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# The effects of osmotic pressure on procaine-induced vacuolation in cell culture

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The effect of increased tonicity on procaine-induced vacuolation in the H.Ep.2 cell has been investigated. An increase in tonicity equivalent to about 1·3% NaCl was found to reduce the formation of the drug-induced vacuoles and also to reduce established vacuolation. There appeared to be no difference in the effectiveness of the osmotic adjusting substances used (sodium chloride, sodium sulphate, glucose, fructose and sucrose) when employed in osmotically equivalent amounts.

A wide variety of weakly basic substances induce cytoplasmic vacuolation in cells in culture (Lettré & Albrecht, 1941, 1943, 1951; Pomerat & Emerson, 1945; Buchsbaum & Kuntz, 1954; Belkin, Hardy & others, 1962). Yang, Strasser & Pomerat (1965) examined the effects of procaine and other substances on Hela cells and chick embryo fibroblasts and discussed possible mechanisms for the vacuolation observed. These workers found that increasing the osmotic pressure of the growth medium by the addition of sucrose did not reduce the rate or extent of the vacuolation.

This paper describes the effects of increased tonicity on procaine-induced vacuolation in human epithelial cells in continuous culture and provides evidence that increased tonicity can influence the course of procaine-induced cell vacuolation.

## EXPERIMENTAL

The human heteroploid epithelial-like cell line H.Ep.2 derived from a carcinoma of the larynx by Fjelde (1955) was used in all experiments. The growth medium in which the cells were maintained throughout all experiments consisted of Medium 199 described by Morgan, Morton & Parker (1950), modified by Salk, Youngner & Ward (1954) and supplemented with 20% bovine serum. Sodium benzylpenicillin and streptomycin sulphate were added to a final concentration of 500 units/ml and 150 $\mu$g/ml respectively. All media and the H.Ep.2 cell line were supplied by Commonwealth Serum Laboratories, Melbourne. Media were adjusted to the required pH with sodium bicarbonate solution and were sterilized by filtration (Millipore 0·47$\mu$m) before use. All experiments were made in 30 ml polystyrene culture flasks (Falcon Plastics) or silicone-stoppered Pyrex T-flasks each containing 5 ml of medium. Each flask was seeded with approximately 750,000 cells and incubated at 37° for 24 h before use.

Experiments were conducted by removing the growth medium from 24 h cultures and replacing it with fresh growth medium alone (controls) or with fresh growth medium plus procaine hydrochloride (3·7mM) and incubating at 37°. The effects of the following osmotic adjustments were observed on both control and procaine-treated cells. In one series, additional solid NaCl was added to the medium to increase the concentration by 0·2, 0·3, 0·4, 0·5, 0·6, 0·7, 0·8, 0·9 or 1% NaCl

(10 flasks were used at each of these concentrations). In other series, osmotic pressure was increased by the addition of anhydrous dextrose to the extent of 1·7, 2·3, 2·8 or 4·7%, fructose 2·3%, sucrose 4·1% or $Na_2SO_4 \cdot 10H_2O$ 1·8% (10 flasks at each concentration). All substances added to the growth medium were added as solids and were dissolved by thorough agitation before the medium was made to volume. The medium was then warmed to 37° and added to the culture flasks.

The progress of vacuolation was followed by phase contrast microscopy (300X). Photographs were taken every hour for the first 24 h, then less frequently, at which times the extent of vacuolation in several random fields of each culture flask was compared with that of cultures treated with procaine alone. Experiments were continued for up to 10 days with the adjustment of pH and replacement of procaine, osmotic adjusting substance and growth medium being made each day. pH measurements were taken before and after media were changed to determine the pH range over which experiments were made. The pH was maintained between 7 and 8 in all experiments; in this range, Yang & others (1959) found the effects of procaine were not pH dependent. Measurements were made with a combination-electrode pH meter immediately after flasks were opened; this minimized the loss of dissolved $CO_2$ from solutions.

## RESULTS

### *Effects of procaine on cells*

In preliminary experiments, the effects of procaine hydrochloride in concentrations ranging from 1–10mM on vacuolation were observed, to determine the concentration producing the most marked vacuolation without producing undue toxic effects. The optimal concentration of procaine was between 3 and 4mM, and as Yang & others (1965) used 3·7mM procaine for Hela cells and chick embryo fibroblasts, it was decided to use this same concentration in all subsequent experiments.

The course of procaine-induced vacuolation in H.Ep.2 cells appeared identical to that described by Yang & others (1965) for Hela cells and chick fibroblasts. The vacuoles started to appear in the perinuclear area and gradually grew in size and number until large vacuoles packed the cells (Fig. 1a). Vacuolation was maximal

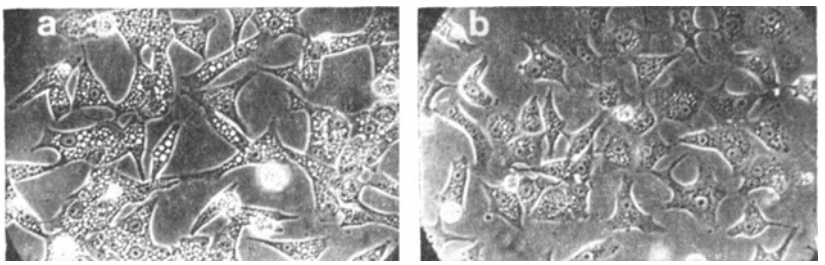


FIG. 1. a. H.Ep.2 cells that have become fully vacuolated after about 24 h exposure to 3·7mM procaine. b. The reduction in vacuolation seen after 30 h exposure to 3·7mM procaine and an additional 0·4% NaCl (bringing the total osmotic pressure of the medium to the equivalent of 1·3% NaCl). Note that occasional cells are vacuole-free.

after about 24 h exposure to procaine (3·7mM) and the cells remained heavily vacuolated for the duration of the experiments. Vacuoles of this nature were never seen in control cultures.

*Effects of increased osmotic pressure on vacuolation*

Procaine-induced vacuolation was markedly reduced both in the extent and size of vacuoles by the addition of 0·3–0·5% NaCl (bringing the total osmotic pressure to the equivalent of 1·2–1·4% NaCl, since the original growth medium was approximately isotonic). The difference between treatment with procaine alone, and procaine with added NaCl was obvious after about 24 h, and was marked after about 30 h. Cells treated with procaine plus NaCl appeared more epithelial in shape and occasional vacuole-free cells were seen in each field. All cells had fewer vacuoles than those exposed to procaine alone. Maximal vacuolation was never attained in the presence of these strengths of NaCl (Fig. 1b). The addition of 0·2% NaCl exerted little effect on the process while the addition of 0·6% or more NaCl produced toxic effects. This toxicity was also noted in control cultures containing an additional 0·6–1·0% NaCl and no procaine.

The addition of 0·3–0·5% NaCl appeared optimal for the modification of the procaine-induced effects. The addition of 1·7, 2·3 and 2·8% dextrose, providing total osmotic pressures equivalent to 1·2–1·4% NaCl (since 0·9% NaCl is iso-osmotic with 5·1% dextrose), also reduced the extent of vacuolation in a similar manner to equivalent strengths of NaCl. Dextrose 4·7% caused cell death within a few hours. The addition of fructose (2·3%), sucrose (4·1%) or $Na_2SO_4 \cdot 10H_2O$ (1·8%), providing total osmotic pressures in the medium equivalent to 1·3% NaCl (since 5·1% fructose, 9·3% sucrose or 4·0% $Na_2SO_4 \cdot 10H_2O$ is iso-osmotic with 0·9% NaCl), also reduced the extent of procaine-induced vacuolation in a similar manner to osmotically equivalent strengths of NaCl.

When the osmotic adjustments listed above were made to cultures that had become fully vacuolated after 24 h exposure to procaine, it was noted after 1–2 days that vacuolation was markedly less than in cultures treated with procaine alone.

While the vacuolation with procaine was very much reduced in media adjusted to an osmotic pressure equivalent to approx. 1·3% NaCl, cells kept under these conditions were less uniform in shape and had a much more granular cytoplasm than untreated controls. These effects became more pronounced as the strength of the osmotic adjusting substance was increased beyond the equivalent of 1·3% NaCl; there was also a concomitant increase in the proportion of dead cells.

## DISCUSSION

Yang & others (1965) found that procaine 3·7mM produced pronounced but reversible vacuolation in Hela cells and chick embryo fibroblasts. We found this also to be true for the H.Ep.2 cell line. Lower strengths of procaine did not produce maximal vacuolation in H.Ep.2 cells and higher strengths proved toxic to the cells within a few hours.

Yang & others (1965) added 10% sucrose to their growth medium to test whether an increased osmotic pressure could reduce drug-induced vacuolation. These workers found no difference in the extent or rate of vacuolation during the 16–18 h the cells were observed. Our study has confirmed that increased osmotic pressure produced no detectable difference during this period, and it was only after about 24 h exposure to procaine and the osmotic adjusting substances that the effect of increased osmotic pressure became obvious. This difference was maintained for the remainder of the experiments (up to 10 days). An increase in osmotic pressure equivalent to

approx. 1·3% NaCl reduced the formation of vacuoles and was also effective in reducing established vacuolation. There appeared to be no difference between the effects of NaCl, glucose, fructose, sucrose or $Na_2SO_4$ when used in osmotically equivalent amounts. A high concentration of dextrose (4·7%) was added to the growth medium in one series because there are reports (Setnikar & Temelcou, 1959; Vaille & Souchard, 1965) that about 10·5% dextrose (instead of the usual 5·1%) is isotonic with human erythrocytes. Since the addition of 4·7% dextrose destroyed the cells within a few hours, it is unlikely that 10·5% dextrose is isotonic with human epithelial cells. It is interesting that a final tonicity equivalent to approx. 1·3% NaCl appeared optimal for reducing procaine-induced vacuolation since Setnikar & Temelcou (1959) found that about 1·3% NaCl was necessary to render procaine solutions isotonic to rabbit erythrocytes (rabbit red cells are normally isotonic with 0·93% NaCl). These workers suggested that procaine increased the permeability of the erythrocyte to NaCl. They also noted that low concentrations of dextrose (0·6 iso-osmolar) not only abolished the increase in permeability produced by procaine but rendered the cell membrane partially impermeable to procaine itself. Hönig, Malm & Persson (1964) found that dextrose appeared to counteract the haemolytic effects of lignocaine hydrochloride on rabbit erythrocytes below a certain concentration of the local anaesthetic. Under the conditions of our experiments, we were not able to detect a specific protective effect of dextrose on procaine-induced cell vacuolation.

It is known that the extent of drug-induced cytoplasmic vacuolation is related to the concentration of vacuolating agent and that the vacuolation is fully reversible even after 24 h exposure to such substances. Vacuolated cells appear to exhibit normal motility and have frequently been observed to undergo mitosis. It has also been established that pH has a marked effect on vacuolation, presumably because of its influence on the ionization state of the vacuolating agent and thus on its availability to the cell (Yang & others, 1965). The present study indicates that osmotic effects can also influence procaine-induced vacuolation but sufficient information is not yet available for the significance of this finding to be fully interpreted.

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REFERENCES

Belkin, M., Hardy, W. G., Orr, H. C. & Lachman, A. B. (1962). *J. natn. Cancer Inst.*, **28**, 187–201.

Buschsbaum, R. & Kuntz, J. A. (1954). *Ann. N.Y. Acad. Sci.*, **58**, 1303–1310.

Fjelde, A. (1955). *Cancer*, **8**, 845–851.

Hönig, J., Malm, L. & Persson, H. (1964). *Acta pharm. suec.*, **1**, 129–138.

Lettré, H. & Albrecht, M. (1941). *Hoppe-Seyler's Z. physiol. Chem.*, **271**, 200–207.

Lettré, H. & Albrecht, M. (1943). *Ibid.*, **279**, 206–208.

Lettré, H. & Albrecht, M. (1951). *Ibid.*, **287**, 58–65.

Morgan, J. F., Morton, H. J. & Parker, R. C. (1950). *Proc. Soc. exp. Biol. Med.*, **73**, 1–8.

Pomerat, C. M. & Emerson, G. (1945). *Proc. Trans. Tex. Acad. Sci.*, (1944), **28**, 91.

Salk, J. E., Youngner, J. S. & Ward, E. N. (1954). *Am. J. Hyg.*, **60**, 214–230.

Setnikar, I. & Temelcou, O. (1959). *J. Am. pharm. Ass.*, (*Sci. Edn.*), **48**, 628–630.

Vaille, Ch. & Souchard, M. (1965). *Annls pharm. fr.*, **23**, 323–330.

Yang, W. C. T., Strasser, F. F. & Pomerat, C. M. (1965). *Expl Cell Res.*, **38**, 495–506.