REFERENCES

- Biörck, G. (1949). Dissertation, *Acta med. scand.*, Suppl. p. 226.
- Bonnichsen, R. K. (1949). *Arch. Biochem.* **12**, 83.
- Bonnichsen, R. K., Chance, B. & Theorell, H. (1947). *Acta chem. scand.* **1**, 685.
- Chance, B. (1943). *J. biol. Chem.* **151**, 553.
- Chance, B. (1947). *Acta chem. scand.* **1**, 236.
- Chance, B. (1949a). *J. biol. Chem.* **179**, 1299.
- Chance, B. (1949b). *J. biol. Chem.* **179**, 1311.
- Chance, B. (1949c). *J. biol. Chem.* **179**, 1331.
- Chance, B. (1949d). *J. biol. Chem.* **179**, 1341.
- Chance, B. (1949e). *J. biol. Chem.* **180**, 947.
- Chance, B. (1949f). *Arch. Biochem.* **21**, 389.
- Chance, B. (1949g). *Arch. Biochem.* **21**, 416.
- Chance, B. (1950a). *Biochem. J.* **46**, 387.
- Chance, B. (1950b). *J. biol. Chem.* **182**, 643.
- Drabkin, D. L. (1941). *J. biol. Chem.* **140**, 373.
- Herbert, D. & Pinsent, J. (1948). *Biochem. J.* **43**, 193.
- Keilin, D. & Hartree, E. F. (1948). *Biochem. J.* **42**, 221.
- Sumner, J. B. (1941). *Advanc. Enzymol.* **1**, 163.

The Site of Hydrochloric Acid Production in the Stomach as Determined by Indicators*

By NORAH M. BRADFORD AND R. E. DAVIES

Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Sheffield

(Received 16 September 1949)

In order to correlate the biochemical function and the morphological structure of acid-secreting gastric mucosa, it is necessary to know exactly where the hydrochloric acid is formed. Bernard (1858) first used microchemical methods in an attempt to locate the site of formation of the hydrochloric acid, and Babkin (1944) reviewed the work on this topic. Although it is generally held that the oxyntic cells secrete hydrochloric acid at concentrations about isotonic with blood (Hollander, 1943), some workers (Hoerr & Bensley, 1936) have stated that a precursor of the acid occurs within the gastric tubules and is hydrolysed to form hydrochloric acid in the foveolae or even the lumen of the stomach.

Frog gastric mucosa can secrete some dyestuffs *in vivo* (Henning, 1932) and hydrochloric acid *in vitro* (Delrue, 1930). Tied bags of isolated frog gastric mucosa were used in this study to find out which dyestuffs are transported across the tissue without interfering with respiration or acid secretion. Observations were made of dye- and acid-secreting oxyntic cells in both amphibian and mammalian gastric mucosa. These established the fact that the contents of the canaliculae of these cells have a pH < 1.4. This shows that acid is formed in the pericanalicular zone of oxyntic cells and is incompatible with the hypothesis that some hypothetical precursor of the hydrochloric acid is secreted by oxyntic cells.

* A part of this work was communicated to the Biochemical Society (Bradford & Davies, 1948).

EXPERIMENTAL

Saline media. Mammalian tissue was incubated in the physiological salt solution (bicarbonate saline) of Krebs & Henseleit (1932), gassed with 5% CO₂ + 95% O₂. For experiments on frog gastric mucosa this solution was diluted with 0.29 vol. of water, when the balance of the O₂ uptake and the CO₂ output was to be measured. The phosphate saline of Krebs (1933) diluted with 0.29 vol. of water was used for measurements of the O₂ uptake of frog gastric mucosa.

The solution used during the preparation of the mucosa was a basal saline similar to amphibian bicarbonate saline but had a HCO₃⁻ content of 0.004M and, under working conditions, pH 7.4. All the salines contained 0.2% (w/v) glucose, and histamine was added at the start of all experiments to a final concentration of 5 × 10⁻⁵M.

Dyestuffs. The following dyestuffs were used. *Basic dyes:* acridine, acriflavine, azur I, azur II, benzene-azo- α -naphthylamine, Bismarck brown, brilliant cresyl blue, brilliant green, chrysoidin, dimethyl yellow, malachite green, methyl violet R.S., methylene blue, neutral red, rhodamine B, rhodamine-6G, safranin-O, thionine, toluene-azo-amino-toluene 2:1:1':4':3' and toluidine blue. *Acid dyes:* brom-thymol blue, congo red, cresol red, fluorescein, methyl orange and phenolphthalein.

Material. Frogs (*Rana temporaria temporaria* L.) and toads (*Bufo bufo bufo* L.) were captured locally and housed in an outdoor froggery under natural conditions. Six-month old polecats and cats were obtained from a local breeder.

Preparation of the tissue. Frog and toad gastric mucosa was washed, isolated and closed with silk thread at both ends according to the method of Davies (1948a). In all cases the pyloric antrum was completely removed and the

Fig. 1.

Fig. 2.

Fig. 1. Living, isolated, toad gastric mucosa secreting toluene-azo-amino-toluene 2:1:1':4':3'. View downwards on to the secretory surface. The dyestuff in the gastric crypts could be followed down into the tubules ($\times 50$).

Fig. 2. Living, isolated, frog gastric mucosa secreting toluene-azo-amino-toluene 2:1:1':4':3'. View downwards on to the secretory surface. Not all the tubules were secreting the dyestuff. Some regions of the mucosa were resting ($\times 50$).

Fig. 3.

Fig. 4.

Fig. 3. Living, isolated, frog gastric mucosa secreting neutral red. Section of tissue and view through the muscularis mucosae. Many of the dots visible in the lower half of the photo are the gastric tubules, seen end on through the muscularis mucosae, filled with crimson neutral red. The rest of the tissue was yellowish. The cut edge at the top has curled over so that the tubules are visible from the side ($\times 60$).

Fig. 4. Living, isolated tubules from polecat gastric mucosa secreting neutral red. The canaliculi of the oxyntic cells contained neutral red in the acid form. Slight alterations of the focus showed the 'basketwork' of canaliculi connected together in the cells ($\times 330$).

tied bag of mucosa made from the corpus of the gastric tissue. The polecats and cats were killed by a blow on the head. The stomach was removed at once, opened and washed in ice-cold Krebs phosphate saline. Mucosa from the corpus was freed from the muscle layers and used directly.

Manometric procedure. The O_2 uptake and the resultant of O_2 uptake and CO_2 output were measured with Warburg manometers in the usual way. Conical cups (20–30 ml. capacity) with a centre well were used. Dyestuffs were added from the side-arm as crystals or in solution as required.

Technique for the observation of tissues

Foveolae and mucous cells. The tied bag of secreting frog or toad gastric mucosa was removed from the Warburg cup, cut open and the contents removed. The silk was cut away and the sheet of tissue laid, nutrient side downwards, in a shallow pool of basal saline, containing 5×10^{-5} M-histamine, on a microscope slide. The mucosa is so thin that illumination from below was satisfactory for the use of the two-thirds objective. It was found that if both sides of the tissue were immersed in the basal saline, the dyes were soon washed out of the gastric foveolae.

Gastric tubules and oxyntic cells. The mucosa was placed, secretory side downwards, on a microscope slide. The slight contraction of the muscularis mucosae then caused the cut edges of the tissue to curl up slightly so that some of the tubules lay in a horizontal plane and could be observed easily. The muscularis mucosae is so transparent that the secreting oxyntic cells could also be watched.

Isolated oxyntic cells. The mucosa was examined by the technique described in the previous paragraph and a region containing many actively secreting oxyntic cells was selected. This region was cut out with very fine scissors and the tiny piece of tissue placed in a drop of basal saline and teased apart with fine needles held in a semi-micro manipulator (Harding, 1939). A coverslip was placed over the drop and pushed down gently to separate the debris. Most preparations contained a few isolated, secreting oxyntic cells which could be observed for a few minutes with a one-sixth objective until they presumably became anoxic due to the lack of fresh supplies of oxygen under the coverslip.

RESULTS

Secretion of basic dyes. The results of the seventy-five experiments with tied bags of frog gastric mucosa are given in Table 1.

It is clear that the isolated tissue is able to transport a variety of dyestuffs from the nutrient medium to the lumen of the stomach. All the basic (chromogen in cation) dyes were found to accumulate in the secretions from 10^{-3} M or saturated solution whichever was weaker. Though quantitative estimations were not made in all cases, it was found that many of the basic dyes were concentrated in the secretions up to twenty times or more. The acid dyes only appeared in smaller concentrations than those in the saline solutions.

The site of formation of the acid. Some of the basic dyes are acid-base indicators and were used to locate

the site of formation of the hydrochloric acid. Neutral red proved especially suitable as it forms solutions in acid which are highly coloured and non-toxic. By microscopical observation of the mucosa it was possible to see that the gastric tubules and the canaliculi of secreting oxyntic cells were red with the acid form of the dye, whilst the cytoplasm of the oxyntic cells and the lower regions of the mucosa were yellowish and slightly alkaline (Pl. 2, Fig. 3). This is in accordance with the fact that these cells secrete as many bicarbonate as hydrogen ions (Davies, 1948a). An increase in the pH of the cytoplasm of oxyntic cells with secretion is expected (Davies & Roughton, 1948).

In all mucosae so far examined the activity occurred in patches. Within these patches some glands were quiescent, others actively secreting, and within the active glands some oxyntic cells were not secreting the dyestuff.

The pH of the secreted acid. The experiments with neutral red showed that the acid is formed with a $pH < 6.8$ (Kolthoff, 1932). This is not very impressive; acridine was therefore used which has a green fluorescence at a pH below 4.85 and a pale blue one above this pH (Kolthoff, 1932). Care is needed to interpret the microscopic picture since filled blood vessels in the mucosa have a greenish colour in ultraviolet light, but the experiments showed that the newly formed acid has a $pH < 4.85$.

The best evidence was obtained with toluene-azobenzene-2:1:1':4':3'. This is red at a $pH < 1.4$ (Kolthoff, 1932), but the free base is very insoluble, and successful results were only obtained by adding the dye in freshly precipitated microcrystalline form obtained by rapid neutralization of a solution of the hydrochloride. Although the red colour could be seen easily in the foveolae and tubules (Pl. 2, figs. 1, 2) this dye has such a low tinctorial value that the colour in the tiny canaliculi, though visible by eye, was too faint for photography. Again care is needed to distinguish occasional blood vessels from tubules full of red dye.

The technique of providing an excess of a very insoluble dyestuff in the form of microcrystals in the nutrient medium, makes it possible to study the secretion of such dyes. Microcrystals are more soluble than coarse crystals because of their higher surface activity. When dye is removed from the nutrient medium by the mucosa, more of the crystals dissolve, thus maintaining the concentration in the medium. In the intact animal such experiments might be impossible because the very small amount of dye in solution in the blood could not be replaced. Injected crystals would block up capillaries and would thus be largely removed from the circulation. The concentration of dye in the blood would then probably fall too low for the dye to be seen in the gastric juice.

Table 1. *Secretion of acid and basic dyes by tied bags of frog gastric mucosa*

Dye	Exp. no.	Chemical class	Conc. in medium (M)	Occurrence in secretion	Secretions acid (A), neutral or alkaline (B)	Average Q_{HCl} (μ l./mg. dry wt./hr.)	Average Q_{O_2} (μ l./mg. dry wt./hr.)	Time in presence of dyestuff (hr.)	Remarks
<i>Basic dyes</i>									
Acridine	1	Acridine	Saturated	+	A	2.2	—	0.5	Fresh microcrystals
Acridine	2	Acridine	Saturated	+	A	0.7	—	2.5	Fresh microcrystals
Acridine	3	Acridine	10^{-3}	+	A	0.1	—	2.0	—
Acriflavine	4	Acridine	10^{-4}	+	A	4.0	—	1.0	—
Acriflavine	5	Thiazine	10^{-3}	+	A	3.5	—	2.5	—
Azur I	6	Thiazine	10^{-3}	+	A	3.7	—	2.5	—
Azur II	7	Azo	Saturated	+	A	—	-1.1	1.5	—
Benzene-azo- α -naphthylamine	8	Azo	Saturated	+	A	2.1	—	1.5	—
Benzene-azo- α -naphthylamine	9	Azo	Saturated	+	A	—	-1.8	1.5	—
Benzene-azo- α -naphthylamine	10	Azo	Saturated	-	B	0	—	1.5	—
Bismarck brown	11	Bisazo	Saturated	+	A	7.1	—	2.5	—
Brilliant cresyl blue	12	Oxazine	10^{-3}	+	A	2.3	—	2.5	—
Brilliant cresyl blue	13	Oxazine	10^{-3}	+	A	2.9	—	2.5	—
Brilliant cresyl blue	14	Oxazine	10^{-3}	+	A	—	-2.25 -3.6	3.0	Large increase in respiration
Brilliant cresyl blue	15	Oxazine	10^{-4}	+	A	—	-3.1 -5.9	2.0	Large increase in respiration
Brilliant cresyl blue	16	Oxazine	10^{-4}	+	A	—	-3.5 -4.5	2.0	Large increase in respiration
Brilliant cresyl blue	17	Oxazine	10^{-4}	+	A	1.3	—	2.5	Acid secretions not increased
Brilliant green	18	Triphenylmethane	10^{-3}	+	A	1.9	—	2.0	Acid secretions not increased
Brilliant green	19	Triphenylmethane	10^{-4}	+	B	0	—	2.0	Acid secretions not increased
Chrysoidin	20	Azo	Saturated	+	A	—	-1.5	2.5	Slight inhibition
Chrysoidin	21	Azo	Saturated	+	B	0	-1.9	2.5	Slight inhibition
Dimethyl yellow	22	Azo	Saturated	+	A	0.2	—	3.0	—
Dimethyl yellow	23	Azo	Saturated	-	B	0	—	2.0	—
Malachite green	24	Triphenylmethane	10^{-3}	+	A	1.2	—	2.0	Secretion stopped after 1 hr.
Methyl violet R.S.	25	Triphenylmethane	10^{-3}	Trace	A	1.2	—	2.5	—
Methyl violet R.S.	26	Triphenylmethane	10^{-3}	Trace	A	—	-1.9	1.5	—
Methyl violet R.S.	27	Triphenylmethane	10^{-5}	-	B	0	—	2.0	—
Methylene blue	28	Thiazine	10^{-3}	+	A	0.1	—	2.5	—
Methylene blue	29	Thiazine	10^{-4}	+	A	5.0	—	1.0	—
Methylene blue	30	Thiazine	10^{-4}	+	B	0	—	1.0	—
Methylene blue	31	Thiazine	10^{-4}	+	A	—	-3.8	2.0	—
Neutral red	32	Azine	10^{-3}	+	A	4.0	—	1.5	—
Neutral red	33	Azine	10^{-3}	+	A	—	-2.1	1.5	—
Neutral red	34	Azine	10^{-3}	+	A	3.6	—	2.0	—
Neutral red	35	Azine	10^{-3}	+	A	2.2	—	1.5	—

Neutral red	36	Azine	10 ⁻³	-	<i>B</i>	0	—	1·0	—
Neutral red	37	Azine	2 × 10 ⁻⁴	+	<i>A</i>	1·2	—	1·5	—
Neutral red	38	Azine	10 ⁻⁴	+	<i>A</i>	—	2·7	3·0	—
Neutral red	39	Azine	10 ⁻⁴	+	<i>A</i>	0·5	—	2·5	—
Neutral red	40	Azine	10 ⁻⁴	+	<i>A</i>	6·5	—	2·0	—
Neutral red	41	Azine	10 ⁻⁴	+	<i>A</i>	5·8	—	2·0	—
Neutral red	42	Azine	10 ⁻⁵	+	<i>A</i>	—	2·8	3·0	—
Neutral red	43	Azine	10 ⁻⁵	+	<i>A</i>	2·1	—	2·0	—
Neutral red	44	Azine	10 ⁻⁶	+	<i>A</i>	—	2·5	3·0	—
Neutral red	45	Azine	10 ⁻⁶	+	<i>A</i>	4·4	—	2·0	—
Neutral red	46	Azine	10 ⁻⁷	-	<i>A</i>	—	3·3	3·0	—
Neutral red	47	Azine	10 ⁻⁷	-	<i>A</i>	3·2	—	2·0	—
Neutral red	48	Azine	10 ⁻⁸	-	<i>A</i>	—	2·1	3·0	—
Neutral red	49	Azine	10 ⁻⁹	-	<i>A</i>	4·5	—	2·0	—
Rhodamine- <i>B</i>	50	Pyronine	10 ⁻³	+	<i>A</i>	0·3	—	2·0	—
Rhodamine-6 <i>G</i>	51	Pyronine	10 ⁻³	+	<i>A</i>	2·9	—	2·5	—
Safranin 0	52	Azine	10 ⁻³	+	<i>A</i>	0·5	—	2·5	—
Thionine	53	Thiazine	10 ⁻³	+	<i>A</i>	0·1	—	2·0	—
Thionine	54	Thiazine	10 ⁻³	+	<i>B</i>	0	2·6	4·0	—
Thionine	55	Thiazine	10 ⁻³	+	<i>A</i>	0·7	—	3·0	—
Toluene-azo-amino-toluene 2:1:1'4':3'	56	Azo	Saturated	-	<i>A</i>	1·8	—	1·0	—
Toluene-azo-amino-toluene 2:1:1'4':3'	57	Azo	Saturated	+	<i>A</i>	1·7	—	2·0	Fresh microcrystals
Toluene-azo-amino-toluene 2:1:1'4':3'	58	Azo	Saturated	+	<i>A</i>	—	3·2	1·5	Fresh microcrystals
Toluene-azo-amino-toluene 2:1:1'4':3'	59	Azo	Saturated	+	<i>A</i>	0·7	—	1·5	Fresh microcrystals
Toluene-azo-amino-toluene 2:1:1'4':3'	60	Azo	Saturated	+	<i>A</i>	3·0	—	2·0	Fresh microcrystals
Toluene-azo-amino-toluene 2:1:1'4':3'	61	Azo	Saturated	+	<i>A</i>	1·7	—	1·5	Fresh microcrystals
Toluene-azo-amino-toluene 2:1:1'4':3'	62	Azo	Saturated	-	<i>B</i>	0	—	1·0	Fresh microcrystals
Toluene-azo-amino-toluene 2:1:1'4':3'	63	Azo	Saturated	+	<i>A</i>	3	—	2·0	Fresh microcrystals
Toluene-azo-amino-toluene 2:1:1'4':3'	64	Azo	Saturated	+	<i>A</i>	2	—	2·5	Fresh microcrystals
Toluidine blue	65	Thiazine	10 ⁻³	+	<i>B</i>	0	—	2·5	—
<i>Acid dyes</i>									
Bromthymol blue	66	Pyronine	10 ⁻³	Trace	<i>A</i>	1·8	—	1·5	—
Bromthymol blue	67	Pyronine	10 ⁻⁴	Trace	<i>B</i>	0	—	2·0	—
Congo red	68	Azo	10 ⁻³	-	<i>A</i>	0·4	—	2·5	—
Cresol red	69	Pyronine	10 ⁻³	+	<i>B</i>	0	—	2·0	—
Cresol red	70	Pyronine	10 ⁻³	Trace	<i>A</i>	—	1·5	2·5	—
Cresol red	71	Pyronine	10 ⁻⁴	Trace	<i>A</i>	0·6	—	3·5	—
Fluorescein	72	Pyronine	10 ⁻⁴	-	<i>A</i>	4·0	—	1·0	—
Methyl orange	73	Azo	10 ⁻³	Trace	<i>A</i>	0·1	—	4·0	—
Methyl orange	74	Azo	10 ⁻⁴	Trace	<i>A</i>	3·2	—	1·5	—
Phenolphthalein	75	Pyronine	10 ⁻³	-	<i>A</i>	3·4	—	2·5	—

Experiments with mammalian mucosa. With due precautions the same technique can be applied to mammalian mucosa. Most types of mammalian gastric mucosa are too thick to be effectively oxygenated *in vitro* (Davies, 1946, 1948b; Davenport & Jensen, 1948; Patterson & Stetten, 1949) but the relatively thin gastric mucosa of the long thin polecat (*Putorius putorius*) can be used for the study of the *in vitro* secretion of acid (Davies, 1948b). It was soon found that the secretion of dyestuffs could also be observed with cat gastric mucosa if the material was cut into small pieces.

Samples of the gastric mucosa from these animals were cut into small pieces with fine scissors and incubated at 37° in solutions containing 5×10^{-6} M-histamine, or 5×10^{-6} M-carbamyl-choline chloride, together with neutral red (Pl. 2, fig. 4), acridine or toluene-azo-amino-toluene 2:1:1'4':3'. In these species too, the contents of the intracellular canaliculi of some of the oxyntic cells had a pH < 1.4. In both amphibian and mammalian oxyntic cells, neutral red sometimes accumulated within the cytoplasm as red spherical granules.

Methyl violet R.S., which changes colour from pH 0.1-3.2 (Kalthoff, 1932) might be expected to give useful information, but unfortunately it was secreted in such tiny amounts by frog, toad, cat and polecat mucosa that no conclusive results were obtained under the experimental conditions used.

Secretion of acid dyes. The acid (chromogen in anion) dyes, congo red and phenolphthalein, were not transported, but bromthymol blue, cresol red, fluorescein and methyl orange were sometimes found in traces inside the mucosal bags especially if their secretions were alkaline or only slightly acid.

DISCUSSION

The site of formation of the hydrochloric acid. Although in the last 90 years more than thirty workers have tried to locate the site of formation of the hydrochloric acid produced by gastric mucosa, Babkin (1944) wrote 'Unfortunately as yet none of the histological, microchemical or physiological investigations of the problem permit one to draw any definite conclusions on the subject'.

Bernard (1858) injected 'lactate de fer', which Sehrwald (1889) claimed was largely ferrous, not ferric, lactate, and potassium ferrocyanide into a rabbit which was killed soon after. A deposit of prussian blue was found only on the surface of the gastric mucosa and none could be detected microscopically in the tubules. He concluded that the acidity of the gastric juice only appeared after the secretion from the tubules had reached the surface and had become mixed with the fluids in the stomach.

Brücke (1859), however, believed that the acid was produced free in the gastric tubules. He pressed rabbit gastric mucosa between litmus paper and found that whilst the free surface was acid, the deeper portions were alkaline. He

concluded that if a free acid was liberated in the gastric tubules, an alkaline fluid of equivalent strength must at the same time pass into the blood and lymph systems. Lepine (1872) confirmed Bernard's results and unsuccessfully attempted to demonstrate the presence of acid using macerated tissue. Edinger (1882) used the indicators tropaeolin, phenolphthalein and Na alizarin. He was unable to find out where the HCl was formed but showed that only small areas of the mucosa were in full secretory activity at any one time. This effect has been confirmed during the experiments described in this paper.

Similar negative results with dyestuffs, under conditions such that the gastric mucosa could not have been secreting when the microscopical observations were made, were recorded by Trinkler (1884), Stintzing (1889), Sehrwald (1889) and Fränkel (1891).

The next major attack on the problem depended largely on the prussian blue reaction. Macallum (1908), Fitzgerald (1910), Hammett (1915) and Collip (1920) claimed to have found occasional deposits of prussian blue, and hence of free HCl, in the gastric tubules and even in the intracellular canaliculi. Harvey & Bensley (1912) criticized Fitzgerald's (1910) conclusions because they found prussian blue deposits in many other places in the body and believed, with Bernard (1858), that the HCl was formed on the surface of the gastric mucosa from some precursor. They in turn were severely criticized by Collip (1920) for using very toxic solutions (25% ammonium ferric citrate and 10% sodium ferrocyanide), which were so concentrated that they precipitated prussian blue when mixed, even in the absence of any acid.

Chambers (1915) injected neutral red into the oxyntic cells of a freshly killed mouse and wrote: 'the results seem to leave no room for doubt that the immediate secretion of the parietal cell with respect to neutral red is neutral if not actually alkaline'. However, his experimental technique was such that the cells he was observing could not have been secreting. Dawson & Ivy (1926) found that a blow on the head of a Pavlov-pouch dog, which lived for a further 40 min., completely stopped the secretion of HCl in response to 2 mg. of histamine given 7 min. before, and no secretory effect was observed following the injection of 2 mg. of histamine 25 min. after the blow. They also showed that the oxyntic cells of freshly killed mammals do not secrete. This has been confirmed by finding that a recovery period is required in both amphibian and mammalian isolated gastric mucosa before acid secretion begins (Davies, 1948a, b).

Further inconclusive experiments on non-acid-secreting mucosa were published by Glässner, Wittgenstein & Hamperl (1925), Zimmermann (1925), Linderstrøm-Lang, Holter & Ohlsen (1935) and Ostroch (1937). The work of Linderstrøm-Lang *et al.* (1935) is of special importance. They cut serial sections of cylinders of pig gastric mucosa parallel to the surface and titrated them for acid. They found a correlation between the number of oxyntic cells and the acid in each slice and calculated the normality of the whole volume of these cells to be 0.15N with respect to acid. This work has been quoted by Hollander (1943) as done on 'actively secreting fundus mucosa'. Linderstrøm-Lang *et al.* (1935) said the experiments were done on mucosa removed from the pig several hours earlier and mentioned the 'many unsuccessful attempts to estimate acid in fresh gastric mucosa'. The observations are unexpected and the explanation is probably that given by Linderstrøm-Lang (1947):

'We are not quite certain about the origin of the acid found in our mucosae, but I think that an invasion of HCl from the contents of the stomach is the main cause.'

Hoerr & Bensley (1936) used a new technique of freezing the fresh mucosa in liquid pentane, air or nitrogen and drying under vacuum. They were unable to detect HCl subsequently in the tubules. However, with this technique any free HCl would be removed along with the water in the tissue. Gersh (1938) also freeze-dried gastric mucosa and found that 'chloride was absent from the cytoplasm of all gland cells' and that 'the distribution of chloride in the actively secreting mucous membrane did not differ essentially from that in the resting tissue'. Since he killed his animals by bleeding under ether anaesthesia it is certain that the mucosa was not actively secreting when frozen. His conclusions are in contradiction to those of Collip (1920) and Hogartz (1932) who found large increases in the chloride content of gastric mucosa during secretion.

Thus the previous work on isolated non-secreting mucosa has not solved the problem of the site of formation of the hydrochloric acid.

Henning (1932) observed the secretion of some dyes by living narcotized frogs with the stomach laid open. He said that neutral red only became red in the orifice of the glands on the surface of the mucosa. Keller & Pisha (1947) said they had repeated this but gave no details. This difference from our results is not easy to explain as insufficient data are available about the conditions in Henning's experiments. He used a white arc light to illuminate the mucosa through the stomach muscles and it is possible that the tissue became too hot and thus stopped secreting.

It is of interest that Teorell & Wersäll (1945), who were investigating the electrical impedance properties of frog gastric mucosa, observed that 'In several cases one could successfully remove the top layer of the mucosa cell layer. In spite of this operation the acid secretion continued with unchanged intensity.' This observation supports the view that the hydrochloric acid is formed as such by the oxyntic cells.

There is much evidence that at high rates of secretion the hydrochloric acid is formed primarily at a concentration isotonic with the blood (Hollander, 1932, 1938; Gilman & Cowgill, 1933; Gray & Bucher, 1941); at lower rates of secretion the primary concentration may be even higher than this (Linde, Teorell & Öbrink, 1947).

The fact that the intracellular canaliculi contain acid at a $\text{pH} < 1.4$ during acid secretion shows that the site of formation of the hydrochloric acid is in the pericanalicular zone of the oxyntic cells, and that no precursor is secreted which turns into hydrochloric acid in the foveolae or the lumen of the stomach as believed by Bernard (1858), Ebstein & Grützner (1874), Osborne (1901), Harvey & Bensley (1912), Zimmermann (1925), Hanke (1926), Hanke & Donovan (1927), Hoerr & Bensley (1936) and Ostrouch (1937).

The mechanism of secretion of dyestuffs. Visscher (1942) claimed that 'by invoking the theory that dyes enter and pass through pores of charged membranes in undissociated form it becomes possible to harmonize the information we have on dyestuff permeation with the well established facts regarding permeation of strong electrolytes'. This theory would account for the accumulation of basic dyes since the coloured cation would be 'captured' in the acid secretion. Höber (1945, p. 603) supported this view as applied to the stomach but pointed out (p. 265) that some cells do show active accumulation of dyestuffs.

The experiments described show that the acid dye, phenolphthalein, was not secreted, whilst the basic dyes brilliant cresyl blue, thionine and acriflavine were found to accumulate in the lumen of the stomach. The pK_a of phenolphthalein is 9.7 (Kolthoff, 1932) whilst the pK_{ab} values of brilliant cresyl blue and thionine are greater than 11.0. Thus phenolphthalein is virtually unionized, whilst brilliant cresyl blue and thionine are virtually completely ionized at the pH of the nutrient medium (7.4) or the secretions (usually 1–3). Acriflavine is a quaternary ammonium salt and therefore cannot exist in an uncharged form at any physiological pH, it also has a pK_{ab} of 12.0 and is not reduced in living tissue to a substance of lower basicity.

It therefore seems that some charged molecules can pass cell membranes whilst others are held back. The great variety of chemical types of basic dyestuffs that are secreted suggests that the most important factor in determining whether they are secreted is their possible existence as positively charged ions. It may be that they are secreted across the canalicular walls by a tertiary process of the type described by Davies & Ogston (1950).

The transport of acid dyes is probably carried out by the mucous or neck cells along with their bicarbonate-containing secretions. These acid dyes were never observed in the living oxyntic cells although they stain the dead cells. The view that the acid dyes are transported in the stomach by cells elaborating alkaline juices is supported by the observation that acid dyes are secreted by pancreatic cells, which are stained when dead by basic dyes (Visscher, 1942). Morrison, Gardner & Reeves (1936) and Morrison, Reeves & Gardner (1936) claimed that some acid dyes were secreted by Pavlov-pouch dogs. This claim has been disputed by Ingraham & Visscher (1935), Varco & Visscher (1941) and Vischer (1942) who used histamine-stimulated Pavlov-pouch dogs. The former workers did not state how they stimulated secretion and it may be that the results are not inconsistent if they were observing a mixed secretion whilst Visscher and co-workers were observing only oxyntic cell secretion following the injection of histamine (Babkin, 1944).

SUMMARY

1. Basic dyes of many chemical types were transported across isolated acid-secreting frog and toad gastric mucosa and were concentrated in the secretions.

2. Some of the dyes were acid-base indicators. Microscopical observations showed that during acid secretion the gastric crypts, tubules and intracellular canaliculi had pH values < 6.8 (neutral red), < 4.85 (acridine in ultraviolet light) and < 1.4 (toluene-azo-amine-toluene 2:1:1':4':3'). Methyl violet, a basic dye with colour changes at still lower pH values, was not

secreted in sufficient amounts to determine the pH of the tubules etc. It is concluded that the site of formation of the hydrochloric acid is the wall of the canaliculi of the oxyntic cells (the pericanalicular zone).

3. In all cases the secretory activity of the tissue occurred in patches, and even within active tubules some oxyntic cells were not secreting the dyes.

4. Some acid dyes were transported, but were not concentrated in the secretions.

5. Similar results were obtained using small pieces of isolated polecat and cat gastric mucosa.

6. The mechanism of secretion of dyestuffs is discussed.

REFERENCES

- Babkin, B. P. (1944). *Secretory Mechanism of the Digestive Glands*. New York: Hoeber.
- Bernard, C. (1858). *Propriétés Physiologique des Liquides de l'organisme*. Paris: Baillière.
- Bradford, N. M. & Davies, R. E. (1948). *Biochem. J.* **42**, xii.
- Brücke, E. (1859). *S.B. Akad. Wiss. Wien*, **37**, 131.
- Chambers, R. (1915). *Lancet-Clinic*, **113**, 363. (Seen in Babkin, 1944; Hollander, 1943.)
- Collip, J. B. (1920). *Univ. Toronto Stud. physiol.* Series. no. 35.
- Davenport, H. W. & Jensen, V. (1948). *Gastroenterology*, **11**, 227.
- Davies, R. E. (1946). *Biochem. J.* **40**, xxxv.
- Davies, R. E. (1948a). *Biochem. J.* **42**, 609.
- Davies, R. E. (1948b). The mechanism of hydrochloric acid secretion by the stomach. Ph.D. Thesis. University of Sheffield.
- Davies, R. E. & Ogston, A. G. (1950). *Biochem. J.* **46**, 324.
- Davies, R. E. & Roughton, F. J. W. (1948). *Biochem. J.* **42**, 618.
- Dawson, A. B. & Ivy, A. C. (1926). *Amer. J. Physiol.* **76**, 158.
- Delrue, G. (1930). *Arch. int. Physiol.* **33**, 196.
- Ebstein, W. & Grützner, P. (1874). *Pflüg. Arch. ges. Physiol.* **8**, 122.
- Edinger, L. (1882). *Pflüg. Arch. ges. Physiol.* **29**, 247.
- Fitzgerald, M. P. (1910). *Proc. roy. Soc. B*, **83**, 56.
- Fränkel, S. (1891). *Pflüg. Arch. ges. Physiol.* **48**, 63.
- Gersh, I. (1938). *Proc. Soc. exp. Biol., N.Y.*, **38**, 70.
- Gilman, A. & Cowgill, G. R. (1933). *Amer. J. Physiol.* **103**, 143.
- Glässner, K., Wittgenstein, H. & Hamperl, H. (1925). *Arch. Verdau.Kr.* **34**, 303.
- Gray, J. S. & Bucher, G. R. (1941). *Amer. J. Physiol.* **133**, 542.
- Hammett, F. S. (1915). *Anat. Rec.* **9**, 21.
- Hanke, M. E. (1926). *J. biol. Chem.* **67**, xi.
- Hanke, M. E. & Donovan, P. B. (1927). *J. biol. Chem.* **74**, xxiv.
- Harding, J. P. (1939). *J. R. micr. Soc.* **59**, 19.
- Harvey, B. C. H. & Bensley, R. R. (1912). *Biol. Bull.* **23**, 225.
- Henning, N. (1932). *Arch. exp. Path. Pharmac.* **165**, 191.
- Höber, R. (1945). *Physical Chemistry of Cells and Tissues*. Philadelphia: Blakiston.
- Hoerr, N. L. & Bensley, R. R. (1936). *Anat. Rec.* **65**, 417.
- Hogartz, W. (1932). *Pflüg. Arch. ges. Physiol.* **230**, 668.
- Hollander, F. (1932). *J. biol. Chem.* **97**, 585.
- Hollander, F. (1938). *J. biol. Chem.* **125**, 161.
- Hollander, F. (1943). *Gastroenterology*, **1**, 401.
- Ingraham, R. C. & Visscher, M. B. (1935). *J. gen. Physiol.* **18**, 695.
- Keller, R. & Pisha, B. V. (1947). *Rev. Gastroent.* **14**, 495.
- Kolthoff, I. M. (1932). *Säure-Basen-Indicatoren*. Berlin: Springer.
- Krebs, H. A. (1933). *Hoppe-Seyl. Z.* **217**, 191.
- Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
- Lepine, R. (1872). *C.R. Soc. Biol., Paris*, **24**, 221.
- Linde, S., Teorell, T. & Öbrink, K. J. (1947). *Acta physiol. scand.* **14**, 220.
- Linderström-Lang, K. (1947). Private communication.
- Linderström-Lang, K., Holter, H. & Ohlsen, A. S. (1935). *C.R. Lab. Carlsberg*, **20**, 66.
- Macallum, A. B. (1908). *Ergebn. Physiol.* **7**, 552.
- Morrison, S., Gardner, R. E. & Reeves, D. L. (1936). *J. Lab. clin. Med.* **21**, 822.
- Morrison, S., Reeves, D. L. & Gardner, R. E. (1936). *Amer. J. digest. Dis.* **3**, 551.
- Osborne, T. B. (1901). *Amer. J. Physiol.* **51**, 80.
- Ostrouch, M. (1937). *Z. Zellforsch.* **26**, 424.
- Patterson, W. B. & Stetten, D. Jr. (1949). *Science*, **109**, 256.
- Schrwald, E. (1889). *Münch. med. Wschr.* p. 177.
- Stintzing, R. (1889). *Münch. med. Wschr.* p. 793.
- Teorell, T. & Wersäll, R. (1945). *Acta physiol. scand.* **10**, 243.
- Trinkler, N. (1884). *Arch. mikr. Anat.* **24**, 174.
- Varco, R. L. & Visscher, M. B. (1941). *Proc. Soc. exp. Biol., N.Y.*, **46**, 295.
- Visscher, M. B. (1942). *Fed. Proc.* **1**, 246.
- Zimmermann, K. W. (1925). *Ergebn. Physiol.* **24**, 281.