Carbonic Anhydrase in the Gastric Mucosa

Thesis

by

Horace Willard Davenport

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy,

California Institute of Technology,

Pasadena, California

1939

Summary

The evidence on the site of formation of hydrochloric acid in the gastric mucosa of mammals is reviewed. The evidence points to the participation of the parietal cells in the formation and secretion of the acid, but it is insufficient to prove that the acid is or is not formed directly in or by the parietal cells.

The discovery of large amounts of carbonic anhydrase in the gastric mucosa is described. A technique of analysis for the enzyme in the gastric mucosa of cats and rats and for determining histologically the number of different kinds of cells in the material analysed is described. A strong positive correlation between the enzyme concentration and the number of parietal cells is taken as proof that carbonic anhydrase is present in the parietal cells in concentration higher than in the red blood cells. Similar evidence is given that carbonic anhydrase is present in small amounts in the cells of the surface epithelium. Evidence is also given that carbonic anhydrase may be present in small amounts in the gastric juice of cats, rats and humans. The enzyme in human gastric juice is different from that in red blood cells.

The bearing of these facts on the theory of the formation of hydrochloric acid is discussed, and it is concluded that the hydration of carbon dioxide to carbonic acid and the subsequent ionization of the acid may be the means by which hydrogen ions are provided. The energy necessary for the formation of hydrochloric acid from blood is estimated, and the prevailing theories of secretion are reviewed. All are found inadequate.

The gastric mucosa of mammals secretes hydrochloric acid. The concentration of acid secreted may be as high as 0.17 molal (Hollander, 1934). This very remarkable property of the gastric mucosa has received considerable attention both experimentally and theoretically. However the exact site of secretion and the mechanism by which hydrochloric acid is formed remains unknown.

In this thesis the evidence concerning the site will be reviewed. Then some new evidence on the presence in the parietal cells of carbonic anhydrase will be presented. Finally a detailed and critical review of the evidence concerning the mechanism will be presented in the light of the old and new experimental facts.

Hydrochloric acid is secreted copiously by the glands of the fundus, but the possibility that other parts of the stomach secrete some acid is not excluded. The cells of the fundic glands belong to two main types, the chief cells and the parietal or border cells. Langley (1881) in his study of the fundic glands determined that the chief cells contain and secrete pepsinogen. He and others (e.g., Heidenhain, 1870) were inclined to attribute the secretion of the acid to the parietal cells on the grounds that the other secretory functions had been associated with other types of cells. This left the secretion of acid and the parietal cells to be associated. Also Greenwood (1885), Macallum (1908) and Fitzgerals (1910)

found that chlorides are more abundant in the parietal cells, suggesting that these cells are involved in the secretion of the acid. Lately Gersh (1938) using a more refined technique has disputed the fact that chlorides are more abundant in the parietal cells. Also it is a well-known fact of pathological anatomy that in diseases involving achlorhydria, as for example pernicious anemia, the parietal cells are missing. This fact however loses some of its force when it is considered that in pernicious anemia all the glands of the stomach undergo degeneration.

Linderstrom-Lang et al. (1925) found that acid of undertermined nature has a distribution in the gastric mucosa roughly similar to that of the parietal cells. They also found that the distribution of chloride is very similar to that of the parietal cells. They concluded that "on the basis of the available material it is only possible to assert that the parietal cells are in all probability the cells which produce hydrochloric acid."

The parietal cells are described as being "situated along the tubule, lying between the basement membrane and the central cells. . . Each of these cells is penetrated by a network of minute passages, communicating with the lumen of the gland by a fine canal, which passes between the central cells . . . The (parietal) cells are sometimes present in the neck of the gland or even at the surface of the mucosa; in these places they are wedged between the ordinary epithelium cells" (Sharpey-Shafer, 1934).

If the parietal cells are concerned with the secretion

of the acid then there are three possibilities. The acid may be formed inside the cells and secreted across the membrane into the canaliculi and thence into the lumen. The acid may be formed at the cell membrane and appear first in the canaliculi. Some precursor of the acid may be found in the parietal cells and be secreted into the lumen where it gives rise to the acid. On the basis of the present evidence it is impossible to decide conclusively among these possibilities.

Claude Bernard (1859) injected ferric lactate and potassium ferrocyanide into the ear veins of a rabbit. These reagents in acid solution form insoluble Prussian blue. At autopsy Bernard found a blue color on the surface of the stomach but no blue color in any of the glands.

Fitzgerald (1910) repeated Bernard's experiment. She found that Prussian blue was deposited in the canaliculi of some of the parietal cells and sometimes in the interglandular lymph and blood vessels and in wandering cells and leucocytes. In some experiments the blue color was not obtained, and when it did appear it was in only some parts of the stomach and in very few of the parietal cells. Fitzgerald concluded that the results showed that acid was present in some of the cells. The negative results she attributed to the possibility that not all of the cells are secreting at one time.

Harvey and Bensley (1912) repeated these experiments and confirmed Fitzgerald. However on the basis of their more extensive experiments they completely denied the validity of her conclusions. After injecting ferric salts and

ferrocyanide into a number of animals they found that the Prussian blue appeared in many places besides the gastric mucosa. They concluded that the reaction probably occured without the help of acid. They sometimes used solutions of concentration as high as 25% from which Prussian blue will precipitate without acid. They also found that the color although present on the surface of the stomach was usually absent from the parietal cells. Harvey and Bensley injured the stomach of a cat after the reagents had been injected. Five hours later the cat was killed and the stomach examined. They concluded that only dead cells were stained with Prussian blue while live ones were not. This suggested to them that the death of the cells or the "lowering of vitality" permitted the penetration of the cells by the reagents. They concluded that "since Prussian blue may be precipitated in so many places, the fact that it is sometimes precipitated in the canaliculi of a few parietal cells in a relatively small part of the stomach in an abnormal condition does not necessarilly prove that free hydrochloric acid is formed under normal conditions in the parietal cells of the stomach as a whole."

Harvey and Bensley also attempted to stain the parietal cells with indicator dyes. Their technique was the following: The animal was killed by a blow on the head, the stomach exposed and a small piece of the mucosa cut out. The mucosa was washed and stained several minutes in the dye solution. When cyanamin was used the parietal cells stained with the red alkaline color of the dye, but injured cells and the small cells of Heidenhain stained the blue acid color.

Similar results were obtained with nile blue and neutral red. This technique is open to sever criticism. Dawson and Ivy (1926) showed that stunning an animal at once stops secretion from a Pavlov pouch. In addition one would expect that in the long interval between the killing of the animal and the examination of the mucosa any acid present would be neutralized by diffusion.

Collip (1920) repeated the Prussian blue experiments using less toxic solutions, and he obtained spproximately the same result. However he accepted Fitzgerald's interpretation and rejected the criticism of Harvey and Bensley. He also showed by microchemical tests that phosphates and probably carbonates were present in the cytoplasm of the parietal cells during rest while chlorides were present only in traces. When the cells were taken from actively secreting stomachs chlorides were found to be more abundant.

Dawson and Ivy (1926) attempted to solve the problem using better technique. They prepared dogs with Pavlov pouches and stimulated them to secrete acid. When Dawson and Ivy injected cyanamin they found that the dye tinted the juice blue but that insufficient quantity was present in the cells to permit determination of the acidity. Pieces clipped from actively secreting mucosa and dipped in the dye solution were stained, but the parietal cells showed the alkaline color. They pointed out however that the color change of cyanamin takes place about pH 3.0 and that their observations show only that the cytoplasm has a pH greater than 3.0. Dawson and Ivy also injected neutral red which is

crimson at pH lower than 6.8. Pieces clipped from the mucosa and examined in less than three minutes showed that the parietal cells were diffusely stained crimson and that the crimson color in the lumen was less conspicuous. Pieces were clipped from the mucosa and immersed in neutral red solution. Dawson and Ivy found in these pieces that the lumen and the canaliculi showed an acid reaction at the moment of staining. The crimson color soon disappeared which they attributed to the rapid diffusion of the acid.

In attempting to repeat the work of Fitzgerald, Dawson and Ivy found that the solutions inhibited secretion. In only one case could they get results similar to those of Fitzgerald. When pieces were fixed in alcohol and immersed in acid no reaction was obtained except at the mouths of the glands. It appears from this that the reagents were not present in the parietal cells and that the negative results have no bearing on the question.

Chambers (1915) injected neutral red into the parietal cells with a micropipette. The dye slowly diffused through the cell and into the canaliculi and lumen. Throughout the cell the dye was alkaline in color and acid only in the lumen, and from this Chambers concluded that the actual secretion of the glands was neutral if not alkaline. The whole experiment must be sceptically considered. The length of time between the death of the animal and the observation must have been several minutes. It is well-known that a fall in blood pressure stops secretion, so it can be expected that excision of the mucosa stopped secretion. If an active

mechanism is stopped the diffusion of the acid will result in its neutralization. Now even if it be granted that during secretion the canaliculi contain 0.17 molal acid, it cannot be expected that after diffusion has taken place for several minutes the acid will remain very highly concentrated in a canaliculus having a diameter of no more than 0.002 mm. That diffusion can take place rapidly is shown by the experiment itself, for the neutral red diffused from the point of injection through the cell and into the lumen.

Hoerr and Bensley (1936) have made further observations on stained mucosa without adding anything to the knowledge of its reaction. However they claim to have seen secretion by excised parietal cells, and they state that the secretion is a viscid material. They also claim that when the material is present in the canaliculi it gives a faintly positive reaction with Millon's reagent and is therefore a protein. Stöhr (1882), Zimmerman (1925), Revell (1912), and Ma (1927) have also observed a protein-like material in the canaliculi of fixed parietal cells.

It is obvious that the site of the formation of hydrochloric acid remains to be demonstrated. It has not been clearly shown that it is formed in or by the parietal cells. On the other hand the hypothesis that the parietal cells form either the acid or its precursor cannot seriously be attacked.

Evidence that there is a relation between blood carbon dioxide and gastric secretion is present in the work of Delhougne (1927) who found a decreased acidity in response

to a test after hyperventilation. Bakaltshuk (1928) found an increased gastric acidity after inhalation of carbon dioxide. Similar results are found in the work of Apperly and Semmens (1928).

Dodds and McIntosh (1923) found that the carbon dioxide content of the blood rises during gastric secretion and that there is a concommitant rise in alveolar carbon dioxide tension. Apperly and Crabtree (1931) found that the concentration of hydrochloric acid during a fractional test meal is determined by the bicarbonate content of the plasma. They also claim that the emptying time of the stomach, and hence the volume of gastric juice secreted, varies with the blood cH. Since the blood cH in turn varies with the ratio H_2CO_3 to $NaHCO_3$ the total acid secreted varies as $NaHCO_3$ times H_2CO_3 divided by $NaHCO_3$ or simply as H_2CO_3.

Brown and Vineberg (1932) studied the acid secretion of anesthetized dogs. The secretion aroused by vagal stimulation was inhibited by hyperventilation with air but not by air-carbondioxide mixtures. Acidosis produced by injection of lactic acid caused a decrease in blood carbon dioxide and inhibited gastric secretion. They argued that the inhibition was not the indirect result of disturbance of the oxygen supply, for secretion was unaffected by some degree of cyanide poisoning. However they also found that the gastric secretion stimulated by histamine was not affected by hyperventilation or acidosis after the secretion was fully established. Acidosis occurring before injection of histamine did inhibit secretion.

These experiments indicate a dependence of gastric

secretion upon the carbon dioxide content of the blood. In the course of speculation as to the nature of the mechanism of secretion these facts suggested that the secretion of the acid might be similar to the familiar chloride shift occurring across the membrane of the red blood cells. The chloride shift takes place in the following manner: when arterial blood passes through the capillaries it loses oxygen and takes up carbon dioxide. The reaction between water and carbon dioxide is relatively slow, and on account of this fact and on account of the inferior buffering power of the plasma very little carbon dioxide is converted into bicarbonate in the plasma. In the cells on the other hand there is the enzyme carbonic anhydrase which catalyses the reaction between carbon dioxide and water, and there is the superior buffering power of the reduced hemoglobin. Consequently in the cells carbon dioxide is rapidly converted to carbonic acid, and the acid is neutralized to bicarbonate. The large and rapid increase of bicarbonate in the cells over that in the plasma causes diffusion of bicarbonate from the cells into the plasma. Since the cell membrane is apparently impermeable to cations, electrical neutrality is maintained by a simultaneous and equivalent diffusion of chloride ions from the plasma into the cells. In the lungs where carbon dioxide is given off the reverse process takes place, and chloride shifts from the cells to the plasma.

It is thought that a modified version of this chloride shift might account for the secretion of chloride ions by the gastric mucosa. Since one of the central elements

of the mechanism in the red blood cells is the enzyme carbonic anhydrase, R. B. Fisher (personal communication) suggested that a search be made for the enzyme in the gastric mucosa. The search and its success together with its bearing on the problem of secretion will now be discussed.

II

Carbonic anhydrase was discovered independently by Stadie and O'Brien (1933) and by Meldrum and Roughton (1932). Its properties have been described by Meldrum and Roughton (1933), and its role in the respiratory cycle has been worked out by Roughton (1935). Meldrum and Roughton have also established the individuality of the enzyme and made a highly purified but non-crystalline preparation. Roughton (1934) has reviewed the literature concerning the enzyme.

The enzyme catalyses the reaction

$$H_2O + CO_2 \rightleftharpoons H_2CO_3$$

in both directions. Its activity is destroyed by brief boiling and by heating to 60° C. for twenty minutes. Its activity is also destroyed by standing at pH 2 or pH 13 for thirty minutes. The enzyme is inhibited by heavy metals and by M/800 HCN. It is also inhibited by carbon monoxide, but there is some doubt as to the reversibility of the inhibition by light.

Booth (1938) has described a specific inhibitor for the enzyme. The inhibitor occurs in the sera of pig, sheep, horse, ox, cat and rat but not in the sera of man, monkey or duck. The inhibitor has the properties of pseudoglobulin, and it has no effect on the uncatalysed rate of reaction.

Two general methods have been used to estimate the activity of the enzyme. The first was devised by Brinkman (1933) and greatly improved by Philpot and Philpot (1936). A solution of carbon dioxide is mixed with a solution of sodium carbonate. The pH of the solution becomes lower in the course of time on account of the formation of carbonic acid

from carbon dioxide and water. The change in pH is followed by a suitable indicator, and the time from the moment of mixing to the attainment of a definite pH is used as a measure of the rate of hydration of carbon dioxide. The method is very crude, and it is suitable only for rough work.

The second method is the accurate one of Brinkman, Margaria and Roughton (1933) improved by Meldrum and Roughton (1933) and by Roughton and Booth (1938). A diagram of the essential parts of the apparatus is given in Figure 1. A boat-shaped vessel having two troughs is blown from Pyrex glass. Its shape is such the 2 ml. of different solutions can be placed in it without mixing until the vessel is violently shaken after which mixing is rapid and complete. In one trough is placed 2 ml of a phosphate solution made by mixing equal volumes of M/5 Na_2HPO_4 and M/5 KH_2PO_4. The pH of the buffer is 6.8. In the other is placed 2 ml. of M/5 $NaHCO_3$ dissolved in M/50 NaOH. The boat is attached by a well-fitting stopper and a short length of pressure tubing to a xylene manometer. The other end of the manometer is connected with a compensation bottle of large volume containing air and a little water. The whole apparatus except the manometer and the motor driving the shaker is enclosed in an efficiently stirred water bath. After allowing ten minutes for thermal equilibrium to be reached the boat is shaken at 300 cycles per minute. Carbon dioxide is evolved from the mixed solutions, and the rate of evolution is measured by reading the manometer at the end of 0, 10, 20, 30, 45 and 60 sedonds and thereafter at 30 second intervals. Provided that the first half of the total carbon

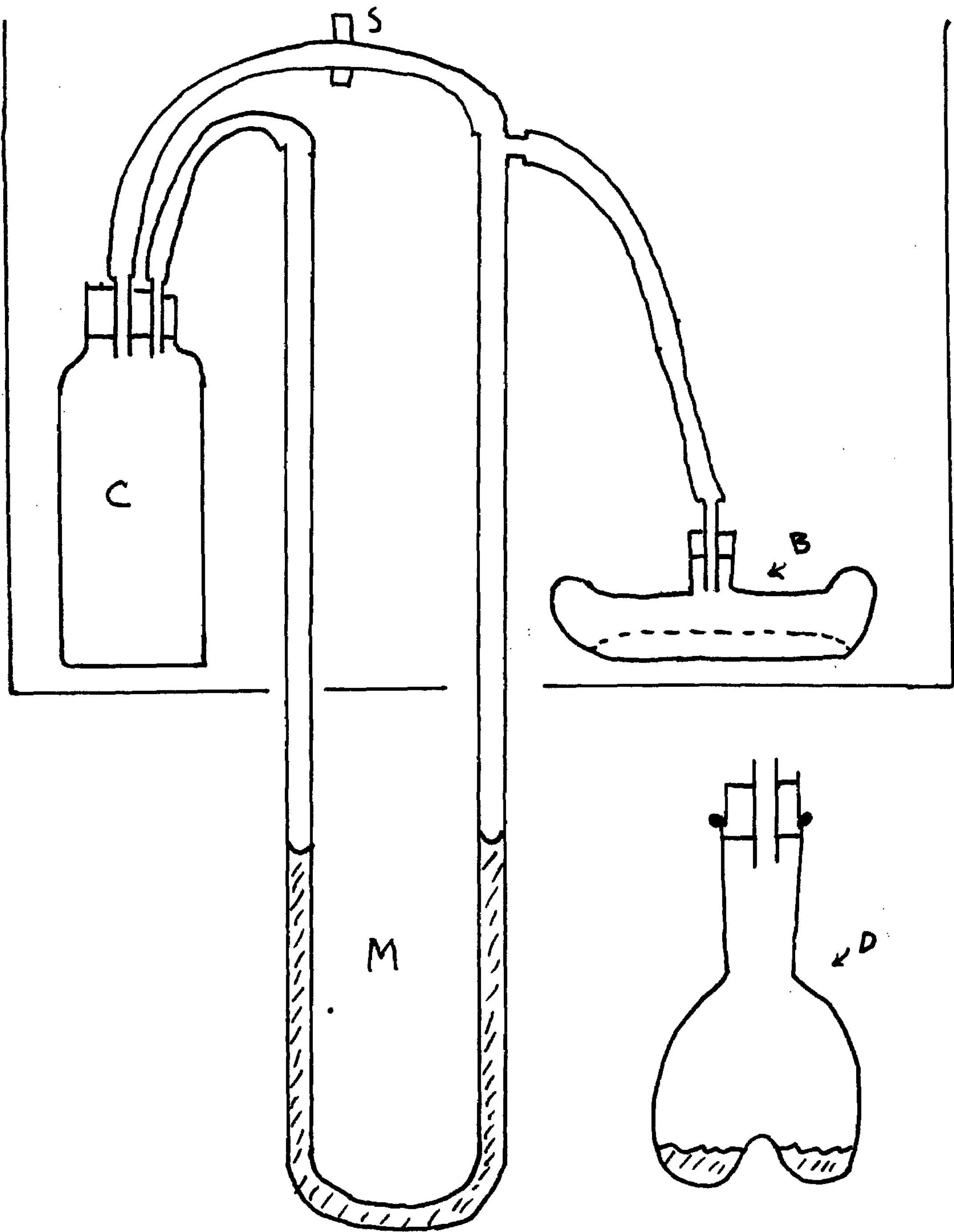


Figure 1

Diagram of carbonic anhydrase apparatus.
B is the boat; D is a transverse section of the boat; C is the compensation bottle; M is the manometer; S is a clamp.

dioxide is not evolved in less than 15 secomds, diffusion from the liquid to the gas phase does not prove to be a limiting factor, and the rate of gas evolution is a true measure of the rate of dehydration of carbon dioxide. A set of curves of the catalysed and uncatalysed reactions is shown in Figure 2.

If R_o be the rate of the uncatalysed reaction and R the rate of the catalysed reaction, it is clear that the fraction $(R-R_o)/R_o$ is a suitable measure of the catalytic activity. Meldrum and Roughton list the following reasons:

a) It is proportional to the amount of catalist added to within 5% provided the rate is not too fast.

b) It is independent of the kind of catalist used.

c) It is independent of the dimensions of the apparatus which need not even be known.

d) It eliminates initial errors due to the time taken for the solutions to mix after the shaker starts.

e) The range of pH change during the period is small and constant.

Meldrum and Roughton defined a privisional unit of activity as the amount of enzyme which, when dissolved in 4 ml. of the phosphate-bicarbonate mixture, gives a value of $(R-R_o)/R_o$ equal to unity at 15° C. The unit is called E.

The distribution of the enzyme has been studied by Brinkman and by Meldrum and Roughton. Brinkman found carbonic anhydrase in the blood of a number of invertebrates. The presence

or absence of the enzyme was independent of the nature of the resipratory pigment. The enzyme has been found in the red blood cells of all vertebrates so far examined. It is not present in cow's milk, urine or plasma. Traces have been found in the sperm of rabbits and in sea urchin eggs. Roughton (1934 and personal communication) found small amounts present in watery extracts of mammalian tissue even when these have been perfused with saline to remove contained blood. He was inclined to attribute the small quantities to traces of blood not removed by the perfusion. Brinkman reported that in the vertebrate pancreas the enzyme could be found free of hemoglobin.

Stimulated by the considerations listed in the first chapter Davenport and Fisher (1938) looked for carbonic anhydrase in the gastric mucosa of mammals. The method of Philpot and Philpot was used to estimate the enzyme concentration. The animals, cats, rabbits and rats, were killed by a blow on the head. At once a canula was inserted into the abdominal aorta, and the visceral blood vessels were perfused with 0.9% saline containing 6% gum arabic. The addition of the gum arabic prevented or reduced the edema occurring during perfusion with plain saline. After a variable length of time the vessels were observed to be free of blood. The gut was excised and the mucosa examined. It was usually observed to be a clear yellow, and any preparation suspected of not being free of blood was rejected. Simple aqueous extracts were tested for activity, and occasionally "crude chloroform" extracts were made according to the method of Meldrum and Roughton.

In Table I the amount of carbonic anhydrase found is listed. The units of activity are roughly those of Meldrum and Roughton. A dry preparation of carbonic anhydrase from the gastric mucosa of a cat was prepared and used to calibrate the method of Philpot and Philpot. The same preparation was tested according to the Meldrum and Roughton method through the courtesy of Dr. Roughton, and the results were used to convert the Philpot units to E.

The results show that there is a remarkably high amount of carbonic anhydrase in the gastric mucosa but practically none of the enzyme in the rest of the gut.

An apparatus for estimating carbonic anhydrase according to the method of Meldrum and Roughton was constructed. It was found to give completely consistent and reproducible results. The apparatus was used at 0° C. and at atmospheric pressure. The activity of preparation was calculated according to the method of Meldrum and Roughton, but no correction was applied to bring the calculated activity to 15° C. This is now the practice of Roughton (e.g., Roughton and Booth, 1938).

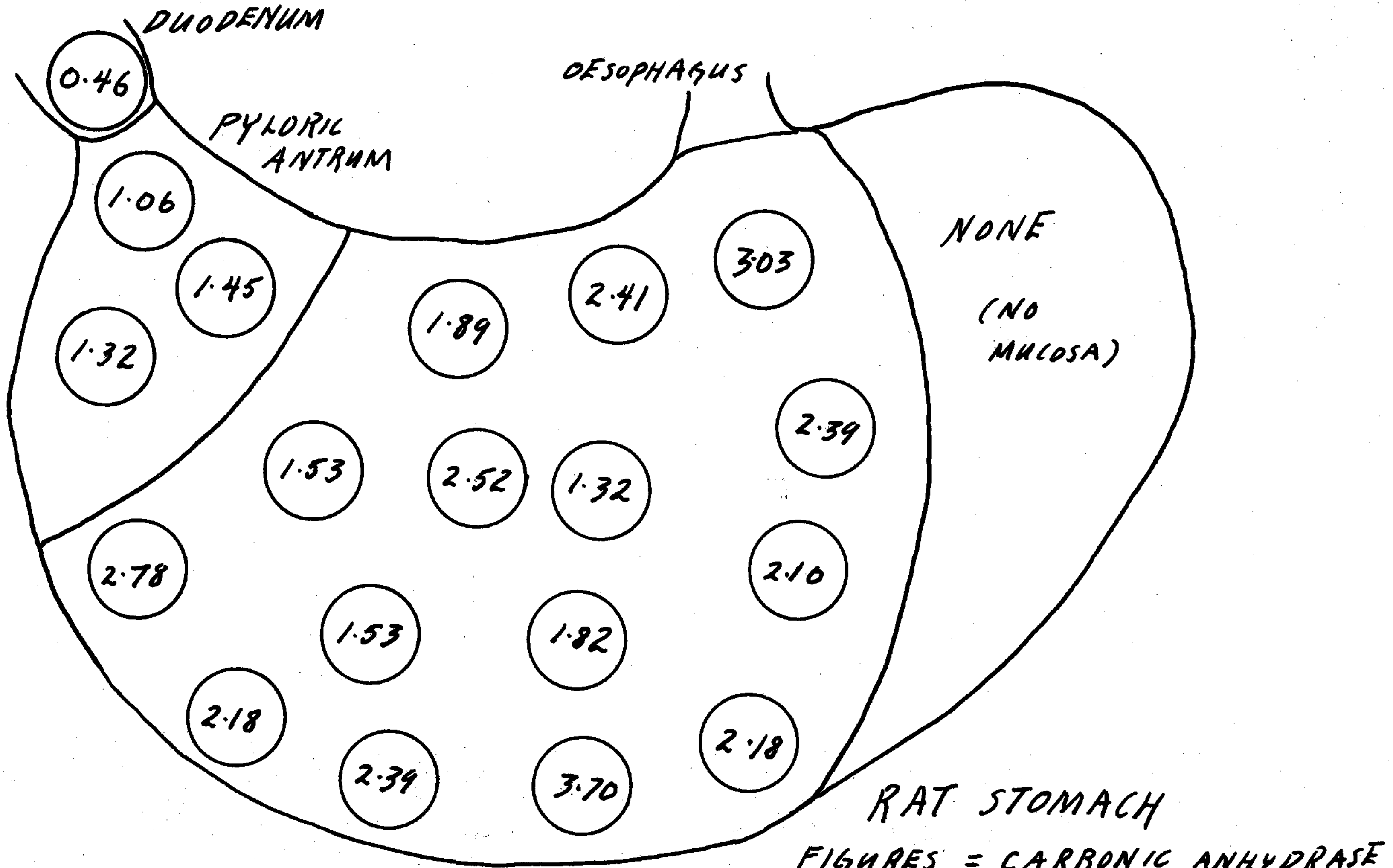
Using this method the distribution of the enzyme in the stomach of the white rat was determined. The animals were killed and the blood vessels perfused in the usual manner. The mucosa was exposed, and cylinders 4.0 mm. in diameter were stamped out with a cork borer. Aqueous extracts of the cylinders of mucosa were made, and the carbonic anhydrase content of the extracts was determined. Similar cylinders were weighed. The distribution of the enzyme is shown in Figure 3.

It can be seen that there is practically no enzyme

Table I

Carbonic anhydrase activity of the gastric mucosa. The units are roughly expressed in E/mg. wet weight.

	Rat	Cat	Rabbit
Stomach			
Cardiac third	2.5	5.8	1.5
Middle third	2.5	4.9	1.7
Pyloric third	2.3	3.4	2.0
Duodenum	0.8	0.6	0.5
Ileum	?	0.8	0.5
Pancreas	+	0.4	--



RAT STOMACH
FIGURES = CARBONIC ANHYDRASE PER MG. WET WEIGHT OF MUCOSA

FIGURE 3.

in the duodenum and relatively little in the pyloric antrum. The greatest concentration of enzyme is in the fundus near the greater curvature.

The earlier observations on the presence of the enzyme in the mucosa of rats, cats, guiena pigs and rabbits were repeated using the method of Meldrum and Roughton. The conclusion that the observed activity was caused by the presence of carbonic anhydrase in the mucosa is supported by the following observations: The extracts catalysed both the hydration and dehydration of carbon dioxide. The addition of the extracts to the phosphate buffer did not change the pH as measured by a glass electrode. The end points of the catalysed and uncatalysed reactions were the same. The activity of the extracts was completely destroyed by bliling thirty seconds, by heating to 60° C. twenty minutes, and by standing at pH 2 or Ph 13 thirty minutes. The activity was inhibited by M/800 HCN which has no effect on the non-enzymatic catalysis of the reaction. It was also inhibited by the specific inhibitor of Booth.

The activity of the extracts was not caused by included blood for the following reasons: Active extracts gave a negative benzidine test. Extracts to which very much blood has been added than would be needed to account for the activity gave strongly positive tests. Extracts from various parts of the mucosa showed the very characteristic distribution of activity, and it is unlikely that the distribution of included blood would be always the same. Some slices of the gastric mucosa of cats were found tb contain 3 to 5 per mm^3.

Meldrum and Roughton found that rat blood contains about 1.7 E per mm^3. Similar values have been found in the course of this work. Cat blood contains about 3.7 E per mm^3. Therefore had the activity of the slices of the gastric mucosa been caused by included blood the amount of blood included would have been equal to the volume of the slices. Upon histological examination of many slices of perfused mucosa small clumps of red blood cells have been seen on only three occasions.

The next research was undertaken to determine in what particular cells of the gastric mucosa the enzyme is present. A series of diagrams has been prepared to show the three main types of cells in the glands of the fundus. In Figure 4 is shown a longitudinal section of a gland of the rat's fundus with approximate dimensions. At the surface of the gland and lining the gastric pit are cuboidal surface cells. Below the neck of the gland are the chief cells and parietal cells. No attempt has been made to differentiate between the so-called neck chief cells and the chief cells. The former are supposed to secrete mucous and the latter pepsinogen. In Figure 5 is shown a longitudinal section of a gland from the cat's fundus. In the cat the gastric pit is very much deeper and well marked. The surface epithelium lining the pits is very characteristic. The cells are tall and very regular, and they are arranged into simple columnar epithelium. They are supposed to secrete mucin of a peculiar type. The rest of the gland is similar to that of the rat. In Figure 6 is given a diagram made from a camera lucida drawing of a cross section of a gland of the fundus of the

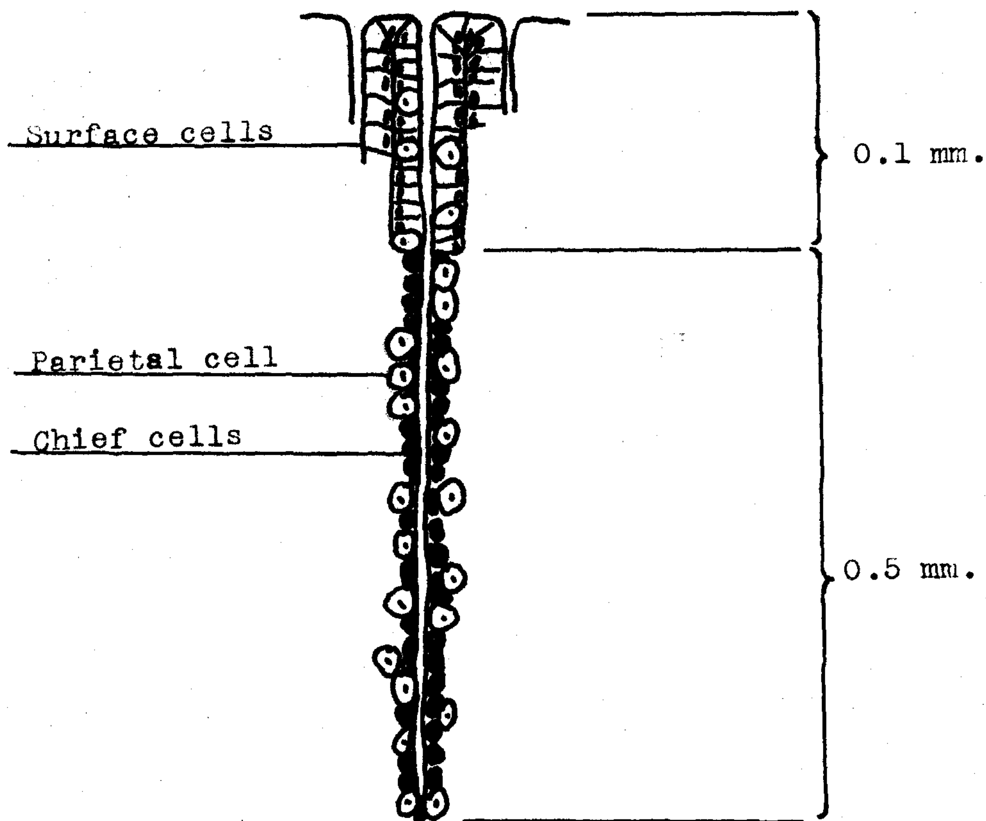


Figure 4

Diagram of a gland of the fundus of a rat's stomach.

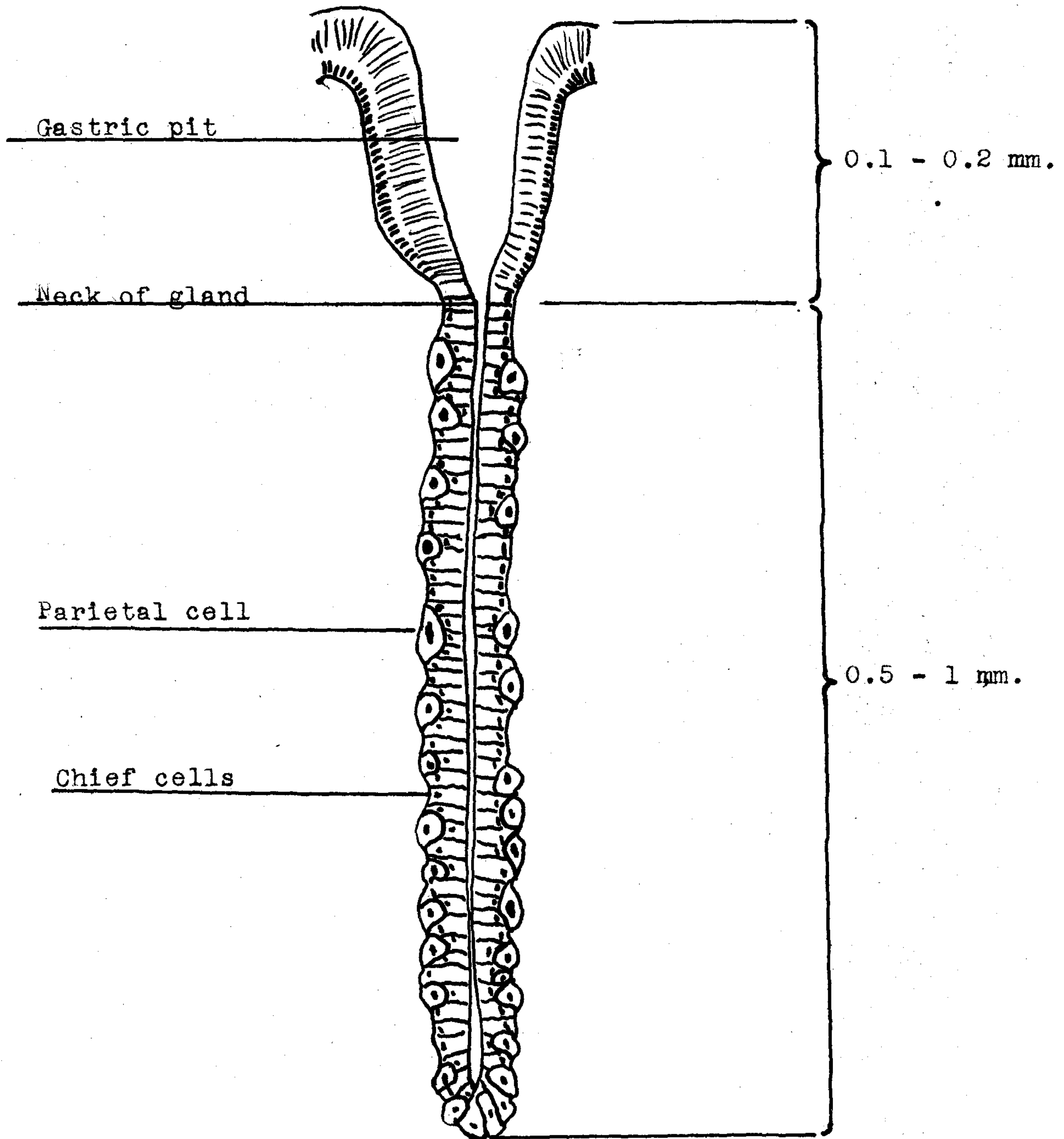


Figure 5

Diagram of a gland from the fundus of a cat's stomach.

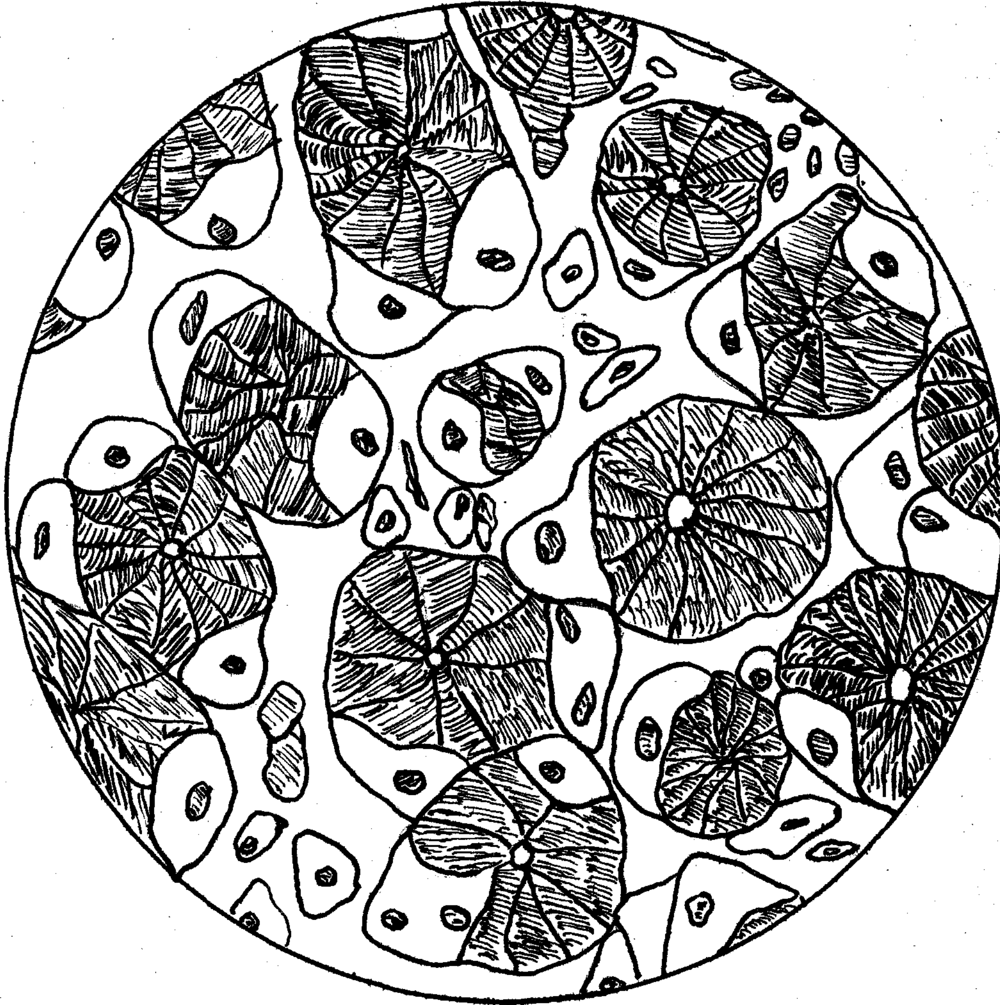


Figure 6

Diagram of a camera lucida drawing of a cross-section of the fundus of a rat's stomach. Section made with a freezing microtome. Chief cells hatched; parietal cells large and clear.

rat's stomach. The chief cells are seen to be numerous and to be arranged around the lumen of the gland. The parietal cells are less numerous, and they are situated at the periphery of the gland.

The best modern method localizing enzymes in particular cell is that applied to the gastric mucosa by Linderstrom-Lang et al. (1925). They stamped out cylinders of the mucosa with a cork borer and mounted the cylinders on the table of a freezing microtome so that the axis of the cylinder was perpendicular to the plane of cutting. They froze the tissue and cut serial sections 0.010 to 0.025 mm. in thickness. Each slice was analyzed for its enzyme content, and the cistribution of the enzyme with respect to the distance from the surface of the mucosa was determined. Using a larger cork borer they also stamped out a ring of mucosa from around the hole left by the cylinder. This was fixed and embedded in paraffin. Serial sections were cut to a thickness of 0.010 mm. in a direction parallel to the frozen sections. These sections were mounted and stained. The cells of any particular type were estimated in each stained section from the count of those cells in a few sample areas chosen from around the hole, and the distribution of the cell population with respect to the distance from the surface of the mucosa was found. The two distributions were compared, and correspondences in maxima and minima were taken as evidence that the enzyme was associated with the particular cell type under consideration.

This method has several obvious disadvantages clearly recognized by Linderstrom-Lang et al. It is assumed that the

lateral distribution of the enzyme is the same as that of the cells. The fixing and embedding of the ring changes its shape and dimensions relative to those of the fresh section, and correction must be made for this. The accuracy of the method depends on the correct numbering of the serial sections. It is difficult to determine which is the first section cut either with a freezing or with a rotary microtome, and consequently the distribution curves are subject to some error with respect to the distance from the surface. Also there is some difficulty in orienting the embedded pieces so that the sections are really parallel. The method does not allow any statistical estimation of the goodness of the relation between the two distributions. Finally the method is extremely laborious.

In order to overcome some of the difficulties a new method was devised which is believed to be considerably better than the older one. A cylinder 4.0 mm. in diameter was stamped from the gastric mucosa and mounted on the table of a freezing microtome in such a direction so that thin cylindrical sections could be cut whose axes were perpendicular to the surface of the mucosa. Thus the slices contained cross sections of the gastric glands. Sections 0.020 mm. thich were cut, and cutting was continued until <u>three consecutive sections</u> were cut which seemed satisfactory. The three sections chosen were apparently uniform in thickness and were without tears. Sections could not be cut from within 0.0 mm. of the surface nor from within 0.1 mm. of the muscularis mucosa coating the back of the mucosa, but otherwise the distribution of the position of these sections was random with respect to the distance from the surface of the mucosa.

The first and third of the consecutive sections were transfered directly from the knife to a small glass mortar made of a Pyrex test-tube fitted with a ground-in pestle. These sections were thoroughly macerated and extracted with exactly 3 ml. of the phosphate buffer used in the rate measurements. The 2 ml. of the extract was added to one trough of the boat, the bicarbonate to the other, and the carbonic anhydrase content was determined.

The second section was mounted on a microscope slide, fixed in Carnoy's solution and stained with Delafield's hematoxylin and aurantia. A camera lucida outline drawing of the section was made, and careful note was taken of the folds, tears and other imperfections. The area of the drawing was determined with a simple planimeter, and from the known magnification the area of the slice on the slide was calculated. From 64 slices the measured area had a mean of 12.9 mm^2. The area calculated for a disc of 4.0 mm. diameter is 12.6 mm^2.

There was considerable variation in the area of the slices. This can be seen from Tables III and V. It depended on the amount of stretching which occured when the slices were straightened on the slide. It is believed that this variation does not affect the final result of the cell counting technique as explained below.

The slice was observed under an ocular of 5X and an oil immersion objective. The total area of such a visual field was measured with a stage micrometer and found to be 0.0407 mm^2. By means of a mechanical stage the slice was moved in a systematic manner from left to right and from top to bottom so that

visual fields could be carefully observed over the whole slice. There was no selcetion of the fields, and the method of moving the stage provided that the fields observed were a fair sample of the whole slice. From 20 to 40 fields were observed on each slice.

In each field the total number of cells of one type was counted and recorded, and the stage was then moved so that another field could be counted. The parietal cells were recognized as large, clear yellow cells with more or less central nuclei, and they were situated at the periphery of the gland as shown in Figure 6. The chief cells were smaller, deep brown in color with a deeply stained reticulum.

The total number of cells counted in all the fields was mulitplied by the total area of the slice and divided by the total area of all the fields counted. The result was taken to be a good estimate of the total number of cells of one type in the slice. The variation in the size of the slice thus canceled out. With considerable stretching the slice would be bigger but the density of the cells in the fields counted would be correspondingly less. The reverse would be true where no stretching took place.

The number of cells of one type in a slice was plotted against the observed concentration of carbonic anhydrase in the two surrounding slices. In doing this it was assumed that the total amount of enzyme in the slices 1 and 3 would be equal to twice the amount in slice 2. Conversely it was assumed that the number of cells in the second slice was equal to the average of the numbers in the two surrounding

slices. These assumptions are inherently very reasonable, and they will be justified in the following discussion of the errors of the technique.

The regression line for the data was calculated. The regression line is the best straight line fitting the data as calculated by the method of least squares. It can generally be seen by inspection whether or not the two variables thus plotted have any real correlation, but there are several statistical tests which can be made. The correlation coefficient was calculated for the data.

The following discussion of the statistics of variation is taken largely from R. A. Fisher's "Statistical Methods for Research Workers," sixth deition, 1936.

Normal variation with one variate may be specified by a frequency formula. The frequency in any infinitestimal range dx may be written as:

$$df = \frac{1}{\sigma\sqrt{2\pi}} e^{-\frac{1}{2}\frac{(x-\mu)^2}{\sigma^2}} dx$$

where $x-\mu$ is the distance of the observation, x, from the center of distribution μ. σ is the standard deviation and measures in the same units the extent to which the individual values are scattered.

Where we have two variates the formula for the normal correlation surface may be written

$$df = \frac{1}{2\pi\sigma_1\sigma_2\sqrt{1-\rho^2}} e^{-\frac{1}{2(1-\rho^2)}\left\{\frac{x^2}{\sigma_1^2} - \frac{2\rho xy}{\sigma_1\sigma_2} + \frac{y^2}{\sigma_2^2}\right\}} dxdy.$$

In this expression x and y are the deviations of the two variates from their means and are not the values of the variates themselves.

σ_1 and σ_2 are the two standard deviations. ρ is the correlation between the variates x an y. The correlation may be positive or negative, but it cannot exceed unity. It is a pure number without physical dimensions.

If $\rho = 0$, the expression for the frequency degenerates into the product of the two factors:

$$df = \frac{1}{\sigma_1\sqrt{2\pi}} e^{-\frac{x^2}{2\sigma_1^2}} dx \quad \frac{1}{\sigma_2\sqrt{2\pi}} e^{-\frac{y^2}{2\sigma_2^2}} dy.$$

This shows that the limit of the normal correlation surface, when correlation vanishes, is merely that of two normally distributed variates varying in complete independence. At the other extreme, when ρ is +1 or -1, the variation of the two variates is in strict proportion, so that the value of either may be calculated accurately from that of the other.

These quantities denoted by the Greek letters σ_1 , σ_2 and ρ refer to the ideal quantities of an infinite population. It is the purpose of statistical calculation to obtain good estimates of these quantities. The estimates are known by the Roman letters s_1 , s_2 and r . r is called the correlation coefficient or the product moment correlation.

The three statistics are then

$$ns_1^2 = \Sigma(x^2)$$

$$ns_2^2 = \Sigma(y^2)$$

$$ns_1s_2 = \Sigma(xy)$$

Here as above x and y are the deviations of the variates from their means. N is the number of degrees of freedom, or one less than the number of pairs of observation in the sample.

By algebraic rearrangement

$$r = \frac{\Sigma(xy)}{\sqrt{\Sigma(x^2)\cdot\Sigma(y^2)}}$$

The actual operations of calculation are as follows. The notation is now changed and x and y refer to the real observed values of the variates. $\bar{x}$ and $\bar{y}$ are the calculated mean values, and hence $(x-\bar{x})$ and $(y-\bar{y})$ are the deviations which have been called x and y by Fisher.

n' is the number of pairs of observations

ⓐ Σx			
ⓑ Σy			
ⓒ Σx^2	$n'\bar{x}^2\ (= \frac{ⓐ^2}{n'})$.	$\Sigma(x-\bar{x})^2$	$(= \Sigma x^2 - n'\bar{x}^2)$
ⓓ Σy^2	$n'\bar{y}^2\ (= \frac{ⓑ^2}{n'})$	$\Sigma(y-\bar{y})^2$	$(= \Sigma y^2 - n'\bar{y}^2)$
ⓔ Σxy	$n'\bar{x}\bar{y}\ (= \frac{ⓐⓑ}{n'}$	$\Sigma(xy-\bar{x}\bar{y})$	$(= \Sigma xy - n'\bar{x}\bar{y})$

The calculations indicated in the left hand column are made, and then those in the middle and right hand column are derived from them. It can be seen that r can then be calculated from the values in the right hand column by the equation

$$r = \frac{\Sigma(xy - \bar{x}\bar{y})}{\sqrt{\Sigma(x-\bar{x})^2\cdot\Sigma(y-\bar{y})^2}}$$

which is the same as the equation for r given above with different notation.

Also the coefficients of the regression line

$$Y = bx + A$$

can be calculated.

$$b = \frac{\Sigma (xy - \bar{x}\bar{y})}{\Sigma (x - \bar{x})^2}$$

and

$$A = \frac{\Sigma y - b \Sigma x}{n'} .$$

In testing the significance of an observed correlation we require to calculate the probability that such a correlation should arise by random from an uncorrelated population. The correlation coefficient does not have a normal distribution in small samples so an estimate of its significance cannot be made by applying the standard deviation. However r can be transformed into a statistic z which has an approximately normal distribution.

$$z = \tfrac{1}{2} \left\{ \ln (1 - r) - \ln (1 - r) \right\} .$$

As r changes from 0 to 1, z will pass from 0 to ∞.

z can either be calculated or found from a table such as Table VB in Fisher. It has a standard error of $\sqrt{\frac{1}{n'-3}}$. In the case of a normal distribution there is less than one chance in a hundred that z lies outside the limits of $\pm$ 2.977 times its standard error. Therefore the limits of z can be calculated and from them the limits of r. There is less than ine chance in a hundred that the true value of r lies outside these limits.

It was expected that this treatment of the data would show whether or not there was any real correlation between the numbers of calls of any one type and the amount of carbonic anhydrase.

Before the data are presented there must be a discussion of the errors of the method. Linderstrom-Lang et al. list the errors to which their method is subject. Since the present method is a modification of their's it is subject to many of the same errors. The list is transcribed below with a discussion of the way the errors are controlled in the present method.

A. Errors in enzymatic estimation.

1. Errors in principle (bad contact between enzyme and sutstrate, destruction of enzyme, incomplete activation). In the present method the maceration of the tissue and its extraction ensures adequate contact between enzyme and substrate. The enzyme is stable for days in the buffer and needs no activation.

2. Errors in technique (variations in section thickness). While this error is important in Linderstrom-Lang's method, it is avoided in this one. Every precaution was taken to obtain uniform thickness. However provided the errors were distributed at random about a mean the error does not enter into the final result.

B. Errors in histological control.

1. Errors in principle.

a. Errors in chosing observation area. Linderstrom-Lang chose areas in the rings close to the central hole and counted cells in less than ten fields.

The method of moving the slice used here and the larger number of areas counted were designed to provide an adequate sample and randomness in the choice of areas.

b. Errors in identifying cells. This error depends upon subjective factors difficult to control.

c. Errors due to loss of cells and other imperfections in the slices. This error is also difficult to control. Sections which were damaged were discarded and only fairly good ones used for counting. In the good sections there was apparently no loss of cells except possibly around the edges. This would probably occur both in the slices used for counting and for enzyme estimation and would not effect the results.

2. Errors in technique.

   a. Microtome errors. Since the sections used for enzyme estimation and for counting were serial sections the errors would be the same in each. If the errors were at random they would not affect the final result.

   b. Errors in drawing and measuring the slice and in counting. Again if the errors were at random they would not affect the result.

There are two fundamental assumptions involved in

this method. The first is that the concentration of enzyme in the cells of any type are distributed at random about a mean. It is not possible to test this assumption, but it seems entirely reasonable.

The second assumption is that the number of cells in the second section is equal to the average of the number of cells in the first and third slices, thereby providing an accurate estimate of the number of cells in those slices. This assumption was tested by counting the parietal cells in several sets of serial sections. The results are given in Table II. It can be seen that the assumption is justified.

In consideration of these facts it seems correct to assume that the number of cells in the slice counted is a good estimate of the number of cells in the slices in which the enzyme was determined. Therefore the degree of correlation between the cell count and the enzyme concentration should be a good test of the hypothesis that the enzyme is present in that cell type.

The first data are derived from the mucosa of the fundus of the white rat.

In Figure 7 the concentration of carbonic anhydrase per slice is plotted against the parietal cell count per slice. The line drawn is the regression line. Its equal is

$$Y = 0.000043\ x + 0.10.$$

Y is the enzyme concentration per slice and x is the number of parietal cells per slice.

The correlation coefficient (r) is $+\ 0.95$.

The statistic z has been calculated as explained above. Its value is 1.832 ± 0.229. Therefore the limits of z are 2.52 and

Table II

Parietal cell counts in serial sections.

Number of slice	Cells found	Mean of two surrounding slices	per cent. difference
1	5610		
2	5750	5680	+1.2
3	4850		
1	6450		
2	6050	6065	+0.2
3	5680	5685	+0.1
4	5340		
1	10350		
2	11000	11950	+8.6
3	13550	12200	-10.0
4	13400	12525	-6.5
5	11500		
		Mean	-1.1

and 1.15. These correspond to limits of r of 0.81 to 0.99. There is only one chance in a hundred that the correlation between the two variables falls outside these limits. As both limits give extremely high correlation, especially for unselected biological material, there is no doubt that the variables are highly correlated.

The data are given in Table III.

The chief cells in ten sections were counted. The values are plotted in Figure 8 and the data are given in Table IV. It can be seen at a glance that there is no correlation between the variables. The calculated correlation coefficient is + 0.15 which for these data is insignificantly different from zero.

It was not possible to distinguish other types of cells, e. g., Heidenhain's cells or the argentaffine cells. Consequently it is not absolutely certain that the carbonic anhydrase is not associated with the other types of cells. However the correlation between the enzyme content and the parietal cell count is so extraordinarily good that such other correlation is unlikely. It is further unlikely that any other type of cell would have perfect correlation with the parietal cells over the whole range of from no cells to about 15,000 cells per slice. This would be necessary if the enzyme were really in another type of cell and the correlation between enzyme and parietal cells were only apparent. The conclusion is therefore almost inescapable that the enzyme is very closely associated with the parietal cells, that in fact it is actually in those cells.

It should be pointed out that this is the best evidence that has so far been offered that a particular enzyme is actually

Table III

Parietal cells in the rat fundus.

No. of slice	Area of slice	No. of fields counted	No. of cells counted	Total number of cells per slice	E per slice
37	12.5 mm^2	20	8	0	0.098
32	7.25	22	222	1870	0.089
31	10.75	40	267	1650	0.245
13	15.25	33	592	6710	0.264
2'	13.25	20	281	4350	0.290
17	13.75	20	363	6150	0.324
6	16.50	27	341	5130	0.340
5'	12.00	24	548	6750	0.343
15	15.00	24	435	6690	0.394
1'	12.50	20	406	6250	0.420
4	14.50	26	489	6700	0.445
10	16.50	23	591	9140	0.463
18	13.25	20	593	9670	0.501
21	16.00	20	508	10000	0.501
3'	14.00	22	679	10600	0.502
1	12.10	39	1087	10670	0.552
16	14.75	30	612	7390	0.561
12	13.25	28	1003	11620	0.616
9	13.50	25	1086	14400	0.695
14	14.00	24	813	11600	0.695
3	18.25	30	1039	15400	0.762
8	13.75	30	1317	14850	0.770

Table IV

Chief cells of the rat fundus.

No. of slice	Cells per slice	E per slice
32	35000	0.089
7	84000	0.098
13	75000	0.264
5	49000	0.343
6	76000	0.340
18	28000	0.501
12	53000	0.616
3	94000	0.762
8	51000	0.770

in a particular cell. The ordinary method used for such conclusions are the dubious ones of histology. Stains of various types are applied to tissues, usually fixed, and from the appearance of the selcetions the conclusions are drawn. With the exception of the work of Linderstrom-Lang ět. al., this has been the method used for the gastric mucosa. Also from the remarks above it should be clear that this method, despite its faults, is more sensitive and accurate than that of Linderstrom-Lang.

There was no detectable difference between 16 pairs of observations taken from rats with full stomachs and 6 pairs taken from rats with empty stomachs. Consequently both sets of observations are pooled in the calculations. It can be concluded that if the enzyme is secreted, it is secreted in amounts relatively small compared with the amount in the cell.

It is seen from Figure 2 that there is a small amount of the enzyme in the pyloric antrum of the rat. The pyloric antrum is anatomically and histologically distinct from the fundus. The glands of the antrum are about half as long as those of the fundus, and they contain no pariteal cells. It was not possible to apply the slicing technique to the antrum mucosa, for the tissue is very friable. Consequently it was not possible to tell directly in what cells the carbonic anhydrase is contained.

The standard error of the intercept (A) on the Y axis ($Y = 0.10$) is ± 0.032. The value of A lies between the limits 0.04 and 0.196. The standard error is very high, being 30% of the estimate of A. The value of A is consequently somewhat indeterminate. However there is only one chance in two hundred of its being zero.

That the intercept on the Y axis is significantly greater than zero means that there is a small amount of the enzyme more or less uniformly distributed throughout the mucosa. If the specific gravity of the mucosa be approximately one, then the amount of carbonic anhydrase not associated with the parietal cells is about 0.4 per mg. This would account for part of the enzyme in the pyloric antrum.

From the above equation one million parietal cells are seen to contain 43 E. The average diameter of the cells is about 0.017 mm., and assuming them to be spherical the volume of one million cells is 2.6 mm^3. There is about 17 E per mm^3. If the red blood cells comprise about half the volume of the blood they have 6 E per mm^3. or less. Therefore the carbonic anhydrase is about three times as concentrated in the parietal cells as in the red blood cells.

Exactly the same technique was applied to the gastric mucosa of the cat. First the distribution of the enzyme was determined. This is shown in Figure 9. There is a large amount of the enzyme in the fundus where the parietal cells are abundant and a small amount in the fundus where parietal cells are absent.

When the freezing microtome technique was used it was found that the slices fell into two well marked groups. The first group was composed of slices cut from the base of the glands and contained only parietal cells, chief cells and the usual connective tissue. The second group contained slices cut from nearer the surface, and in addition to parietal cells and chief cells contained more or fewer gastric pits made up of long tapering surface cells. The two groups will be considered separately.

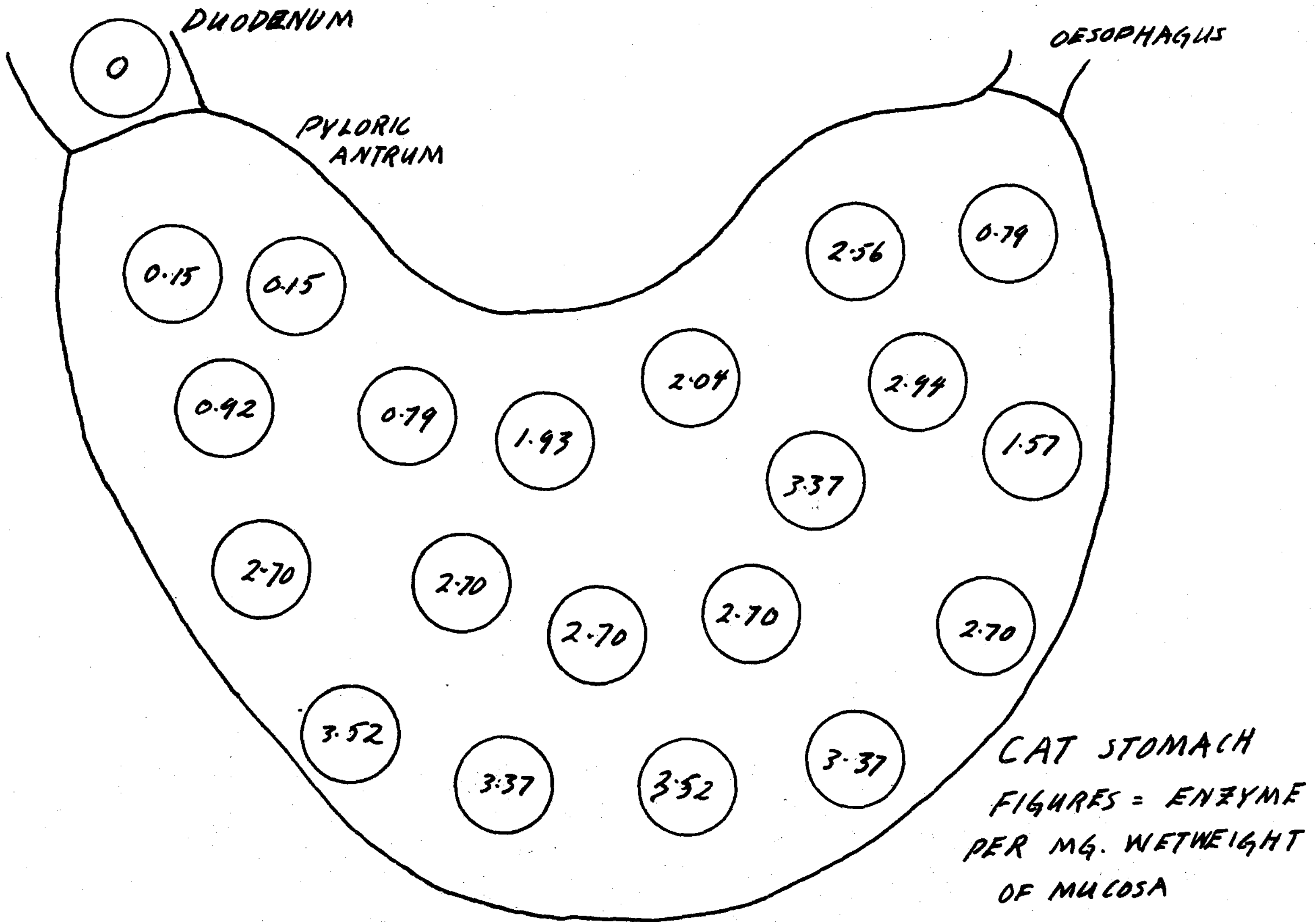


FIGURE 9

In the group containing parietal cells and chief cells the praietal cells were counted in the usual manner. In Figure 10 the parietal cell count is plotted against the enzyme concentration. The line drawn is the regression line. Its equation is

$$Y = 0.000102\,x - 0.036$$

where Y is the enzyme concentration per slice and x is the number of cells per slice. The correlation coefficient is +0.95. The limits are exactly the same as those found for the rat correlation coefficient, +0.81 to +0.99. The correlation coefficient is again extremely high, and the remarks made as to its significance in the case of the rat also apply here. The enzyme is in all probability in the parietal cells.

The data are given in Table V.

The slope of the curve is much steeper than that of the rat curve. There are fewer parietal cells in the cat slices, but there is also two and one half times as much carbonic anhydrase in the cells. By a calculation similar to that made for the rat it is found that the carbonic anhydrase is five to six times as concentrated in the parietal cells as in the red blood cells.

The intercept on the Y axis is negligibly different from zero. In these slices there is probably no enzyme in any other type of cell.

In the group containing surface cells as well as parietal and chief cells the enzyme concentration was obviously not proportional to the number of parietal cells.

This led to the supposition that a certain amount

Table V

Parietal cells of the cat fundus. Slices containing no gastric pits.

No. of slice	Area of slice	No. of fields counted	No. of cells counted	Total number of cells per slice	E per slice
C3	-	-	0	0	0.026
C24	4.25*	10	146	1530	0.070
C51	14.25	30	263	3040	0.198
C50	14.50	34	359	3770	0.216
C21	10.75	38	226	1920	0.243
C52	10.50	21	276	3380	0.277
C40	8.25	20	270	2750	0.291
C53	14.50	26	304	4180	0.293
C47	10.25	20	289	3850	0.413
C55	14.50	20	312	5650	0.428
C19	15.25	28	421	5650	0.488
C22	11.00	20	376	5080	0.531
C43	11.50	30	572	5400	0.620
C42	15.50	30	595	7550	0.620
C20	11.00	36	925	6950	0.660
C54	15.50	20	363	6900	0.705
C17	13.00	25	640	8200	0.816
C16	13.00	30	652	7000	0.853
C23	13.50	28	789	9350	0.853
C45	12.50	36	758	9100	0.950

of carbonic anhydrase might be in the surface cells. This was tested in the following manner. The parietal cells were counted in the usual way, and from the equation above the most probable amount of enzyme in that number of parietal cells was calculated. This was then subtracted from the total amount of enzyme found, thereby giving the residual carbonic anhydrase, Then the surface cells were estimated. The number of cells in observational fields was counted, and it was gound that owing to the regularity of the gastric pits the number per field was remarkably close to the average of 160 per field. From this average and from the area occupied by the gastric pits the total number of surface cells per slice was calculated. This method is inferior to that of direct counting, but it was made necessary by the fact that the areas containing surface cells was much more fragile than that containing parietal and chief cells and was consequently much less perfect. Direct counting was impossible in a number of slices. The data are given in Table VI. In Figure 11 the residual enzyme is plotted against the estimated number of surface cells. The broken line is the parietal cell curve plotted on the same scale for reference. The fewness of the data and their obvious imperfection prevent any elaborate calculation as to their significance. The errors involved are not only those of the previous curves but additional ones introduced by the method of counting and the poorness of the slices. However it is clear that the excess enzyme is accounted for by the presence of the enzyme in the surface cells. There is approximately a tenth as much as in the parietal cells.

Table VI

Residual carbonic anhydrase and surface cells.

No. of slice	Total area	Surface cell area	Parietal cell area	Total parietal cell count	CA in parietal cells	Total C.A.	Residual C.A.	Total surface cells
C1	12.50	6.25	6.25	2700	0.238	0.440	0.202	24700
C4	10.25	6.75	3.50	1180	0.084	0.483	0.399	26600
C5	9.75	7.00	2.75	2070	0.173	0.483	0.310	27800
C7	11.25	4.75	6.50	2800	0.249	0.390	0.141	18700
C8	12.25	12.25	0	0	0	0.483	0.483	49300
C15	11.75	5.50	6.25	7200	0.694	1.011	0.317	21600
C25	10.75	1.00	9.75	1920	0.159	0.243	0.084	3900
C41	7.75	1.50	6.25	460	0.012	0.081	0.069	5900
C64	11.25	5.25	7.00	2100	0.178	0.453	0.275	20700
C68	12.75	4.50	8.25	1000	0.070	0.307	0.237	17700

This conclusion is supported particularly well by the highest point on the curve and by a number of slices that were so damaged that they could not be included in the table. In these slices there were no parietal cells at all, yet there was considerable amount of the enzyme.

There was no difference between pairs of observations made on fasting cats and on cats in the midst of digesting a large meal. As for the rat, it can be concluded that if the enzyme is secreted by the cat gastric mucosa it is in amounts small in comparison with the amount in the cells.

That the enzyme may also be in the surface cells of the rat gastric mucosa is indicated in Figure 12 where the enzyme concentration is plotted against distance from the surface. These curves were obtained by stamping out cylinders and slicing serial sections to a thickness of 0.020 mm. with a freezing microtome. Beginning with the first good section every section was analysed for its enzyme content. The distance from the surface was found by keeping track of the number of the section in the series. The blue line in the figure is the parietal cell distribution found from counting serial sections.

The right hand portion of the enzyme curve is similar to that of the parietal cells as expected, but the left hand portion is not. There is more enzyme near the surface than can be accounted for by the presence of parietal cells.

Again attempts were made to apply the slicing and counting technique to the surface of the mucosa of the rat. Unfortunately by the time an evenly cut section was obtained the surface cells had been cut off. In sections cut obliquely

to the surface it was found that the surface cells fell off. This is also often true even of paraffin sections.

While the evidence from cats cannot be applied directly to rats, it is good supporting evidence. Taken in conjunction with Figure 12 it allows the conclusion to be drawn with reservations that the enzyme is also present in small quantities in the surface cell of the rat gastric mucosa.

It is also possible that the enzyme is confined to one or more types of cells in the pyloric antrum, and that the constant A in the equation for the rat parietal cells is not sufficient to account for all the enzyme in the antrum. In Figure 13 the concentration of the enzyme in the pyloric antrum of the rat is plotted against the distance from the surface of the mucosa. The three curves are similar. There is a high concentration at the surface, a drop to low concentration and another rise followed by a drop. "In the glands of the pyloric canal . . . the secreting tubules possess cells of only one kind. These appear to correspond with the 'mucoid' cells of the fundus glands . . . They are also quite unlike the epithelium cells of the surface and ducts, which is formed, as elsewhere, of long tapering cells. (Sharpey-Shafer, 1934)." If the enzyme were in these two types of cells in different amounts their distribution would account for the shape of the curves in Figure 13. Attempts to apply the slicing technique to the mucosa of the pyloric antrum were unsuccessful for the reason that the mucosa is too thin and friable. Good sections could not be obtained. Consequently the point must remain unsettled. The small amount

of the enzyme to be accounted for is relatively unimportant in comparison with the large amount in the parietal cells.

It was thought possible that there might be some carbonic anhydrase in the gastric juice. In order to test this supposition cats and rats were used. A tube was passed into the stomach and some phosphate buffer at pH 6.8 was introduced in order to neutralize the acid that would inactivate any enzyme present. Fifteen minutes later the stomach contents were withdrawn and analysed for the enzyme. In all cases a small amount of the enzyme was found. This enzyme had all the characteristics of that found in the blood or gastric mucosa.

Unfortunately this cannot be taken as proof that the enzyme is secreted into the gastric juice. More or less traumatic blood may have been present in the samples. Even with humans willing to swallow the tube this is often the case. In fact several rat samples were discarded on account of obvious blood, but none was seen in the cat samples. Also it is possible that in passing the tube some surface cells were scraped off. According to Maximow (1934) even under physiologic conditions many of the surface cells are desquamated and perish. The presence of blood or surface cells would account for the small amount of enzyme found. It will not be possible to settle this point definitely until samples are obtained from animals with suitable gastric fistulas.

Numerous experiments were performed on one human subject. No other normal humans could be persuaded to swallow the tube. A soft rubber duodenal catheter was swallowed only

to a length sufficient to allow it to enter the stomach. The subject became very expert at swallowing the tube, and it is unlikely that the samples contained any traumatic blood. None was ever observed.

The tube passed, and 100 - 200 ml. of phosphate buffer was introduced into the stomach. After 30 minutes the stomach contents were withdrawn. The experiment was performed 14 times with the subject fasting and 3 times after a bland, enzyme-free meal had been taken. The gastric juice was always found to contain more or less of a substance which accelerated the hydration and dehydration of carbon dioxide. On account of the introduction of the buffer into the stomach and the consequent dilution of the gastric juice it is not possible to obtain a good estimate of the amount of the substance present in the gastric juice. However the order of magnitude was about 300 E per 30 minutes sample. An attempt was made to prove the substance identical with blood carbonic anhydrase.

The substance was destroyed by 30 seconds boiling, by 20 minutes heating to 60° C., by 30 minutes standing at pH 2 or pH 13, and it was inhibited by M/800 HCN. It differed from the carbonic anhydrase in the subject's own blood in not being inhibited by rat serum. This could be caused by one of two things. Either the substance in the gastric juice is identical with blood carbonic anhydrase or it is not. In the first case it is necessary to postulate another factor in the gastric juice which either destroys or masks the inhibitor from rat serum. In the second case the substance might be an enzyme similar in action to that of blood carbonic anhydrase but differing from it in

that it is not inhibited by the specific serum factor.

The following experiment was designed to distinguish between these possibilities. The results are given in Figure 14. A certain amount of carbonic anhydrase from the subject's own blood was tested for activity (A). Four times that amount was found to have four times the activity (B). Activity A was completely inhibited by rat serum, and activity B was inhibited 58% by the same amount of serum (C and D). Then a partially purified preparation of the gastric juice substance was tested for activity (E). This activity was entirely unaffected by the addition of rat serum (F). The activity of the gastric substance and the blood carbonic anhydrase was found to be additive (G). Then rat serum was added to the mixture of gastric substance and blood carbonic anhydrase. The amount added was sufficient to inhibit 58% of the activity. However it was found that exactly that fraction of the activity caused by the blood carbonic anhydrase (36%, H) was inhibited. This experiment shows that the gastric juice does not contain a factor preventing the action of the inhibitor. Therefore the substance in blood and gastric juice must be different. Consequently the substance in gastric juice cannot be derived from traumatic blood that must have a different origin.

Obviously more work must be done on this human gastric factor. It is necessary to make extracts of human gastric mucosa and to observe the distribution of the substance as was done in the case of the cat and rat. It has not been possible to do this on account of the scarcity of material. A few observations have been made on the samples of gastric juice obtained from

hospital patients. The results are shown in Table VII. It can be seen that abundant substance was found in a case of hyperacidity and none in a case of pernicious anemia having complete achlorhydria.

Table VII

Human gastric juice substance.

Subject	Source	Diagnosis	Treatment	Presence of substance	Destroyed by 30' at 100C.	Inhibited by M/800 HCN	Inhibited by Rat serum
H.D.	-	Normal acidity	-	++	Yes	Yes	No
A.B.	Huntington Mem. Hospital	Hyper-acidity	Alkaline powders	++++	Yes	Yes	No
M.S.	"	P.A. with achlor-hydria	none	-			
			½ hr. after histamine	-			

III

Roughton points out that it is advantageous to the animal that carbonic anhydrase is confined to the red blood cells. As a result carbonic acid is formed most rapidly and to the greatest extent only at a place where there is a very efficient buffering mechanism, thereby preventing acidosis in the tissues. The presence of carbonic anhydrase in such high concentration in the parietal cells means that any carbon dioxide formed in those cells would be converted to carbonic acid with extreme rapidity. This fact cannot fail to be important for the formation of hydrochloric acid. It is probable that considerable amounts of carbon dioxide are formed in the parietal cells from metabolites, and it is equally probable that no buffering mechanism comparable with that of the blood is present. If this be the case the formation of some acid *de novo* in the parietal cells can be accounted for.

This does not explain the secretion of acid, for it is necessary to account for the high concentration of the acid and the fact that it is hydrochloric and not carbonic acid. However it is obviously a fundamental fact which must be considered seriously in any discussion of the theories of acid secretion.

The first theory to be reviewed is that of Bensley and his colleagues (Harvey and Bensley, 1912; Bensley and Hoerr, 1936). Bensley believes that a protein hydrochloric is secreted by the parietal cells and that in the gastric pits it is transformed into hydrochloric acid and neutral protein

by the secretion of water from the surface cells. The evidence supporting this theory is that Bensley and his colleagues have seen the secretion of protein-like material by the parietal cells and that the presence of acid in the parietal cells has not been unequivocably demonstrated.

This theory is inherently extremely implausible. There is no protein detectable by the Biuret test in the acid secretion of the gastric mucosa (Hollander, 1934). The acid in this secretion is 0.17 molal. If each protein molecule were associated with one thousand molecules of HCl and if the protein had a molecular weight of 35,000 one would expect at least 35 grams of protein to be present with one mol of acid. This would be about 6 grams per liter of gastric juice which would be easily detectable. In order to save the hypothesis one must also assume reabsorbtion of the protein.

The protein hydrochloride is supposed to be converted into 0.17 molal HCl and neutral protein simply by dilution. In making the protein hydrochloride in the parietal cells it is presumed that the reverse process takes place. This requires the initial presence of the acid in the cells which defeats the purpose of the theory.

An experiment by Osborne (1901) is thought to have some bearing on this problem, and since it is frequently quoted (e.g., Bodansky, 1938) it is worth while disposing of it. Osborne dissolved edestin in a sodium chloride solution and then precipitated it by passing carbon dioxide through the solution. The precipitate was edestin hydrochloride, and the supernatant solution contained sodium bicarbonate. Since the isoelectric

point of edestin is 6.9 and since a solution of carbon dioxide must have been more acid than this it is not surprising that the precipitate was hydrochloride. The bearing of this on the secretion of acid is remote.

The theories of Donnan have often been used in connection with the secretion of acid. If one starts with the following system secretion of acid will take place.

Initial State

Substance	R^+	Cl^-		H_2O
Concentration	C_1	C_1		pure

Final State

Substance	R^+	Cl^-	OH^-		H^+	Cl^-
Concentration	C_1	$(C_1 - x)$	(x)		(x)	(x)

Such systems have been constructed _in vitro_ and have been found to support Donnan's deductions. Donnan is of the opinion, and he is echoed by others (e.g., Bodansky, 1938; Gortner, 1938), that given the proper ampholyte this mechanism could account for the secretion of acid by the parietal cells.

This secretion requires that the membrane be permiable to snall anions. If this were the case one would expect that the secreted fluid would contain in addition to chloride all the anions present in the cell, as for example phosphate, sulphate, lactate, etc. This is not the case. Also it is necessary to assume that the membrane between the cell and the blood capillaries is very different, for otherwise acid would equally well be secreted into the blood. Obviously the theory as it stands is much too simple.

The most telling criticism of the theory is derived from a consideration of the nature of the ampholyte. Presumably it is a protein positively changed. Actually all the proteins of the cell must be concerned in the equilibrium, and there must be a protein present in sufficiently high concentration and with a sufficient number of positive charges so that the weighted mean of all the proteins is similar to that required by the theory. From the well-known equations of Donnan we have

$$\frac{(Cl^-)_j}{(Cl^-)_c} = \frac{(H^+)_c}{(H^+)_j}$$

where the subscripts c and j represent cell and gastric juice respectively. Substituting the values in the final state as noted above we have

$$\frac{(Cl^-)_j}{(Cl^-)_c} = \frac{x}{C_1 - x}$$

and

$$\frac{(H^+)_c}{(H^+)_j} = \frac{\frac{K_w}{x}}{x} = \frac{K_w}{x^2}$$

therefore

$$\frac{x}{C_1 - x} = \frac{K_w}{x^2}$$

or

$$x^3 = K_w(C_1 - x)$$

or

$$C_1 = \frac{x^3}{K_w} + x$$

Now x is about 0.17 molal and hence

$$C_1 = \frac{[0.17]^3}{10^{-7}}$$

or

$$C_1 = 4.9 \times 10^5$$

C_1 must also be the concentration of the ampholyte. Egg albumin (Schmidt, 1938) has 27 positively charged groups per molecule, and other proteins have similar numbers. If we assume the reasonable value of 50 positive charges per molecule the concentration of the amoholyte must be approximately 10^4 which is fantastic.

The most popular theory has been that of Maly (1878). He assumed that the acid is formed according to the equation

1) $NaH_2PO_4 + NaCl \rightleftharpoons Na_2HPO_4 + HCl.$

The sodium dihydrogen phosphate is reformed:

2) $Na_2HPO_4 + H_2O + CO_2 \rightleftharpoons NaH_2PO_4 + NaHCO_3.$

These two equations can be added with the result:

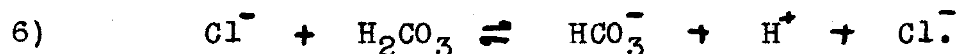
3) $CO_2 + H_2O + NaCl \rightleftharpoons NaHCO_3 + HCl.$

Since we have the two equations

4) $CO_2 + H_2O \rightleftharpoons H_2CO_3$

and 5) $H_2CO_3 \rightleftharpoons H^+ + HCO_3^-.$

we can write the master equation as



6) $Cl^- + H_2CO_3 \rightleftharpoons HCO_3^- + H^+ + Cl^-.$

It is generally assumed that there is some mechanism which separates the products, HCL being secreted and bicarbonate passing into the blood stream.

Before considering this theory it will be worth while to calculate the amount of work done in forming one liter of gastric juice. Any theory must provide the mechanism from which the energy may be derived, and it is well to know the magnitude of the requirement.

The values are taken from Hollander (1934) and Peters and Van slyke (1931).

The concentration of the constituents of the gastric juice as secreted by the parietal cells is supposed to be

$$H^{+} = 0.17 \text{ molal}$$

$$Cl^{-} = 0.17 \text{ molal}$$

$$H_2O = 55.22 \text{ molal.}$$

The specific gravity of such a solution is less than 1.003, and the mol fraction of water is 0.994.

The concentration of these constituents in the plasma is

$$H^{+} = 5 \times 10^{-8} \text{ molal}$$

$$Cl^{-} = 0.10 \text{ molal}$$

$$H_2O = 90.151\% \text{ by weight.}$$

From the data given by Mathews (1927) and by Hawk and Bergeim (1927) it is found that the number of mols dissolved in one liter of plasma is 0.313. The specific gravity of plasma is 1.0312. The weight of water is one liter of plasma is 929.6 grams, the number of mols 51.6, and the mol fraction 0.994.

The general expression for calculating the free energy change in transporting N mols of a substance from an infinite volume of plasma to an infinite volume of gastric juice is

$$\Delta F = NRT \ln \frac{N \text{ gastric juice}}{N \text{ plasma}} .$$

Considering first the water it is seen that the free energy change is zero since the mol fractions of water in the two solutions is the same. Also it can be concluded that the osmotic pressures of the two solutions are the same. This conclusion can also be reached by comparing the known osmotic pressure of plasma with that of a solution of 0.17 HCl.

The energy necessary to transfer 0.17 mols of Cl^- from 0.1 molal to 0.17 molal can be written

$$\Delta F = (0.17)\ (1.99)\ (310)\ (2.3)\ \log \frac{0.17}{0.10} \text{ calories.}$$

$$\Delta F = 55.5 \text{ calories.}$$

The energy necessary to transfer 0.17 mols of H from 5×10^{-8} to 0.17 molal can be written

$$\Delta F = (0.17)\ (1.99)\ (310)\ (2.3)\ \log \frac{0.17}{5 \times 10^{-8}} \text{ calories.}$$

$$= (0.17)\ (1.99)\ (310)\ (2.3)\ (6)\ \log 3.4 \text{ calories.}$$

$$\Delta F = 772 \text{ calories.}$$

Therefore the total energy necessary to form a liter of gastric juice from plasa is about 828 calories.

The first of the three possible mechanisms is that proposed by Dawson and Ivy (1926) who postulated that the parietal cells secrete a dilute solution of hydrochloric acid of pH between 3.0 and 6.8. This is concentrated to 0.17 molar by reabsorption of water in the lumen of the glands or in the gastric pits.

The final acid solution has an osmotic pressure equal to that of plasma. Consequently if a more dilute solution were separated from plasma by a membrane permeable only to water it would be concentrated to the extent necessary. Also it is

conceivable that by mass action as expressed in equation 6 above the parietal cells could form a solution whose pH was between 3.0 and 6.8.

The serious difficulty of this theory is the postulated production of a dilute solution containing only HCl. This means that there must be some mechanism for holding back the salts of the blood and for concentrating the water. The dilute solution could not be in osmotic equilibrium with the plasma, and energy would be needed to concentrate the water. This cannot be derived from the difference in hydrostatic pressure between blood in the capillaries and the dilute solution, for the difference can hardly be more than 20 mm. Hg. There must be some sort of a machine for drawing energy from another source for concentrating the water. It is not impossible to conceive that such a machine exists. In fact one such must ve present in the salivary glands where a dilute salt solution is secreted against a pressure which may ve experimentally raised to a value greater than the capillary pressure. However there is no evidence that it exists in the parietal cells, and it must remain a supposition.

The second possibility is that the ordinary order of activity may not hold in this system. The mass action equation for equation 6 may be written

$$\frac{[HCO_3^-][H^+]}{[H_2CO_3]} = K.$$

From the data of Thiel and Stronecker (1914) the true K is found to be equal to 2×10^{-4}. Hence if H is 0.17 which is what we want to make it

$$\frac{[HCO_3^-]}{[H_2CO_3]} = 1.2 \times 10^{-3}$$

The values of the other concentrations in the cell can only be guessed at. The value of [HCO_3^-] must be something higher than that in arterial blood, and for the sake of the argument it can be assumed to be equal to that in venous blood. Hanke et al. (1931) found that in the gastric venous blood of dogs it was about 18 to 21 millimols per liter. Taking an average value of 20 millimols per liter for the bicarbonate in cell [HCO_3^-] is equal to 0.02. Substituting this in the equation, [H_2CO_3] comes out to be equal to 17 mols per liter.

This is a fantastically high value and probably an impossible one. The values in brackets above are equal to the concentration of the substance multiplied by the activity coefficient. In the case of H_2CO_3 the activity coefficient must be very high indeed if the mechanism proposed above is correct. Presumably the carbon dioxide is formed by the oxidation of some metabolite, possibly hexose pyruvic acid or lactic acid and is at once converted into H_2CO_3 by the carbonic anhydrase of the cell. Perhaps in the immediate vicinity of the locus of this reaction the activity of H_2CO_3 is actually as high as 17. The energy raising the H_2CO_3 to this activity would be derived from the oxidation of the metabolite, and if the proper means of filtering off the HCl were present the rest if the process could take place without the addition of any energy. There is of course no evidence at all to support this supposition, but it must be pointed out that there is no reason *a priori* such a supposition should be dismissed without consideration.

The final method begs the question altogether. It is merely assumed that the gastric mucosa contains a machine of completely unknown composition which concentrates HCl by an unknown mechanism. This view is made attractive only by the obvious faults of the specific mechanisms proposed above. Until some conclusive experimental method is devised this counsel of despair must be given certain consideration.

References

Apperly and Crabtree (1931) J. Physiol. 73, 331.

Apperly and Semmens (1928) cited by Apperly and Crabtree (1931).

Bachaltshuk (1928) Berl. klin. Wschr. 7, 1551.

Bernard (1859) cited by Harvey and Bensley (1912).

Bodansky (1938) Introduction to Physiological Chemistry, New York, pp. 165-169.

Booth (1938) J. Physiol. 91, 474.

Brinkman (1933) J. Physiol. 80, 171.

Brinkman, Margaria and Roughton (1933) Phil. Trans. Roy. Soc. Lond. A 232, 65.

Brown and Vineberg (1932) J. Physiol. 75, 345.

Chambers (1915) quoted by Hoerr and Bensley (1936).

Collip (1920) Univ. Toronto Studies, Physiol. Series, no. 35.

Davenport and Fisher (1938) J. Physiol. 94, 16P.

Dawson and Ivy (1926) Amer. J. Physiol. 76, 158.

Delhougne (1927) Berl. klin. Wschr. 6, 804.

Dodds and McIntosh (1923) J. Physiol. 57, 139.

Fisher (1936) Statistical Methods for Research Workers, Sixth Edition, London, pp. 176-213.

Fitzgerald (1910) Proc. Roy. Soc. B 83,

Gersh (1938) Proc. Soc. Expt. Biol. and Med. 38, 70.

Gortner (1938) Outlines of Biochemistry, New York, p. 344.

Greenwood (1885) J. Physiol. 5, 195.

Hanke, Johannesen and Hanke (1931) Proc. Soc. Expt. Biol. and Med. 28, 698.

Harvey and Bensley (1912) Biol. Bull. 23, 225.

Hawk and Bergeim (1927) Practical Physiological Chemistry, Philadelphia, p. 594.

Heidenhain (1870) Archiv. f. mikr. Anat. 6,

Hoerr and Bensley (1936) Anat. Record 65, 417.

Hollander (1934) J. Biol. Chem. 104, 33.

Langley (1881) Phil. Trans. Roy. Soc. Lond. 172, part 3.

Linderstrom-Lang et al. (1935) Glick, Holter, Linderstrom-Lang and Ohlsen (1935) Compt. rend. Lab. Carlsberg 20, no. 11. Also Linderstrom-Lang and Holter (1934) Ergebnisse der Enzymfsch. 3, 309.

Ma (1927) Anat. Record 35, 337.

Macallum (1908) Ergebnisse der Physiol. 7, 628.

Maly (1878) Zeit. f. physiol. Chem. 1, 325.

Mathews (1927) Physiological Chemistry, New York, p. 478.

Maximow (1934) A textbook of Histology, Philadelphia, p. 385.

Meldrum and Roughton (1932) Proc. Roy. Soc. Lond. B 111, 296.

Meldrum and Roughton (1933) J. Physiol. 80, 113.

Osborne (1901) Amer. J. Physiol. 5, 180.

Peters and Van Slyke (1931) Quantitative Clinical Chemistry, I Baltimore.

Philpot and Philpot (1936) Biochem. J. 30, 2191.

Revell (1912) cited by Harvey and Bensley (1912).

Roughton (1934) Ergebnisse der Enzymfsch. 3, 289.

Roughton (1935) Physiol. Rev. 15, 241.

Roughton and Booth (1938) Biochem. J. 32, 2049.

Schmidt (1938) The Chemistry of the Amino Acids and Proteins, Baltimore, p. 888.

Sharpey-Shafer (1934) Essentials of Histology, London, pp. 356-365.

Stadie and O'Brien (1933) J. Biol. Chem. 100, (Proc.) lxxxviii.

Stöhr (1882) Archiv. f. mikr. Anat. 20, 221.

Zimmermann (1925) Ergebnisse der Physiol. 24, 281.