and 7.12 respectively. Animals given either 100 or 150 µl. of 0.1N HCl, as well as the control animals (Tables XII and XIII), survived for more than 72 hr. Table XII shows that loss of hemolymph due to sampling does not effect pH, and Table XIII shows that there is not a significant change in pH caused by the use of distilled water rather than saline for injecting acid.

TABLE X Comparison of hemolymph pH using two types of glass electrodes, as an estimate of the error due to loss of carbonic acid.

Animal	Micro-electrode reading	Anaerobic reading	Difference
1	7.37	7.274	0.096
2	7.17	7.070	0.100
3	7.13	7.122	0.008
4	7.08	7.036	0.044
5	7.08	7.098	-0.018
6	7.13	7.121	0.009
7	7.23	7.178	0.052
8	7.08	7.110	-0.030
Mean	7.17	7.13	0.033
S.D.	0.10	0.0727	
S.E.	0.0354	0.0257	

TABLE XI The pH of hemolymph with time after injection of 100 µl. of 0.1N HCl into unstarved adult male locusts.

Animal	pH				Final condition of animal
	1-2 hr	6-7 hr.	24 hr.	72 hr.	
1	7.08	6.98	6.95	-	weak
2	7.12	7.20	7.31	7.40	strong
3	6.68	6.97	7.09	7.14	strong
4	6.90	6.72	6.78	6.87	weak
Mean	6.95				

TABLE XII The pH of hemolymph of control (uninjected) locusts with time.

Animal	pH			
	0 hr.	6 hr.	24 hr.	72 hr.
1	7.43	7.38	7.35	7.36
2	7.22	7.42	7.56	7.30
3	7.28	7.34	7.46	7.32
4	7.33	7.37	7.40	7.44

TABLE XIII The pH of hemolymph with time after injection of 150 µl. of distilled water into adult unstarved male locusts

Animal	pH			
	1-2 hr.	6 hr.	24 hr.	72 hr.
1	7.53	7.54	7.54	7.64
2	7.44	7.33	7.45	7.53
3	7.48	7.22	7.40	7.57
4	7.48	7.38	7.47	7.52

(b) pH of fecal pellets of animals injected with acid

The pH of fecal pellets of animals injected with acid was measured to investigate whether the degree of acidification of excreta (and hence perhaps buffer) reflects the acid load of an animal. That is, excess acid might be lost via the excreta, in which case the rectal acidification could well be important in the pH regulation of the animal as a whole. An alternative is that the acidification may simply be coupled to water, Na^+ and K^+ transport in the rectum.

To determine if excess acid was in fact lost in the excreta, six animals were injected with 100 µl. of 0.1N HCl, this being an amount that the animal was known to tolerate. Fecal pellets were collected before the injection and for 72 hr. thereafter. Neither food nor water was given to the animals during the collection time. Hemolymph samples were taken from the leg joint of unanaesthetized animals at the end of the experiment, and pH measured to determine whether any recovery had occurred. The results are given in Table XIV.

In all animals examined except animal 2, the excreta became an average of 0.3 units more acid after the injection of HCl; moveover the excreta of that exception was already more acid than the others. The two animals from which the least number of fecal pellets were collected (no. 1 and no. 6) had the lowest final hemolymph values: pH 6.88 and 6.66. However, the other four animals seem to have been able to excrete the acid in fecal pellets and showed a recovery to normal hemolymph values. The initial hemolymph pH was estimated

TABLE XIV The pH of fecal pellets of animals injected with 100 µl. of 0.1N HCl, followed over 72 hr. Distilled water added for moisture except for underlined values.

Hours after injection	Animal					
	1	2	3	4	5	6
0	6.23	5.20	7.70	5.98	6.40	5.99
1-5	5.55	-	6.65	5.75	6.18	5.50
20-30		5.68	4.72	5.02	5.50	4.53
	-	6.00			4.79	
		5.48				
40-50	-	-	5.51	5.19	5.41	-
			5.08		5.48	
			6.40		5.50	
60-72	-	-	5.32	-	5.28	-
			5.82		5.38	
					5.48	
					5.52	
Hemolymph-terminal value	6.88	7.02	7.22	7.26	7.27	6.66

previously at pH 7.17 (Table X), and the amount of acid injected lowers the pH to about 6.95 (Table XI). The values of hemolymph pH measured at the end of 72 hr. (6.66-7.27) were comparable to those in Table XI (6.87-7.40), as expected since the amount of acid injected was the same for both groups of animals. That the two animals which showed the least recovery to normal hemolymph pH values also excreted the least number of fecal pellets may indicate that the amount of material in the gut at the time of acid injection limits the amount of acid eliminated in the excreta. At least two animals (no. 3 and no. 5) show a trend of progressively more acid pellets and then a return to less acid values, presumably as the acid load is reduced. All animals were very active at the end of the experiment, regardless of the degree of recovery of hemolymph pH values to a normal level.

These preliminary observations do suggest that the level of acidification of the excreta reflects the acid load of the animal. The excretory system appears to be one way available to the animal for eliminating excess acidity.

DISCUSSION

In investigating the acidification of rectal fluid, the pH of different regions of the gut was measured to see if the normal acidity of rectal contents was due to introduction of acid from some anterior region. The values found agree roughly with those found in the literature, many of which have been determined colorimetrically rather than electrometrically. For example, Hastings and Pepper (1943) studied the body fluids of seven orthopterans and found that the regurgitated digestive juices from six species of grasshoppers fell within the pH range of 5.2 to 5.8. This is comparable to the value of 5.46 found for the mid ventriculus of the locust where the brown fluid occurs. Swingle (1931) found that the majority of insects studied, representing seven orders, had a slightly acidic digestive tract, and that the pH usually increased from the mouth to the foregut and anterior region of the midgut, and then decreased from the posterior region of the midgut through the hindgut. The locust follows this pattern, at least for the areas studied from midgut to rectum. Payne (1961, cited by Uvarov, 1966) studied pH in the alimentary canal of Schistocerca gregaria; he gave values of 5.4 for foregut, 6.2-7.1 for midgut and 7.0 for hindgut. Hindgut includes the ileum, colon and rectum, so it is difficult to compare the one figure for hindgut pH to those for ileum and rectum as given in Table 1. Values of 6.0 to 6.2 are not uncommon in the hindgut of grasshoppers and locusts (Uvarov, 1966).

The alkalinity in the region of Malpighian tubule entry may be due to their fluid contribution to the gut here. It is known that the Malpighian tubules elaborate the primary excretory fluid in Schistocerca (Phillips, 1965), and that this fluid is modified by the rectum. One feature of the alteration is a change in the hydrogen-ion concentration. Ramsay (1956) working on excretion by the Malpighian tubules of the stick insect, Dixippus morosus (Orthoptera) found that the difference in pH between urine and serum is somewhat variable but in all cases the output of the Malpighian tubules was alkaline to serum but became acid in the rectum. Measurements made of pH of rectal fluid expressed through the anus were between 3.5 and 4.5 in the stick insect. Rectal pH values found here were higher than both those given by Ramsay above and by Phillips (1961) who found the pH of rectal contents to be 4.73 ± 0.32 and the pH of excreted feces to be 5.30 ± 0.36 for the locust. These are to be compared to pH values of 5.91 ± 0.44 and 5.95 ± 0.55 respectively in the present study. The differences for the same experimental animal may be attributable to differences in the electrode system used for determination, or to differences in diet of the animals. In the case of Dixippus, there is a species difference to consider as well.

There was no significant difference between the pH of gut contents of control animals and those fed tetracycline in the diet to kill off the gut flora. A change in either the positive or negative direction was conceivable since Fletcher and Haub (1933, cited by House, 1965) did find that the pH

of the alimentary canal of Phormia regina (Diptera) larvae is slightly higher in those reared nonaseptically than in those reared aseptically. In Blatella germanica (Orthoptera), Wigglesworth (1927) explained acidity in the crop as due to the activities of microorganisms acting on sugar: with a carbohydrate food the pH was 4.8, with protein 6.3. No differences of this magnitude were found in Schistocerca by treatment with antibiotic, and so it has been concluded that bacterial fermentation does not account for the observed acidification in the rectum, nor for variation in pH along the length of the alimentary canal. Rather the pH of gut contents appears to reflect the pH of secretions into the gut, the product of absorptive processes by the gut lining plus the products of digestion.

The acidity in the rectum is not due to the introduction of acid from any anterior region of the gut, since the rectum was always acid to all other regions posterior to the Malpighian tubules and since the same degree of acidification was achieved by the ligated rectum. There was some indication that the hindgut in general may acidify the alkaline material which enters it, as evidenced by a change in pH from 7.94 to 7.13, the latter value taken from the anterior part of the hindgut (anterior ileum) of control animals. Alternatively there may be some forward movement of acid from the rectum; or the decrease from pH 7.94 to 7.13 could also mean that the area under consideration fails to maintain the original gradient, the change resulting from net diffusion of hydrogen ions across the gut wall with the hemocoel.

The same acid pH is attained if a more alkaline or more acid buffer is introduced into the rectum. This suggests that the epithelium is permeable to hydrogen ions, and agrees with the results of Phillips (1961). He, too, rejected the possibility that 'equilibrium is not attained because the rectal wall is relatively impermeable to hydrogen ions (which seems unlikely in view of the rapid absorption of water and salts from the rectum).' Acidification occurred even when an antibiotic was included in the injected buffer which agrees with the results of Phillips (1961) although he used terramycin rather than tetracycline. Both are wide-spectrum antibiotics. Furthermore, there was found to be neither significant nor sufficient slow release of hydrogen ions by fecal material over the experimental period to acidify injected buffer solutions. Phillips had found previously that the pH of rinsings did not change more than 0.1 unit over a 3 hr. period after their removal from the rectum. Evidence supports the hypothesis that the observed acidification is due solely to the activity of the rectal wall.

Failure of the preparation to acidify after repeated injection was not correlated with altered hemolymph pH; nor did the preparation die within the experimental period as evidenced by its ability to maintain the pH gradient over an extended period of time. Phillips (1964b) found that the transrectal potential in the same <u>in vivo</u> preparation was maintained undiminished over a 3 hr. period. This indicates that

failure was not due to changes in potential across the membrane versus time, and is in agreement with the finding that the preparation survives for at least this length of time. It is possible also that the adjustment of acidification is located in the tissue of the rectum itself and that this has somehow been effected by the experimental procedure.

Another possibility is that the intima is acting as an exchange resin and after a number of runs the supply of hydrogen ions for exchange on the cuticular membrane may be exhausted. Some doubt is thrown on the idea by calculations of the amount of acid required to acidify the injected buffer solution. With the stronger buffer, the preparation could be used still for two or three runs, i.e. equivalent to the release of 1.2 µM HCl. However in three runs of the weaker buffer, this capacity would not have been exceeded, i.e. 0.21 µM HCl. Also, even if the intima were to act as an exchange resin, the maintenance of the gradient would still depend on the activity of the epithelium, since the system is in a state of dynamic equilibrium. In view of this, the only other possibility that suggests itself is that the chances of heat damage as the preparation is sealed with hot wax increase with each successive injection.

Phillips suggested in his study, though he did not study the possibility experimentally, that the acidity resulted from preferential absorption of the basic form of a buffer pair. It has been suggested that the acidity of the gut contents might be due to phosphoric acid (Hobson, 1931); and in the bee, Hoskins and Harrison (1934) showed that the concentration of

phosphate in the midgut (0.046M) is nearly five times that of the hemolymph (0.01M) whereas the excreta contain only 0.005M. Some work has been done by Lindsay and Craig (1942) on the uptake of labelled phosphate by the gut epithelium of insects. They found that radiophosphate became concentrated in the midgut of all insects studied, but was absent from the foregut and hindgut of most. They looked at the waxmoth, mealworm, cockroach, and firebrat. The concentration refers to tissue content rather than body fluids, and was interpreted by the authors as indicating that the midgut epithelium was the main tissue concerned with fat mobilization. No information was given by these workers, however, as to the site of phosphate reabsorption in the gut.

The concentrations of phosphate in the body fluids of Schistocera follow the pattern as found in Dixippus morosus by Ramsay (1956). He found that the concentration of phosphorus in the Malpighian tubule fluid was 140 mg.-atoms/l., and that the concentration of phosphate was greater in this fluid than in the hemolymph or serum. Other figures for Dixippus morosus (Ramsay, 1955a) are, serum 39 meq./l. and Malpighian tubule fluid 51 meq./l. Similarily in the locust, the Malpighian tubule fluid contained 14.5 ± 5.65 mM/l. phosphate as compared to a hemolymph value of 6.24 ± 0.40 mM/l. The concentration of phosphate in the rectal fluid was considerably higher than either of these at 47.9 ± 5.89 mM/l. for the locust. Other figures found in the literature for hemolymph phosphate concentrations in Exopterygotes are:

Sutcliffe (1962) Odonata: *Aeschna grandis* larvae...4 meq./l.
Wood (1957) Phasmida: *Carausius (Dixippus) morosus* adults...16 meq./l.

Phosphate values for Endopterygotes range from 2.8 meq./l. to 10.3 meq./l. (Florkin and Jeuniaux, 1964).

The high concentration of phosphate in the rectal fluid may be a result either of phosphate secretion into the lumen or of rectal water absorption. The first possibility was eliminated by this investigation. Although there is a high concentration of phosphate in the rectal fluid, it cannot be concluded from this fact that little phosphate is reabsorbed in this region of the gut. The large concentration gradient resulting from water reabsorption favours movement of phosphate out of the rectum into the hemolymph. The actual quantity that is reabsorbed would depend on the rate of entry of material into the rectum as well as how long the material remained there. Also, in collection of rectal fluid, samples were presumably only obtained from animals in which all water had not yet been reabsorbed, and nothing is known about relative rates of absorption of water and phosphate. This fact may partly account for the variation in determinations for this body fluid.

Phosphate is present in the rectal fluid in sufficient concentration that preferential absorption of one form of this ion could alter the pH. At the normal rectal pH of about 6, the phosphate would be found predominantly as the acid form, $H_2PO_4^-$. This could be the result of selective reabsorption of the alkaline form leading to acidification. Transport

systems for both the monovalent and divalent forms are known to occur biologically: in the fertilized sea urchin egg the penetrating form is the $HPO_4^=$ ion (Chambers and Whiteley, 1966); and in yeast and bacterial cells there is transport of phosphate as $H_2PO_4^-$ ion (Goodman and Rothstein, 1957; Mitchell, 1954).

Doubt is cast on the theory of selective reabsorption of the basic form of the phosphate buffer pair by experiments in which a Tris-HCl buffer was used rather than McIlvaine's buffer (phosphate-citrate). Acidification persisted in five out of ten animals with the Tris buffer, i.e. in the absence of the phosphate buffer system. The pH values at equilibrium were the same regardless of the buffer components. Other evidence comes from measurements of phosphate concentrations of acidified buffer, done to study the possibility of phosphate secretion. It can be calculated that if the pH of 2mM phosphate buffer was lowered from pH 7.2 to 6.5 by removal of $HPO_4^=$ only, then the final phosphate concentration would be 1.2mM. Changes in phosphate concentration during pH change are not of this magnitude.

A further limitation is imposed upon reabsorption of the basic form of phosphate by the cuticular intima. Tracer studies indicate that the intima is probably acting to restrict the movement of the larger divalent $HPO_4^=$ ion and any uptake of phosphate would be in favor of the monovalent form. The acidity of the rectum would facilitate any phosphate reabsorption

which does occur in this organ, since the intima is virtually impermeable at higher pH values to the basic form. The acidity is also important in nitrogen excretion being responsible for precipitation of uric acid (Wigglesworth, 1931b). The low pH of the rectal contents also means that weak acids which dissociate in the hemolymph can be excreted at least in part in the undissociated form (i.e. carrying out H^+), as described for the vertebrate kidney (White, Handler and Smith, 1964).

Movement of acid phosphate into the lumen of the rectum, either actively or passively was eliminated as a mechanism of acidification. Even when the gradient favored movement of $H_2PO_4^-$ out of the rectum rather than inwards (0.02M buffer), acidification occurred. When the phosphate concentration in the weaker buffer was checked after acidification and compared to the pH value of the same sample, there was no correlation. Hence any evidence for involvement of the phosphate buffer system appears to be lacking.

The bicarbonate buffer system is also widespread biologically, and its involvement in the acidification has been considered, though only partially. Phillips (1961) on the basis of his experimental evidence considered it 'highly possible that acidification in the locust rectum is due to active secretion of hydrogen ions into the lumen, or reabsorption of bicarbonate from carbonic acid in the rectal fluid (which in effect leads to the same result as active secretion of hydrogen) or both'.

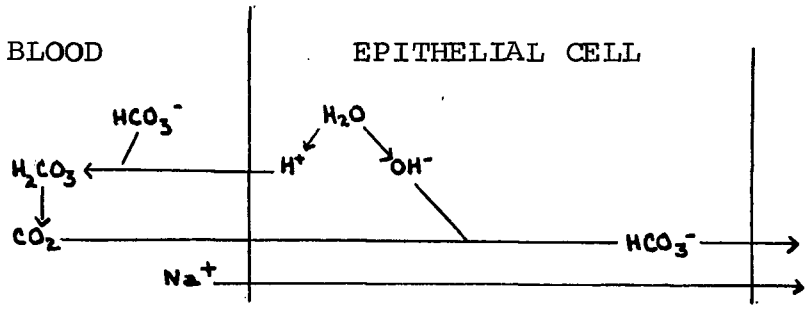
It has been shown that little of the acidity of rectal fluid can be attributed to a high carbon dioxide tension in the lumen, by experiments in which acidified buffer was allowed to equilibrate with air. This refutes the hypothesis suggested originally by Phillips (1961) that the metabolic activity of the rectal epithelium produces a high partial pressure of carbon dioxide, and hence carbonic acid, in the epithelium and lumen of the rectum. The lumen is not in equilibrium with a high carbon dioxide tension in the epithelium. Phillips (1961) also rejected this hypothesis on the basis of the same experiment. In addition, results with the anaerobic electrode system showed that there was only slight error due to carbon dioxide loss during these experiments. This does not however eliminate involvement of the bicarbonate buffer system. If in fact there were active reabsorption of bicarbonate from carbonic acid and if the reabsorption were sufficiently rapid to produce a low concentration of carbonic acid, this would be indistinguishable from hydrogen ion secretion.

There are many examples of involvement of the bicarbonate system in acidification to be drawn from vertebrates. The pH gradients across the intestinal epithelium can be considerable. Wilson (1962) reviews studies carried out on acid-base changes across the wall of the intestine. In most animals, the contents of the jejunum are acid to the blood while contents of the ileum are more alkaline. Evidence suggests that these gradients are the result of the active transport of

HCO_3^- and H^+.

The rat intestine *in vitro* has been shown by Wilson (1954) to develop a pH gradient across its wall such that the lumen is acid compared to the serosal side. Parsons (1956) investigated pH changes across the rat intestine *in vivo* and showed that when a saline solution containing 25 mM HCO_3^- is placed in the jejunum, the bicarbonate ion was almost completely removed in 4 hr. In the ileum and colon, the bicarbonate concentration rises under similar conditions.

Wilson and Kazyak (1957) did experiments in which inverted sacs of hamster ileum were incubated with a bicarbonate-saline solution on both sides of the intestinal wall. They found that the pH and HCO_3^- fell on the serosal side and rose on the mucosal side. Furthermore the loss from the mucosal side was equal to the gain on the serosal side. Studies of fluid and ion movement indicated that the ileum secreted an isotonic solution of Na^+ (or K^+) HCO_3^- from the serosal to the mucosal side. It was noted that the carbon dioxide tension increased in the serosal solution while transport was occuring. This observation was taken as an indication that the mechanism of transport involves exchange of a hydrogen ion for some other cation across the serosal border of the epithelial cell. The following mechanism was postulated by them:



From all these studies, it seems as though the more acid side has a high carbon dioxide tension whenever a pH gradient is maintained across the intestinal wall. This holds both for the solution in the lumen of the jejunum in acid secretion *in vivo* (Parsons, 1956) and the solution of the serosal side in alkaline secretion by the ileum *in vitro* (Wilson and Kazyak, 1957). Wilson (1962) considers it reasonable to suppose that a similar mechanism is involved in both parts of the small intestine, one which involves the exchange or transfer of hydrogen ions across one border of the epithelial cell.

A second example is the reabsorption of bicarbonate in the vertebrate kidney. Pitts (1963) describes the mechanism in the proximal tubule which is specialized for reabsorption of most of the bicarbonate which enters the glomerular filtrate. Sodium and bicarbonate ions enter the proximal tubule in the glomerular filtrate. Na^+ is constantly pumped out of the tubular cells and actively extruded into the peritubular fluid. The resulting low intracellular concentration of Na^+ means that Na^+ ions of the filtrate diffuse into the cell. H^+ ions from the interior of the cell are exchanged for Na^+ ions in the tubular lumen. The H^+ associates with HCO_3^-

of the filtrate and forms carbonic acid which in turn decomposes into CO_2 and water (slowly, since not enzymatically catalyzed). The CO_2 diffuses into the cell and is hydrated under the influence of carbonic anhydrase. Subsequent dissociation furnishes HCO_3^- which diffuses down a concentration gradient into the peritubular fluid. According to this scheme the reabsorption of bicarbonate is indirect i.e. via conversion to CO_2 in the tubular lumen. The essential process or central process is the exchange of intracellular hydrogen ions for sodium ions in the tubular fluid. Whether the movement of H^+ ions is passive or requires a pump is not certain (Pitts, 1963). About 90% of filtered bicarbonate is reabsorbed in the proximal tubule of the kidney. There is a further mechanism in the collecting duct of the vertebrate kidney specialized to reabsorb the remainder of the filtered bicarbonate against a high gradient. Acidification of urine to a pH below 6 requires almost total removal of bicarbonate.

Phillips (1961) found that there would have to be a total net influx of at least 0.07 µequiv. H^+ or 0.4 µequiv. H^+/hr. to account for the observed acidification of buffer in the ligated rectum of the locust. A somewhat higher value of 1.4 µequiv. H^+/hr. was estimated in this investigation. At least for the former estimate, the net influx of hydrogen ions is of the same order of magnitude as net sodium chloride absorption from the rectum in the absence of a concentration gradient, so that there may be an exchange of hydrogen ions

for sodium ions as in the vertebrate kidney. Or there could be a potassium-hydrogen ion exchange; potassium reabsorption in the rectum is 10 times that for sodium (Phillips, 1964b, 1965). Potassium transport may be linked to the movement of hydrogen ions in the midgut of Hyalophora cecropia (Haskell, Clemons, and Harvey, 1965), as suggested by inhibition of transport at moderately alkaline pH values, as well as the inhibitory effects of carbon dioxide and carbonic anhydrase inhibitors.

In the vertebrate system if carbonic anhydrase is inhibited then there is not acidification, and also more Na^+ is excreted. This supports the idea the H^+ is exchanged for Na^+ and that cellular carbon dioxide is the source of the secreted protons (White, Handler, and Smith, 1964). Carbonic anhydrase has been reported in the tissues of several insects (Sobotka and Kann, 1941; Anderson and March, 1956; Buck and Friedman, 1958). It is said to occur in the soluble fractions of the cells (Gilmour, 1961). No inhibitor studies have been attempted in this study since their value is questionable in an introductory investigation of cellular mechanism. For example, Parsons (1956) conducted some studies with 'Diamox', a carbonic anhydrase inhibitor. He found that in the ileum of the rat its effect was to cause the direction of net movement of carbon dioxide to change to absorption so that the ileum became more acid (pH 7.2) than in the untreated animals (pH 7.5). In the jejunum its effect was to inhibit the absorption of total carbon dioxide so that the contents of the jejunum became more

alkaline (pH 7.0) than in the untreated animals (pH 6.5). However he cautions that the effects of 'Diamox' lead to a general depression of the capacity to absorb water and Na^+ and Cl^-, and that the effects of 'Diamox' reported above are not necessarily due to its action as an inhibitor of the enzyme carbonic anhydrase since carbonic anhydrase is not even present in the mucosa of the small intestine of the rat.

Hydrogen ion secretion has been demonstrated also for yeast cells (e.g. Conway, 1954; Conway and O'Malley, 1955), into the lumen of the stomach of vertebrates (e.g. Davenport, 1957, 1967; Davies, 1957), from frog skin (Huf et al. 1951, Fleming, 1957).

In this study no measurements of bicarbonate concentrations were made because of technical problems encountered with the technique of microdiffusion (Shaw, 1955; Conway, 1957). Hence no differentiation can be drawn at this stage between hydrogen ion secretion as such, and bicarbonate reabsorption from carbonic acid leaving behind hydrogen ions.

The concentration of HCO_3^- in the hemolymph of one Exopterygote, Odonata: Aeschna grandis larvae, is given as 15 meq./l. by Sutcliffe (1962). The estimate for two Endopterygotes is the same: 15 meq./l. for Megaloptera: Sialis lutaria larvae (Shaw, 1955; Sutcliffe, 1962); 17 meq./l. for Diptera: Gasterophilus intestinalis larvae (Levenbook, 1950a). According to Levenbook (1950b) the concentration of bicarbonate is usually low for terrestrial insects; the

figure for Gasterophilus blood is much higher than in the hemolymph of many other insects e.g. eight times that of Prodenia eridania.

Representative values found in the literature for rectal concentration of HCO_3^- were: Sialis lutaria larvae, 91 mM/l. (Shaw, 1955; Staddon, 1955); Notonecta glauca adult, 75 mM/l. (Staddon, 1963).

The hemolymph of insects is usually very slightly acid (Wigglesworth, 1965). The locust was found to have a hemolymph pH of 7.13, in agreement with Phillips (1961). The buffer capacity, or resistance to alteration in pH, of the hemolymph is said to be greater on the acid side of neutrality, and probably due to bicarbonates, inorganic phosphates, proteins, amino acids, and perhaps also urates (Wigglesworth, 1965). Protein and bicarbonate are the most important quantitatively according to Levenbook (1950b). The fact that the buffer curve for insect blood is usually smooth and has few, if any, inflexion points has been taken by workers including Levenbook as an indication of the involvement of a whole series of overlapping buffer systems in which no one of these dominates.

The buffer capacity of the hemolymph of insects shows certain characteristic features, as based on studies of the larvae Pieris rapae and Heliothis armigera (Craig and Clark, 1938), the adult Mormon cricket Anabrus simplex (Pepper, Donaldson, and Hastings, 1941), and various other Orthoptera

(Hastings and Pepper, 1943) and reviewed by Levenbook (1950b). The main difference from the situation found in vertebrates is that the buffer capacity is always lowest in the region of the normal pH of the blood rather than being optimally buffered at this pH as in most other animals. This results in a U-shaped curve when buffering capacity is plotted against pH (Levenbook, 1950b). Vertebrate blood buffers less efficiently on either side of its normal pH and the buffer value curve is ∩-shaped. It does appear correspondingly that insects can withstand a wider variation in pH than mammals. The locust does show a remarkable tolerance of excess acidity, as evidenced by observations on animals injected with hydrochloric acid. The range of pH in which the animal is still relatively active is wider than for example the blood of man. The latter is regulated at pH 7.4, and if the pH falls to 7.0, acidotic coma and death ensue, while at a pH of 7.8 life ends in tetany (Giese, 1962).

Levenbook (1950b) has suggested a possible advantage of the U-shaped buffer curve to the insect. The lactic acid released into the blood as a result of muscular exertion by the active insect would increase the free CO_2 content of the blood at the expense of bicarbonate. Activity is also associated with an increase in cellular respiratory rate and this, too, would increase the carbon dioxide tension in the blood. Wigglesworth (1935) and others showed that an increase in the lactic acid in the blood, or a lowered pH due to an excess of

carbon dioxide causes the animal to increase ventilation of the tracheal system. This is a response also found in vertebrates and is done in an effort to remove the excess carbon dioxide by increasing its rate of diffusion through the respiratory system. In the opinion of Levenbook, the insect tracheal system is not as efficient in this respect as is the vertebrate lung. Hence the carbon dioxide content of the hemolymph might reach a concentration that would injure the tissues, but for the fact that the hemolymph shows an increased buffering capacity in these regions. Miller (1964) also suggests that the U-shaped curve has a relationship to water conservation, allowing discontinuous release of carbon dioxide.

The enzyme carbonic anhydrase in the erythrocyte of the mammal plays a central role in the buffering efficiency of the blood. Carbonic anhydrase has been found in the hemolymph of only one insect species, the larva of Chironomus (Brinkman et al., 1932). Levenbook and Clark (1950) confirmed the absence of carbonic anhydrase from insect blood in experiments on Gasterophilus and Locusta migratoria.

Ramsay (1955a) injected solutions of NaCl and KCl into the hemolymph of Dixippus to alter the concentrations and then followed the process of return to normal. He found that for potassium regulation, the return to normal had progressed half way in about one day. The adaptive response for sodium on the part of the excretory system of Dixippus seemed

feeble, especially when compared to Rhodnius. In the latter animal excess sodium taken in with a meal is gotten rid of in 3 hr. (calculated by Ramsay, 1955a, from Wigglesworth, 1931a). Hence the response of an animal to elevated sodium concentrations in the hemolymph varies, and this may also hold for hydrogen-ion regulation. However in the locust injected with 10^{-5} μM HCl, the rate of return of hydrogen-ion concentration to normal had progressed about one-half way in one day, and compares to Ramsay's results for potassium in Dixippus (1955a).

For animals with hydrochloric acid injected into the hemolymph, the excreta collected subsequently were more acid than prior to injection. It appears probable that rectal acidification might play a role in eliminating excess acidity of the animal. The normal pH of excreta is similar to the equilibrium values attained on injecting solution into the ligated rectum. These lower values after acid injection suggest the rectum has adjusted to a new more acidic equilibrium. In the case of vertebrates, to deal with strong acids such as hydrochloric or sulfuric the following occurs (Pitts, 1963). The acids are excreted fully neutralized, and this is accomplished without loss of sodium by combining the acid with ammonium ions instead. The net reaction as given by Pitts (1963) is

$$Na_2SO_4 + 2H_2CO_3 + 2NH_3 \longrightarrow \underset{\text{excreted}}{(NH_4)_2SO_4} + \underset{\text{reabsorbed}}{2NaHCO_3}$$

There is an enzyme, glutaminase, present in the kidney which catalyzes the hydrolysis of glutamine to form glutamic acid and NH_3. The ammonia diffuses across the tubular lining into the glomerular filtrate. Here it combines with H^+ derived from intercellular H_2CO_3, and is exchanged for Na^+. Ammonia excretion thus serves to elevate extracellular HCO_3^- and also returns Na^+ to the plasma which otherwise would have been associated with an anion in the urine. There seems then to be a basic difference between the vertebrate treatment of strong acids in excreting them neutralized, and the observation that in the locust the excreta become noticeably more acid when strong acid is injected into the hemolymph. This is the only evidence gathered to support the idea that rectal acidification might be important in the pH regulation of the animal as a whole.

SUMMARY

1. Acidity in the rectum of the locust is not due to the introduction of acid from any anterior region of the gut.

2. Bacterial activity does not account for pH changes along the length of the gut.

3. Average pH values found were: midgut 5.46 ± 0.39; Malpighian tubule region 7.59 ± 0.72 and 7.94 ± 0.49; ileum 7.13 ± 0.47; rectum 6.19 ± 0.45 and 5.91 ± 0.44.

4. The same acid pH is attained whether a more alkaline or more acid buffer is introduced into the ligated rectum. Acidification of injected buffer occurs even in the presence of antibiotic, and hence the phenomenon is not due to bacterial activity. This suggests that the epithelium is permeable to hydrogen ions and that the pH gradient is maintained by the rectal wall, i.e. a case of dynamic equilibrium.

5. There is not sufficient slow release of hydrogen ions by fecal material to account for the acidification.

6. Phosphate concentration increases from Malpighian tubule region to rectum. Serum concentration is lower than both of these.

7. Rectal pH does not favor absorption of the basic form of phosphate buffer; and since acidification occurs also with a Tris-HCl buffer, absorption of the basic form of a phosphate buffer pair seems unlikely to be the only mechanism of acidification.

8. The intima of the rectum is most permeable to

phosphate at pH 4 and least permeable at pH 8. It may be acting as a molecular sieve to exclude the larger divalent $HPO_4^{=}$ ion in favor of $H_2PO_4^{-}$.

9. Acidification takes place with 0.02M phosphate buffer when the gradient is against passive movement of the acidic component of the phosphate buffer into the rectum.

10. There is no active secretion of the acid component of the phosphate buffer pair which could account for acidification of rectal contents.

11. Acidity is not due to a high partial pressure of carbon dioxide in the acidified buffer. However, whether or not the bicarbonate buffer system is otherwise involved has not been demonstrated.

12. Schistocerca can tolerate an injection of about 100 µl. 0.1N HCl into the hemolymph, and shows some ability to regulate this dose. Excess acid may be lost in the excreta and aid recovery.

13. Failure of acidification after a number of runs is not correlated with measurable hemolymph pH changes due to buffer injection into the rectum, nor is it due to death of the preparation. Failure is probably due to heating damage.

14. Calculations of the amount of acid secreted cast doubt on the theory of the cuticular intima acting as an ion exchange resin.

15. Either hydrogen ions or some acid molecule which dissociates are actively secreted into the rectum, or bicarbonate is absorbed from the lumen.

LITERATURE CITED

Anderson, A.B., and R.B. March, 1956. Inhibitors of carbonic anhydrase in the American cockroach Periplaneta americana (L.). Can. J. Zool., 34:68-74.

Brindley, G.S., 1962. Beats produced by simultaneous stimulation of the human eye with intermittent light and intermittent or alternating electic current. J. Physiol., 164:157-167.

Brinkman, R., R. Margaria, N.U. Meldrum, and F.J.W. Roughton, 1932. The CO_2 catalyst present in blood. J. Physiol. 75:3P-4P.

Buck, J.B., 1953. Physical properties and chemical composition of insect blood, p. 147-190. In, K.D. Roeder (ed.) Insect Physiology. Wiley, New York.

Buck, J.B., and S. Friedman, 1958. Cyclic CO_2 release in diapausing pupae. III. CO_2 capacity of the blood: carbonic anhydrase. J. Insect Physiol., 2:52-60.

Chabun, P., 1967. Unpublished observations.

Chambers, E.L. and A.H. Whiteley, 1966. Phosphate transport in fertilized sea urchin eggs. I. Kinetic aspects. J. Cell. Physiol., 68:289-308.

Conway, E.J., 1954. Some aspects of ion transport through membranes. Symp. Soc. Exp. Biol., 8:297-324.

Conway, E.J., 1957. Microdiffusion Analysis and Volumetric Error. Fourth edition, Lockwood, London. 465 p.

Conway, E.J. and E. O'Malley, 1955. Evidence for a redox pump in the active transport of cations. Intern. Rev. Cytol., 4:377-396.

Craig, R. and J.R. Clark, 1938. The hydrogen ion concentration and buffer value of the blood of larvae of Pieris rapae (L.) and Heliothis obsoleta (F.). J. Econ. Entomol., 31:51-54.

Davenport, H.W., 1957. Metabolic aspects of gastric acid secretion, p. 295-302. In, Q.R. Murphy (ed.) Metabolic Aspects of Transport across Cell Membranes. Univ. of Wisconsin Press, Madison.

Davenport, H.W., 1967. Stimulation of gastric motility by acid. Gastroenterlolgy, 52:198-204.

Davies, R.E., 1957. Gastric hydrocholoric acid production--the present position, p. 277-293. In, Q.R. Murphy (ed.) Metabolic Aspects of Transport across Cell Membranes. Univ. of Wisconsin Press, Madison.

Fiske, C.H. and Y. Subbarow, 1925. The colorimetric determination of phosphorus. J. Biol. Chem., 66:375-400.

Fleming, W.R., 1957. On the role of hydrogen ion and potassium ion in the active transport of sodium across the isolated frog skin. J. Cell. Comp. Physiol., 49:129-152.

Florkin, M. and C. Jeuniaux, 1964. Hemolymph: composition, p. 110-152. In, M. Rockstein (ed.) Physiology of Insecta. Academic Press, New York Vol. 3.

Giese, A.C., 1962. Cell Physiology. Second edition, Saunders, Philadelphia, 592 p.

Gilmour, D., 1961. Biochemistry of Insects. Academic Press, New York. 343 p.

Gomori, M.D., 1942. A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. J. Lab. Clin. Med., 27:955-960.

Goodman, J. and A. Rothstein, 1957. The active transport of phosphate into the yeast cell. J. Gen. Physiol., 40:915-923.

Goodwin, T.W. and S. Srisukh, 1951. Biochemistry of locusts. 5. The green pigment of the haemolymph and integument of solitary locusts (Locusta migratoria migratorioides, R. and F., and Schistocerca gregaria, Forsk.). Biochem. J., 48:199-203.

Haskell, J.A., R.D. Clemons, and W.R. Harvey, 1965. Active transport by the Cecropia midgut. I. Inhibitors, stimulants, and potassium transport. J. Cell. Comp. Physiol., 65:45-55.

Hastings, E. and J.H. Pepper, 1943. Studies on body fluids of seven Orthopterans, their pH, buffering capacity, and effect on solubility of fractionated insecticides. J. Econ. Entomol., 36:857-864.

Hobson, R.P., 1931. Studies on the nutrition of blowfly larvae: I. Structure and function of the alimentary tract. J. Exp. Biol., 8:109-123.

Hoskins, W.M. and A.S. Harrison, 1934. The buffering power of the contents of the ventriculus of the honeybee and its effect upon the toxicity of arsenic. J. Econ. Entomol., 27:924-942.

House, H.L., 1965. Digestion, p. 815-858. In, M. Rockstein (ed.) Phsyiology of Insecta. Academic Press, New York. Vol. 2.

Huf, E.G., J. Parrish, and C. Weatherford, 1951. Active salt and water uptake by isolated frog skin. Amer. J. Physiol., 164:137-142.

Jones, J.C., 1964. The circulatory system of insects, p. 1-107. In, M. Rockstein (ed.) The Physiology of Insecta. Academic Press, New York. Vol. 3.

Levenbook, L., 1950a. The composition of horse bot fly (Gasterophilus intestinalis) larva blood. Biochem. J., 47:336-346.

Levenbook, L., 1950b. The physiology of carbon dioxide transport in insect blood. Part III. The buffer capacity of Gasterophilus blood. J. Exp. Biol., 27:184-191.

Levenbook, L. and A.M. Clark, 1950. The physiology of carbon dioxide transport in insect blood. Part II. The effect of insect blood on the rate of hydration of CO_2. J. Exp. Biol., 27:175-183.

Lindsay, E. and R. Craig, 1942. The distribution of radiophosphorus in wax moth, mealworm, cockroach and firebrat. Ann. Ent. Soc. Amer., 35:50-56.

Miller, P.L., 1964. Respiration --aerial gas transport, p. 558-615. In, M. Rockstein (ed.) The Physiology of Insecta. Academic Press, New York. Vol. 3.

Mitchell, P., 1954. Transport of phosphate across the osmotic barrier of Micrococcus pyogenes: specificity and kinetics. J. Gen. Microbiol., 11:73-82.

Parsons, D.S., 1956. The absorption of bicarbonate-saline solutions by the small intestine and colon of the white rat. Quant. J. Exp. Physiol., 41:410-420.

Pepper, J.H., F.T. Donaldson, and E. Hastings, 1941. Buffering capacity and composition of the blood serum and regurgitated digestive juices of the mormon cricket (Anabrus simplex Hald.). Physiol. Zool., 14:471-475.

Peters, J.P. and D.D. Van Slyke, 1932. Quantitative Clinical Chemistry. Williams and Wilkins Co., Baltimore.

Phillips, J.E., 1961. Studies on the rectal absorption of water and salts in the locust, Schistocerca gregaria, and the blowfly, Calliphora erythrocephala. Ph.D. Thesis. Univ. of Cambridge.

Phillips, J.E., 1964a. Rectal absorption in the desert locust, Schistocerca gregaria Forskal. I. Water. J. Exp. Biol., 41:15-38.

Phillips, J.E., 1964b. Rectal absorption in the desert locust, Schistocerca gregaria Forskal. II. Sodium, potassium and chloride. J. Exp. Biol., 41:39-67.

Phillips, J.E., 1964c. Rectal absorption in the desert locust, Schistocerca gregaria Forskal. III. The nature of the excretory process. J. Exp. Biol., 41:69-80.

Phillips, J.E., 1965. Rectal absorption and renal function in insects. Trans. Roy. Soc. Can., Series IV, 3:237-254.

Phillips, J.E., 1967. Unpublished observations.

Pitts, R.F., 1963. Physiology of the Kidney and Body Fluids. Year Book Medical Publishers Incorporated, Chicago. 243 p.

Ramsay, J.A., 1950. Osmotic regulation in mosquito larvae. J. Exp. Biol., 27:145-157.

Ramsay, J.A., 1953a. Exchanges of sodium and potassium in mosquito larvae. J. Exp. Biol., 30:79-89.

Ramsay, J.A., 1953b. Active transport of potassium by the Malpighian tubules of insects. J. Exp. Biol., 30:358-369.

Ramsay, J.A., 1955a. The excretory system of the stick insect, Dixippus morosus (Orthoptera, Phasmidae). J. Exp. Biol., 32:183-199.

Ramsay, J.A., 1955b. The excretion of sodium, potassium and water by the Malpighian tubules of the stick insect, Dixippus morosus, (Orthoptera, Phasmidae). J. Exp. Biol., 32:200-216.

Ramsay, J.A., 1956. Excretion by the Malpighian tubules of the stick insect, Dixippus morosus (Orthoptera, Phasmidae): Calcium, magnesium, chloride, phosphate and hydrogen ions. J. Exp. Biol., 33:697-709.

Savage, A.A., 1956. The development of Malpighian tubules of Schistocerca gregaria (Orthoptera). Quart. J. Microsc. Sci., 97:599-615.

Shaw, J., 1955. Ionic regulation and water balance in the aquatic larva of Sialis lutaria. J. Exp. Biol., 32:353-382.

Sobotka, H. and S. Kann, 1941. Carbonic anhydrase in fishes and invertebrates. J. Cell. Comp. Physiol., 17:341-348.

Staddon, B.W., 1955. The excretion and storage of ammonia by the aquatic larva of Sialis lutaria (Neuroptera). J. Exp. Biol., 32:84-94.

Staddon, B.W., 1963. Water balance in the aquatic bugs, Notonecta glauca L. and Notonecta marmorea Fabr. (Hemiptera; Heteroptera). J. Exp. Biol., 40:563-571.

Stobbart, R.H. and J. Shaw, 1964. Salt and water balance: excretion, p. 190-258. In, M. Rockstein (ed.) The Physiology of Insecta. Academic Press, New York. Vol. 3.

Sutcliffe, D.W., 1962. The composition of haemolymph in aquatic insects. J. Exp. Biol., 39:325-343.

Swingle, M.C., 1931. Hydrogen ion concentration within the digestive tract of certain insects. Ann. Entomol. Soc. Am., 24:489-495.

Treherne, J.E., 1957. Glucose absorption in the cockroach. J. Exp. Biol., 34:478-485.

Treherne, J.E., 1958. The absorption of glucose from the alimentary canal of the locust Schistocerca gregaria (Forsk.). J. Exp. Biol., 35:297-306.

Treherne, J.E., 1959. Amino-acid absorption in the locust Schistocerca gregaria Forsk.). J. Exp. Biol., 36:533-545.

Uvarov, B., 1966. Grasshoppers and Locusts. A Handbook of General Acridology. Cambridge University Press, 481 p. Vol. 1.

White, A., P. Handler and E.L. Smith, 1964. Principles of Biochemistry. Third edition, McGraw-Hill, New York. 1106 p.

Wigglesworth, V.B., 1927. Digestion in the cockroach. I. The hydrogen ion concentration in the alimentary canal. Biochem. J., 21:791-796.

Wigglesworth, V.B., 1931a. The physiology of excretion in a blood-sucking insect, Rhodnius prolixus (Hemiptera, Reduviidae). I. Composition of the urine. J. Exp. Biol., 8:411-427.

Wigglesworth, V.B., 1931b. The physiology of excretion in a blood-sucking insect, Rhodnius prolixus (Hemiptera, Reduviidae). III. The mechanism of uric acid excretion. J. Exp. Biol., 8:443-451.

Wigglesworth, V.B., 1932. On the function of the so-called 'rectal glands' of insects. Quart. J. Micr. Sci., 75:131-150.

Wigglesworth, V.B., 1935. The regulation of respiration in the flea, Xenopsylla cheopis, Roths. (Pulicidae). Proc. Roy. Soc. Lond. B., 118:397-419.

Wigglesworth, V.B., 1965. The Principles of Insect Phsyiology. Sixth edition, Methuen, London. 741 p.

Wilson, T.H., 1954. Concentration gradients of lactate, hydrogen and some other ions across the intestine *in vitro*. Biochem. J., 56:521-527.

Wilson, T.H., 1962. Intestinal Absorption. Saunders, Philadelphia. 263 p.

Wilson, T.H. and L. Kazyak, 1957. Acid-base changes across the wall of hamster and rat intestine. Biochim. et biophys. acta, 24:124-132.

Wood, D.W., 1957. The effect of ions upon neuromuscular transmission in a herbivorous insect. J. Physiol., 138:119-139.

APPENDIX

A. Inorganic phosphate precipitation

That significant bacterial incorporation of labelled phosphate had not occurred during the tracer experiments was checked using MacKay and Butler's modification of Mathison's method (Peters and Van Slyke, 1932). The solutions required were as follows:

(1) Concentrated HCl.

(2) Magnesium citrate mixture of Fiske. To 265 gm. of citric acid dissolved in 350 cc. of hot water, 13 gm. of magnesium oxide free from carbonate were added with stirring. The solution was cooled and 330 cc. of strong ammonia water (specific gravity 0.90) added. This was cooled and diluted to one liter.

(3) Concentrated ammonia water (specific gravity 0.90).

(4) Dilute ammonia solution. One volume of strong ammonia water was diluted to 15 volumes with water.

B. Measurement of inorganic phosphate by colorimetric method

The method used to measure inorganic phosphate concentrations colorimetrically was that of Gomori (1942). Solutions required were:

(1) 10N H_2SO_4- 282 cc. concentrated H_2SO_4 into 600 cc. water made up to one liter.

(2) Molybdate-sulfuric reagent - two parts 5% sodium molybdate ($Na_2MoO_4\,2H_2O$), one part 10N sulfuric acid, one part water.

(3) Reducing agent - one gram 'elon' (metol) in 100 cc. 3% sodium bisulfite. The latter solution was made freshly every three days.

(4) 5% trichloroacetic acid (TCA).

(5) Standard - 0.5mM KH_2PO_4 .