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THE EXPERIMENTAL PRODUCTION OF WATERY VACUOLATION IN THE ACINAR CELLS OF THE SUBMANDIBULAR GLAND

BY R. L. TAPP AND O. A. TROWELL

From the Physiological Laboratory, University of Cambridge, and the M.R.C. Radiobiological Unit, Harwell

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SUMMARY

1. Watery vacuolation in the acinar cells of the rat submandibular gland is described. The vacuoles are cytoplasmic, membrane-walled, and 2–20 μ in diameter. They are visible in living cells and appear to contain a watery fluid.

2. Vacuolation occurred regularly in the following experimental situations: (1) *in vitro*—under anoxic conditions (2) post mortem—in animals killed by anoxia, and (3) *in vivo*—during secretion.

3. By *in vitro* experiments it was shown that vacuolation occurs only when the cells are both anoxic and exposed to an excess of extracellular fluid containing calcium and bicarbonate. It was further shown that vacuolation is reversible in oxygen and that both its development and recovery are temperature dependent.

4. Evidence is presented that the vacuolation is not a degenerative or necrotic change, that it is accompanied by the entry of fluid into the cells, and that it is not caused by simple osmosis.

5. The mechanism of vacuolation and its possible relation to secretion are discussed. It is suggested that vacuolation represents an imbalance between the ingestion and secretion of water and salts.

6. Similar vacuoles, apparently produced by the same mechanism, were observed in the acinar cells of the parotid gland and the pancreas of the rat.

7. The close similarity of this vacuolation to that previously described in rat liver cells was noted.

INTRODUCTION

This paper describes a type of watery vacuolation that can be experimentally induced in the acinar cells of the submandibular gland of the rat. To produce vacuolation, two main conditions must be satisfied: the tissue

must be anoxic, and the cells must have access to an excess of fluid containing both calcium and bicarbonate ions. Under such conditions fluid enters the cells and forms large watery vacuoles in the cytoplasm. Such vacuolation is reversible provided that oxygen is supplied to the tissue before the cells die.

It was also found that similar vacuolation occurs when the cells secrete under physiological conditions. It is generally accepted that during secretion water from the blood passes through the secreting cells, but very little is known of the mechanisms involved or how they are set in motion. Since experimentally-induced vacuolation appeared to be a precise and controllable phenomenon, it was thought that study of it might throw some light on the process of secretion.

Vacuolation has been investigated in three different experimental situations: (1) *in vitro*—under anoxic conditions (2) post mortem—in animals killed by anoxia, and (3) *in vivo*—during secretion.

METHODS

The experiments were performed on male rats aged 3 months. The animals had free access to food and water, except in the refeeding experiment when they were starved for 48 hr beforehand.

For the *in vitro* experiments the rats were usually killed and bled out by opening the chest and incising the heart, under anaesthesia. The anaesthetic used was either open ether or 50 % CO_2 in O_2. Neither of these was likely to induce anoxia by respiratory depression. In all the major experiments both methods of anaesthesia were tried and the results were always identical. In one experiment the submandibular gland was removed directly from the anaesthetized animal, and in two others the animals were killed by coal gas and by cervical dislocation.

After excision, the submandibular gland was placed in a pool of culture medium (or the solution under test) on a cavity slide and carefully cut into approximately cubical pieces (2 mm^3) with cataract knives. In the simpler experiments, which were to study the effect of immersion in unoxygenated medium at room temperature, 2 ml. volumes of medium were set out in stoppered glass weighing bottles and 3–4 cubes of tissue transferred to each. Experiments showed that the head of fluid overlying the tissue did not affect the results. In the more complex experiments, in which it was necessary to control both the oxygenation of the tissue and the temperature of incubation, the cubes of tissue were 'cultured' by the organ culture method of Trowell (1959), using the Perspex culture chambers of Trowell (1954). The gas phase used was, for the bicarbonate-buffered media, 5 % CO_2 in either O_2 or N_2 (depending on whether oxygenation or anoxia was required), and for the phosphate-buffered media, pure O_2. The basic physiological medium used in these experiments was the chemically-defined culture medium T8 (Trowell, 1959). In some experiments this was modified by reducing the $NaHCO_3$ concentration to 10·7 mM so as to give a final pH (with 5 % CO_2) of 7·0. In other experiments the $NaHCO_3$ was replaced by 10 mM sodium phosphate buffer ranging in pH from 6·0 to 8·2. Other media used were TC 199 (Difco), fresh rat serum, isotonic NaCl, Tyrode, and Tyrode minus one of the following components: K, Ca, Mg, HCO_3, or glucose.

For post mortem studies rats were also killed by exposure to nitrogen or coal gas, by injection of Nembutal or by tying the trachea under ether anaesthesia.

In all cases the 'cultured' tissue cubes or the samples removed from the animal were fixed in formol-sublimate (1 vol. formol to 9 vols. of 6 % mercuric chloride). In some experiments samples were also fixed in Carnoy and Susa fixatives. Paraffin or ester wax sections, 4 μ thick, were stained with haemalum.

RESULTS

1. *Vacuolation* in vitro

Histological description. Plate 1, figs. 1 and 2 show normal submandibular gland acini. Plate 1, figs. 3–5 show experimentally-induced vacuolation: the vacuoles occur in the acinar cells, and they may be found anywhere within the cytoplasm but usually between the nucleus and the central lumen of the acinus. They range in diameter from 2 to 20 μ, although the majority are between 5 and 8 μ, which is about the diameter of the nucleus. In extreme cases, an irregularly shaped vacuole may appear to occupy and distend the entire acinar cell. Up to eight vacuoles may occur in any one cell, but usually there are between one and three. They are spherical, or almost spherical, in shape and are bounded by a distinct membrane. Where a number of vacuoles occur together they may be flattened on one side or polyhedral, from packing. Occasionally vacuoles have a very irregular shape suggesting that they have formed by the fusion of contiguous smaller ones.

In wax sections most of the vacuoles appear empty; some of them, however, particularly the small ones, are partly filled with a homogeneous material which stains lightly with haemalum (Pl. 1, fig. 4). The vacuoles are similarly preserved by fixation in formol-sublimate, Carnoy or Susa.

If very small pieces of vacuolated submandibular gland (unfixed) are mounted in serum or Tyrode and lightly compressed under a cover glass, the vacuoles can be observed in the living cells (Pl. 2, fig. 1). They are faintly pink in colour and have refractile properties which indicate an aqueous, not a fatty, content. If a cell containing vacuoles is ruptured by increasing the pressure on the cover-slip, the vacuoles may be observed to float out of the cellular debris as distinct entities. It is clear, therefore, that vacuolation cannot be ascribed to any artifact of fixation.

Preliminary observations in organ culture. Trowell (1959) reported that pieces of submandibular gland taken from 4-week-old rats survived fairly well in organ culture (in T8 and oxygen) for up to 6 days. The submandibular gland of the rat is not, however, fully developed at 4 weeks: it does not attain full histological maturity until about 10 weeks (Jacoby & Leeson, 1959). But when we cultured the fully mature glands of 12-week-old rats, following exactly the method of Trowell (1959), we were surprised to find that survival was poor: some acini were necrotic by the end of the second day and most of them had disappeared by the fourth day. Cultures examined after shorter intervals revealed that at 1, 3 and 6 hr

after explantation the acinar cells in the central part of the culture were vacuolated, whereas at 9 and 12 hr this vacuolation had disappeared and the cells looked healthy. These observations suggested that vacuolation was caused by some step in the setting up of the cultures and that it was reversible.

In the normal course of setting up organ cultures there is an interval of up to 1 hr between the death of the animal and the final setting up under fully oxygenated conditions at 37° C, and during most of this time the cultures are standing in unoxygenated T8 at room temperature. The first step, therefore, was to see if vacuolation could be produced by simply immersing the cultures in T8 at room temperature.

Vacuolation in unoxygenated T8. Pieces of submandibular gland were cut up in a pool of T8 and then transferred to 2 ml. of T8 (unoxygenated) in a stoppered weighing bottle and allowed to stand at room temperature (20° C). Sample pieces were fixed after standing for 15, 30 and 45 min and 1, 3 and 6 hr. Slight vacuolation was already present after 15 min and this developed rapidly during the first hour; but between the first and the sixth hours there was only a slight further increase (Table 2).

Usually, vacuolation occurred first at the centre of the tissue cube and then spread peripherally, but it stopped short of the edge and the very outermost acini were generally spared. Occasionally, however, the distribution was more irregular and vacuoles might be present at the edge. The vacuoles present after 15 min were already almost as large as those seen after 1 hr, from which it appeared that the early growth in size of the vacuoles must have been very rapid, though it is possible that smaller early vacuoles passed undetected because they could not be identified among the mucoid droplets which normally fill the cytoplasm (Pl. 1, fig. 2).

To check that the method of killing the animal was not a factor involved in vacuolation, a number of animals were killed in the following ways: bleeding out under anaesthesia (ether or 50% CO_2); cervical dislocation under anaesthesia; coal gas. Pieces of gland were also removed under anaesthesia. In every case vacuolation occurred after 1 hr in T8.

Another possibility was that vacuolation was caused by the mechanical trauma of cutting the gland into pieces, either as a result of stimulating parasympathetic nerves within the gland, or as a direct effect on the acini. The T8 vacuolation experiments were repeated using either rats that had been atropinized before being killed (0·3 mg subcutaneously) or atropinized T8 (30 μg/ml.), and in neither case was vacuolation impeded. In another experiment it was shown that vacuolation does *not* occur when the gland is cut up dry and the fragments are kept under moist conditions in a sealed vessel for 1 hr at room temperature: immersion in an excess of T8 is evidently a necessary condition for vacuolation. These

experiments between them seemed to rule out both mechanical trauma and parasympathetic nerve stimulation as primary causes of vacuolation.

The next step was to see to what extent vacuolation could be produced in media other than T8.

Vacuolation in other media. These experiments were performed by immersing small tissue cubes in 2 ml. volumes of fluid in weighing bottles as before, in most cases at room temperature, but in a few cases at 0° C, as shown in Table 1.

TABLE 1. The degree of vacuolation found in pieces of submandibular gland (2 mm^3) after 1 hr immersion in various unoxygenated media. The figure in brackets after the medium is the number of pieces examined

0 = no vacuolation
(+) = vacuolation detectable at centre
+ − +++ = increasing spread of vacuolation

Medium	Temperature (° C)	pH	Vacuolation
T8 (24)	20	7·8	+++
T8 (3)	0	7·8	0
T8 + atropine (3)	20	7·8	+++
T8 + acetazolamide (9)	20	7·8	+++
Serum (28)	20	8·0	+++
Serum (12)	0	8·0	0
Serum + atropine (8)	20	8·0	+++
T8, reduced HCO_3 (8)	20	7·2	++
TC199, reduced HCO_3 (12)	20	7·0–7·6	++
Tyrode (48)	20	7·6	+++
Tyrode (12)	0	7·6	0
Tyrode − glucose (24)	20	7·6	+++
Tyrode − HCO_3 (12)	20	4·9–5·4	(+)
Tyrode − HCO_3 + Na_2HPO_4 (16)	20	7·5–7·7	(+)
Tyrode − Ca (24)	20	7·6	(+)
Tyrode − Mg (8)	20	7·6	+++
Tyrode − K (8)	20	7·6	+++
NaCl + $NaHCO_3$ + $CaCl_2$ (8)	20	7·5–7·7	+++
Distilled H_2O (8)	20	5·1–5·3	0
0·9 % NaCl (24)	20	5·0–5·6	(+)
0·9 % NaCl + 10 mM phosphate buffer (8)	20	7·5	0
10 mM phosphate buffer + pilocarpine (4)	20	7·2	0
Serum + 10 mM phosphate buffer (4)	20	7·7–7·8	+++
Serum + 10 mM phosphate buffer (4)	20	7·5	++

In the first place it may be noted that (at 20° C) vacuolation occurs equally well in T8, serum, or Tyrode, so there is no question of a toxic or unnatural component of T8 being involved.

The most important fact to be deduced from the results in Table 1 is that vacuolation is very markedly reduced in media which lack either bicarbonate or calcium ions. In such solutions slight vacuolation was seen at the centre of the pieces but it seemed reasonable to attribute this to retained extracellular fluid. The addition of acetazolamide (1 mM) to T8 did not prevent vacuolation, so carbonic anhydrase cannot be critically involved. The omission of potassium, magnesium or glucose did not affect

vacuolation, and it developed fully in a solution containing only NaCl $CaCl_2$ and $NaHCO_3$.

The T8, serum and Tyrode used were rather alkaline (pH 7·6–8·0) from loss of CO_2; but in the experiments with TC199 and T8 with reduced bicarbonate and with serum mixed with phosphate buffer, in all of which the pH was more acid, vacuolation was not greatly diminished. An attempt to explore the whole pH range from 5·3 to 8·8, in phosphate-buffered T8, proved unrewarding because vacuolation failed to occur in the absence of bicarbonate. But this experiment did show that abnormal pH *per se* could not cause vacuolation.

It was concluded that T8 has no specific effect and that vacuolation can be expected in any physiological medium that contains the usual amounts of calcium and bicarbonate. The results in Table 2 show that the time course of vacuolation in serum or Tyrode was the same as in T8.

TABLE 2. The time course of vacuolation in various media.
Scoring as in Table 1

Medium	Time after immersion (min)						
	10	15	30	45	60	180	360
T8	.	+	+	++	+++	++++	++++
Serum	.	+	+	++	+++	++++	++++
Tyrode	0	+	+	++	++++	++++	++++
0·9% NaCl	0	(+)	(+)	(+)	(+)	.	.

Vacuolation did not develop in hypotonic solutions (10 mM phosphate buffer) or in distilled water, although a noticeable increase in cell volume occurred. This indicated that *osmotic* entry of water was not itself the cause of vacuolation. Water does, however, enter the cells when vacuoles develop. This was shown in the following way. Pairs of sections, vacuolated and control, taken from the same gland, were projected on to white paper and the outlines of the acini and nuclei were drawn. The acini were then cut out and weighed, the weight divided by the number of nuclei giving the average area per cell measured as milligrams of paper. Eight such measurements, each taken from a separate experiment, showed that during vacuolation the average cell area increased by between 15 and 60%, although usually the increase was between 30 and 40%. The results of two typical experiments are shown in Table 3.

Addition of atropine to the T8 or serum did not inhibit vacuolation, again ruling out a parasympathetic effect. In the absence of calcium and bicarbonate, pilocarpine did not cause vacuolation; neither did it cause secretion, as far as could be judged histologically.

The observation that no vacuolation developed in T8, serum, or Tyrode at 0° C was important because it indicated that physiological rather than purely physicochemical factors were involved.

The roles of anoxia and excess fluid. When vacuolation occurred *in vitro* it developed first at the centre of the tissue cube and then spread peripherally; even after an hour the outer rim of acini was usually spared. This suggested that anoxia might have been a necessary condition for vacuolation to occur in all the experiments so far described. To test this possibility, pairs of Trowell chambers were set up with culture dishes and T8, one of each pair being gassed with 5% CO_2 in O_2 and the other with 5% CO_2 in N_2. Three pairs were set up, at 37, 20, and 2–3° C, and in each case the chambers were pre-equilibrated with gas and adjusted to the right temperature before tissue was explanted. The tissue cubes were prepared and set up in the chambers as quickly as possible to minimize the preliminary period of anoxia. Pieces were removed from each chamber after 1 and 6 hr, fixed, and examined for vacuolation. The results are shown in Table 4.

TABLE 3. The average cell area in sections of normal and vacuolated submandibular gland. Areas measured in arbitrary units (mg paper). In both cases vacuolation had developed in Tyrode at 20° C

	Total area of cells	No. of nuclei	Area per cell	% increase in cell area
Normal	12,110	146	83	.
After vacuolation	15,320	128	120	44
Normal	6,980	68	103	.
After vacuolation	12,180	81	150	47

TABLE 4. The degree of vacuolation in submandibular cultures, after 1 or 6 hr under aerobic or anaerobic conditions at various temperatures. Three cultures were used to determine each point. Scoring as in Table 1

Gas phase	5% CO_2 in O_2			5% CO_2 in N_2		
Temperature	2–3	20	37	2–3	20—	37
1 hr	(+)	(+)	0	(+)	++	++
6 hr	(+)	0	0	(+)	++	++

A marked degree of vacuolation was found only in the chambers gassed with nitrogen, which confirmed the hypothesis that anoxia is a necessary condition for vacuolation. The slight vacuolation found in the oxygenated chambers indicated that some vacuolation occurred during preparation and equilibration of the cultures, even though this was done as quickly as possible; but it rapidly disappeared at 20 or 37° C in oxygen.

Anoxia alone, however, does not cause vacuolation, for none developed when pieces of tissue, or a whole salivary gland, were removed from the body and allowed to stand in an empty, dry weighing bottle for 1 hr at 20° C. Excess fluid, over and above the usual quantity of interstitial fluid, is needed also. This conclusion was further supported by the post mortem observations recorded in the next section.

Reversibility of vacuolation. The presence of central vacuoles in cultures fixed shortly after explantation and their absence later suggested that vacuolation was reversible. This was also indicated by the results in Table 4. To prove it more directly, pieces of tissue were immersed in T8 for long enough to produce acinar vacuolation (½–1 hr) and were then explanted into Trowell chambers that had been pre-equilibrated with 5% CO_2 in O_2, and adjusted to temperatures of 37, 20, and 2–3° C. Explants were removed from the chambers at various intervals after explantation, fixed, and the degree of vacuolation compared with the vacuolated controls fixed at the time of explantation. The results are given in Table 5.

TABLE 5. The reversibility of submandibular vacuolation. Tissue cubes were stood in T8 for ½ or 1 hr (i.e. until vacuoles had developed). They were then explanted into oxygenated culture chambers at various temperatures. Three cultures were used to determine each point. Scoring as in Table 1

	Extent of acinar vacuolation				
	½ hr T8 before explantation		1 hr T8 before explantation		
Time after explantation (hr)	20° C	37° C	2–3° C	20° C	37° C
0 (control)	++	++	+++	+++	+++
½	+++	+++	+++	+++	+++
1	++	++	+++	+++	++
2	++	+	+++	++	+
6	+	(+)	++	+	(+)

In assessing the degree of vacuolation, attention was paid to the possibility that degenerating acini might appear to recover if their vacuoles merged with the cytoplasm. Only the largest pieces of tissue showed any necrotic acini, however, and these were at the centres of the explants which would have been anoxic for the longest period. In the control cultures, fixed after the preliminary exposure to T8 and before transfer to oxygen, the outermost rim was usually not vacuolated (as has already been described). The recovery that occurred in oxygen took the form of a steady widening of this outer non-vacuolated rim. In other words recovery proceeded from without inwards, as might be expected.

The results in Table 5 show that almost complete recovery is possible provided that the period of anoxic stress is not longer than about an hour. The recovery was temperature dependent, indicating that resumption of normal aerobic metabolism was probably the key factor.

The effects of temperature. It has already been shown that vacuolation is prevented if the medium is cooled to 0° C (Table 1), and that recovery is dependent on temperature (Tables 4 and 5).

There is also some indication that the development of vacuolation is temperature dependent. At 0° C no vacuolation occurred; at 20° C vacuolation appeared within 15 min and reached full development in about 1 hr;

and when vacuolation developed immediately post mortem in a gland which was at or near 37° C, vacuoles appeared within a few minutes of death and reached a maximum within about 20 min (see later).

Vacuolation in parotid gland and pancreas. Pieces of parotid gland and pancreas removed from 3-month-old rats and stood in T 8 or serum for 1 hr at room temperature developed acinar vacuolation essentially similar to that observed in the submandibular gland. It was shown that both anoxia and excess fluid were required and that no vacuolation occurred at 0° C; and we have no reason to suppose that the mechanism is any different from that seen in the submandibular gland.

2. *Vacuolation post mortem*

*Histological description.*The vacuolated acini were identical in appearance with those produced *in vitro*: but their distribution was quite different. Post mortem vacuolation was never as severe as that seen *in vitro* and it was always confined to the outermost part of the gland, affecting only those acini that were adjacent to the capsule (Pl. 2, fig. 2). For this reason we have called it 'subcapsular vacuolation'. This distribution is not the result of uneven fixation for it was seen in glands that had been cut in half or into pieces immediately before fixation.

Experiments. It has been shown that vacuolation *in vitro* is dependent on anoxia and the presence of excess fluid containing calcium and bicarbonate. Since in the intact animal a certain volume of interstitial fluid is constantly maintained between the acini, it seemed possible that vacuolation would occur *in situ* during the period of anoxia that develops post mortem. But the amount of interstitial fluid available after death depends on the post mortem blood pressure in the capillaries, and this depends on how the animal is killed. To investigate this possibility a number of animals were killed in a variety of ways and in each case the glands were allowed to remain in the body for various periods after death before they were fixed. In some experiments the glands were fixed whole and in others they were cut into halves or smaller pieces. The results are given in Table 6. The gland can also be made anoxic in the anaesthetized animal by ligating the common carotid artery, and an experiment done in this way is included at the foot of Table 6.

The most important finding to emerge was that vacuolation could occur *in situ* after death, but only when death was caused by asphyxiation (nitrogen, coal gas, tracheal clamp): vacuolation did not occur if death was caused by bleeding or by Nembutal poisoning. Death by asphyxiation is followed by marked venous congestion and Trowell (1946) has shown that in the rat the venous pressure rises when the animal is asphyxiated, and remains above the normal level for several hours after death. Under

these conditions the formation of interstitial fluid would be enhanced. On the other hand, after death by bleeding or from Nembutal poisoning the venous pressure is low, and the interstitial fluid would tend to be withdrawn into the vessels. It seems reasonable to suppose, therefore, that although in all cases the glands were anoxic, the excess fluid necessary for vacuolation was present only after asphyxiation. Vacuolation did not occur when the carotid artery was ligated. Here again the gland was anoxic but because of the low blood pressure within it no excess interstitial fluid would be formed. All the above results are consistent with those obtained *in vitro*.

TABLE 6. The degree of subcapsular vacuolation in submandibular glands that had been left *in situ* for various periods of time after death. Scoring as in Table 1. The figures in brackets are the numbers of animals used

Method of killing	Time after death (min)				
	0	15	30	60	120
50 % CO_2; bled out	0 (3)	0 (1)	0 (1)	0 (3)	.
Ether; bled out	0 (2)	.	.	.	0 (2)
Ether; Nembutal	0 (1)	.	.	.	0 (1)
Nitrogen	+ (3)	++ (3)	++ (3)	++ (3)	++ (1)
Coal gas	+ (2)	++ (2)	++ (2)	++ (2)	++ (1)
Ether; trachea clamped	+ (2)	++ (2)	++ (2)	++ (2)	++ (1)
Urethane; carotid ligated	0 (1)	.	.	0 (1)	0 (1)

The subcapsular distribution of the vacuolation cannot be explained in terms of uneven venous congestion. Thick frozen sections of a gland taken from an asphyxiated animal and stained by the benzidine method for blood (Sjöstrand, 1934) did not show any clear difference in the degree of vascular congestion of the central and subcapsular zones. The explanation remains obscure.

The vacuolation developed more quickly than it did at 20° C *in vitro*, probably because of the higher temperature. Vacuoles appeared within a few minutes, reached a maximum at 15–30 min and then slowly declined.

3. *Vacuolation* in vitro, *during secretion*

Histological description. The vacuoles which developed during secretion were similar to those produced *in vitro*, but on average were smaller in size (3–6 μ). The vacuolation was not found uniformly throughout the gland; it usually occurred at foci involving a group of neighbouring acini (Pl. 2, fig. 3). More such foci were found at the periphery of the gland than in the centre.

Experiments. In each of four experiments, six rats were starved for 48 hr, after which one was anaesthetized and bled out. The remaining animals were then allowed to feed and were killed after 5, 10, 20, 40 and 60 min of refeeding, respectively. Immediately after death in each case

the submandibular glands were removed, cut in half and fixed. The distention of the stomach gave some indication of the amount of food each animal had consumed. For the first half hour the animals appeared to feed voraciously and usually their stomachs were well distended within this time. They continued feeding during the second half of the experimental period, but at a reduced rate.

Focal vacuolation, as described above, was found in all the refed animals. It was most marked after 5 and 10 min refeeding which probably corresponds to the period when secretion was maximally stimulated.

In three experiments, pilocarpine was injected intraperitoneally (1–3 mg/kg) into an anaesthetized rat, and as soon as the first saliva appeared in the mouth (4–5 min) the submandibular glands were removed and fixed. Here again, well marked vacuolation was found in all cases.

Minor vacuolation in 'normal' animals. Very slight vacuolation was sometimes found in normal glands. The vacuoles were 2–7 μ in diameter and occurred in scattered foci, each involving 2–3 adjacent acini. These foci were few and far between and could only be found by careful searching.

The number of foci was variable, but usually there were more in animals that had been killed during the hours of darkness, and in those that had been anaesthetized with ether rather than with 50% CO_2 in O_2. It seems likely that these foci represented secretory activity caused by feeding and grooming on the one hand, and by ether stimulation on the other.

DISCUSSION

It is proposed to discuss first the mechanism of vacuolation and then its relation to secretion.

The complex conditions required for vacuolation allow some simple mechanisms to be excluded. It cannot easily be explained by the dilution or removal of some hypothetical 'anti-vacuolation' factor present in or around anoxic cells, because the diluting fluid had to contain both calcium and bicarbonate. Nor can it be explained by a simple osmotic uptake of water, because this could be produced in other ways (e.g. distilled water) without causing vacuolation; and the marked temperature dependence of vacuolation also speaks against it. It is possible of course that water which enters the cytoplasm by osmosis is, in the presence of both Ca and HCO_3 ions, segregated into vacuoles; but we have no evidence on this point.

It seems more likely that the fluid is taken in by pinocytosis. Pinocytosis is an active process stimulated by substances, including salts, that bind on to the mucoid coating of the cells (Holter, 1959, 1963). Oxygen uptake is not increased during pinocytosis (Holter, 1963) and it is likely that, as in phagocytosis (Takikawa, Ohta & Hibino, 1963), the energy is obtained

by glycolysis. If so, pinocytosis would continue during anoxia and could account for the uptake of fluid during vacuolation. As will be mentioned later, there is electron microscopic evidence that the closely similar vacuoles which develop in anoxic liver cells arise by pinocytosis. Both calcium and bicarbonate may well be required for pinocytosis, but there is no definite evidence available.

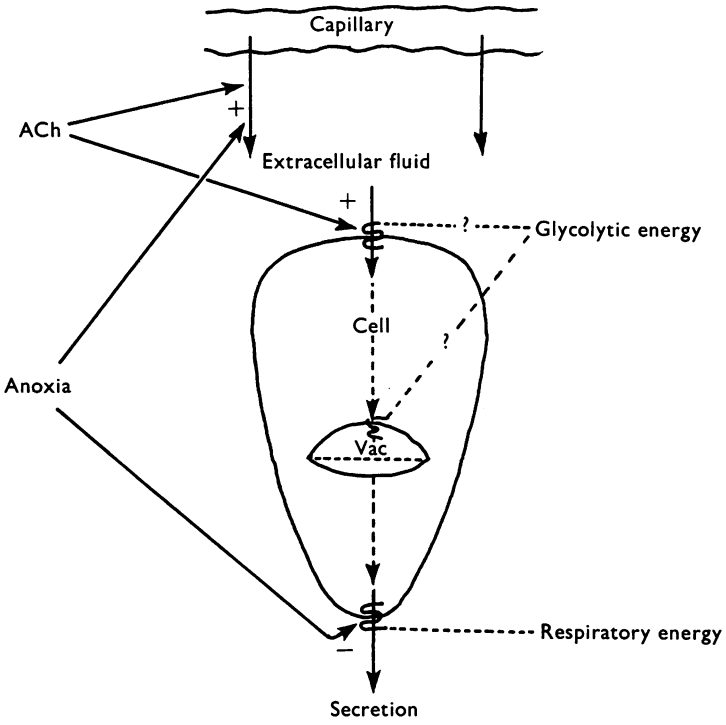
As regards the part played by bicarbonate, the carbonic anhydrase inhibitor acetazolamide did not affect vacuolation, though concentrations of the same order practically abolish bicarbonate output by the pancreas (Birnbaum & Hollander, 1953). This seemed surprising, but it has been suggested that *salivary* carbonic anhydrase is refractory to acetazolamide (Chauncey & Weiss, 1958). It is also possible that the enzyme is present in amounts far exceeding physiological requirements: in red cells the amount of carbonic anhydrase can be reduced by 97 % without diminishing the rate of carbonic acid formation (Davenport, 1946).

At this point it is pertinent to recall that Trowell (1946) described a histological picture of watery vacuolation in rat liver cells exactly similar to that described here in the acinar cells of the submandibular gland. Furthermore, he showed that the vacuolation occurred only when the liver was anoxic and the intrasinusoidal blood pressure was maintained at or above its normal level of 3–4 cm water. If we assume that the sinusoid distension was simply providing an excess of extracellular fluid, the conditions become identical with those found necessary for submandibular vacuolation. Nevertheless, we have found that small pieces of rat liver kept in unoxygenated T8 or Tyrode do not vacuolate, so there are evidently still some differences between the two situations, not yet resolved.

In the case of the vacuoles in the liver it has been shown under the electron microscope that they are bounded by a thin smooth membrane and that they appear to originate by pinocytosis at the vascular pole of the cell (Oudea, 1963; Brewer & Heath, 1965). This origin is further supported by the fact that haemoglobin (Trowell, 1946), the sodium salt of fluorescein (Hanzon, 1952), colloidal mercuric sulphide (Oudea, 1963) and thorotrast (Brewer & Heath, 1965) enter the vacuoles when perfused through the blood vessels. Comparable experiments on the submandibular gland have not yet been performed.

Turning now to the question of secretion, the observation that vacuolation occurred during the normal process of secretion suggested that it might be possible to interpret anoxic vacuolation in terms of the secretory mechanism. Text-figure 1 indicates a very simplified concept of the normal secretory process and the probable effects of anoxia on its several parts. It is convenient to distinguish two components of the secretory process:

(1) the *intake* of fluid into the basal region of the cell, from the extracellular fluid; and (2) the *output* of fluid (secretion) from the apical region. We postulate that *some* intake is a spontaneous and automatic activity of the cell, probably pinocytotic. The mechanism involved is probably stimulated by acetylcholine and pilocarpine but the *quantity* of fluid ingested is limited by the amount of extracellular fluid available. During secretion the blood vessels are dilated and their permeability is increased, so that more extracellular fluid is provided. The output of fluid from the cell is dependent on respiratory energy and is automatic in the sense that the cell puts out whatever volume of fluid is taken in.



Text-fig. 1. A diagrammatic concept of the processes of secretion and vacuolation.

Vacuolation is considered to result from an accumulation of fluid within the cell because of an imbalance between intake and output. During normal secretion output more or less balances input, but during the intense secretion which occurs on refeeding after a fast, or during pilocarpine stimulation, output may temporarily lag behind and some vacuolation occurs. Anoxia leads to an arrest of output (respiration) and also to vasodilation and increased capillary permeability which increases the volume

of extracellular fluid available. Both these factors contribute to a serious imbalance and gross vacuolation ensues.

In support of this hypothesis it may be mentioned that micropuncture studies on 'primary' secretion taken from the intercalated ducts of male rats (Schögel & Young, 1966) have shown that the concentrations of both sodium and potassium are very similar to a plasma ultrafiltrate: unless equilibration is occurring within the acinus or the intercalated duct, these observations suggest a mechanism such as pinocytosis rather than an active ionic pump. It is also known that in a number of species the rate of secretion varies directly with the rate of blood flow through the gland (Burgen & Emmelin, 1961).

It has been shown (in the cat) that the secretory responses of the submandibular gland to acetylcholine and noradrenaline (Douglas & Poisner, 1962, 1963) and to pilocarpine, methacholine and carbachol (McCarthy & Sheehan, 1966) fail to occur in the absence of calcium. This is in line with our finding that *in vitro* vacuolation is also calcium dependent.

The watery vacuolation demonstrated here is reversible and it can occur transitorily under certain normal physiological conditions. It is not therefore a degenerative or necrotic change, and it can most readily be interpreted as an abortive form of secretion.

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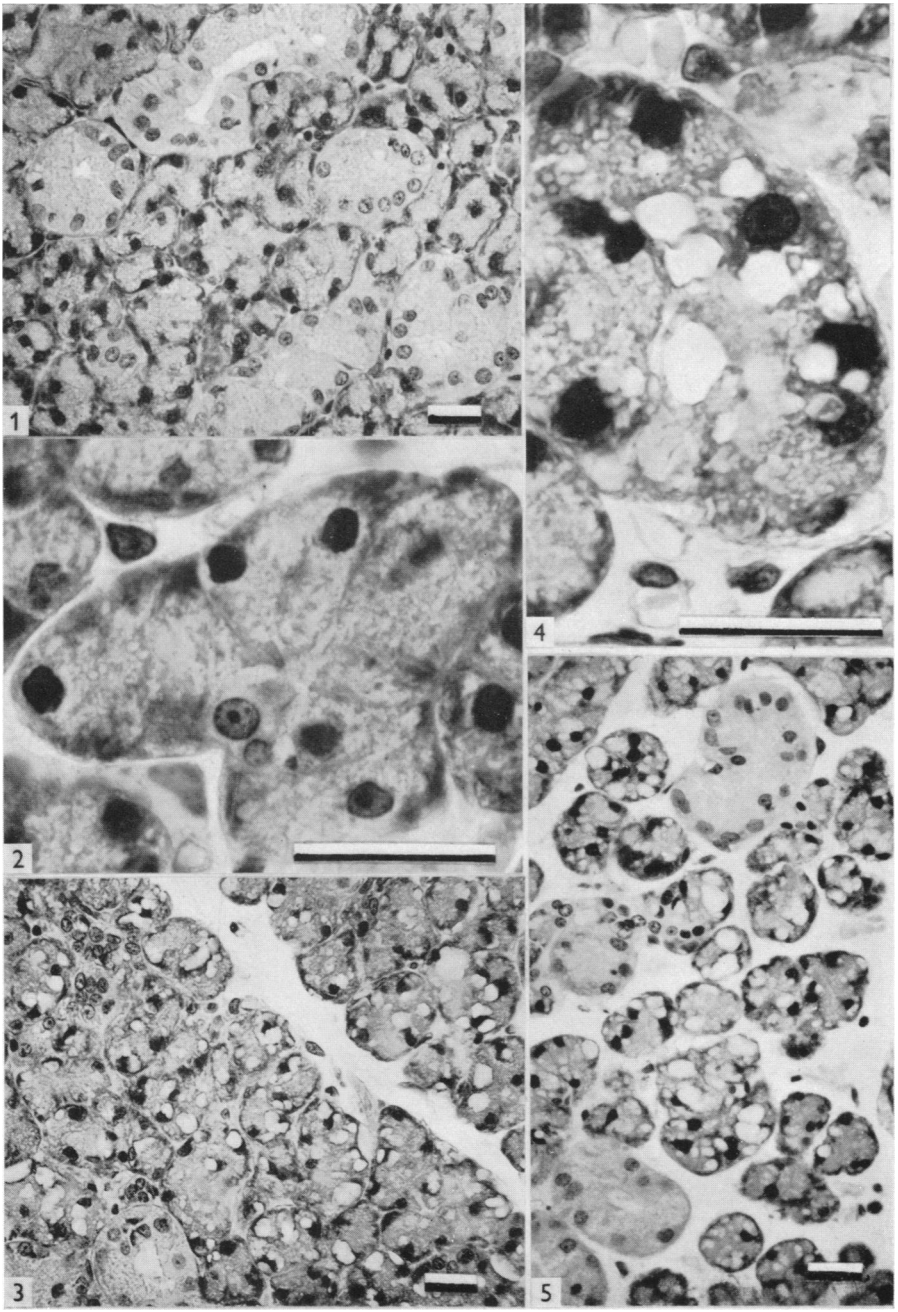
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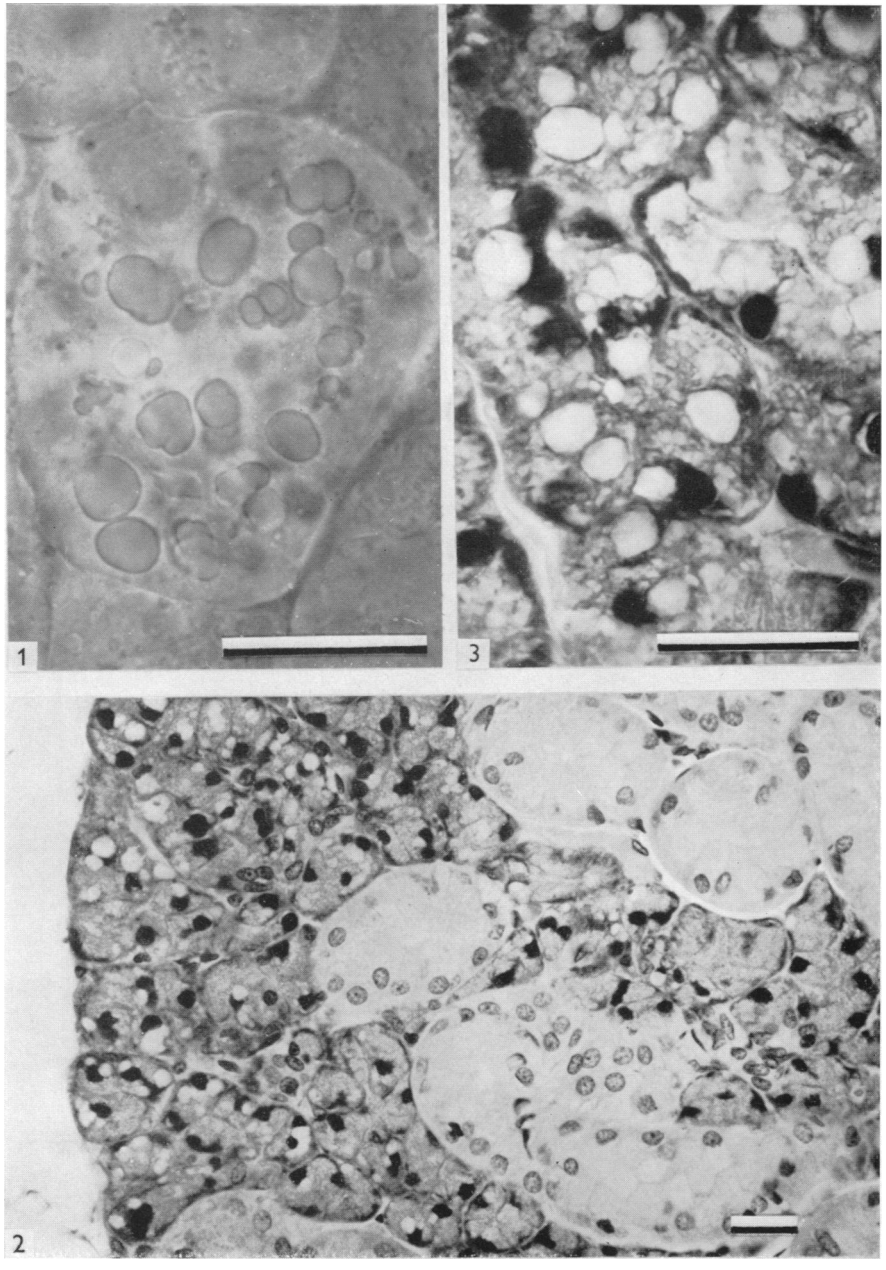
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EXPLANATION OF PLATES

Photomicrographs of submandibular gland. All scale bars represent 25 μ. All except Pl. 2, fig. 1: Formol-sublimate fixation, wax sections, haemalum.

PLATE 1

Figs. 1 and 2. Normal. Figs. 3 and 4. After 1 hr in unoxygenated Tyrode solution. Fig. 5. After 1 hr in unoxygenated T8.

PLATE 2

Fig. 1. Unfixed tissue after 1 hr in unoxygenated Tyrode solution. Fig. 2. Gland removed 15 min after asphyxial death: subcapsular vacuolation. Fig. 3. Gland removed 5 min after refeeding: a vacuolated area.