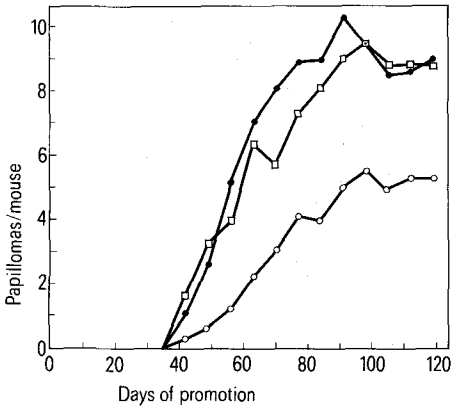
the simplest explanation is that acetic acid treatment results in the death of a proportion of the initiated cells. Previous studies have shown that a single application of 400[8] or 500[4] µmoles of acetic acid to mouse skin causes a marked initial inhibition of thymidine incorporation into DNA. This result suggests that acetic acid is cytotoxic at these doses.



Development of papillomas in mice initiated with DMBA and promoted with croton oil. 1 week after initiation with 25 µg DMBA, groups were treated 5 times at a rate of 2 applications per week with either acetone (●), 167 µmoles of acetic acid (□) or with 500 µmoles of acetic acid (○). 4 days after the final acetic acid or acetone treatment, all groups were promoted with croton oil (see materials and methods).

Application of a higher dose of acetic acid to mouse skin (1000 µmoles) causes superficial ulceration[4].

In a similar way, cell death could explain the observation that acetic acid inhibited 12-0-tetradecanoyl phorbol-13-acetate promotion in mouse epidermis[4].

The results emphasise the dangers inherent in interpreting experiments showing the inability of substances like acetic acid to act as efficient promoters. Such experiments leave open the possibility that these hyperplastiogenic substances induce all of the biochemical changes in skin necessary for promotion, but do not assay as promoters because of cytotoxicity. Consequently the observation that not all hyperplastic agents act as tumour promoters[4,5] does not, by itself, eliminate the possibility that epidermal hyperplasia is a sufficient condition for promotion.

1 Acknowledgment. The author thanks Mr M. Froscio for excellent technical assistance.
2 This investigation was supported by the Australian Research Grants Committee, the Australian Tobacco Research Foundation and the University of Adelaide Anti-Cancer Foundation.
3 R.K. Boutwell, Prog. exp. Tumor Res. *4*, 207 (1964).
4 T.J. Slaga, G.T. Bowden and R.K. Boutwell, J. nat. Cancer Inst. *55*, 983 (1975).
5 T.J. Slaga, J.D. Scribner, S. Thompson and A. Vaije, J. nat. Cancer Inst. *57*, 1145 (1976).
6 T.G. O'Brien, Cancer Res. *36*, 2644 (1976).
7 A.W. Murray and M. Froscio, Cancer Res. *37*, 1360 (1977).
8 L. Krieg, I. Kühlmann and F. Marks, Cancer Res. *34*, 3135 (1974).

Effect of tannic acid on the Ehrlich ascites tumor cells

E.C. Chew[1]

Department of Anatomy, University of Hong Kong, Hong Kong (Hong Kong), 20 March 1978

Summary. Tannic acid was used to fix and stain Ehrlich ascites tumor cells in suspension. An increase in surface microvilli and cytoplasmic blebs in the tumor cells was observed. The mechanism of tannic acid induced surface morphological changes in tumor cells and the formation of a precipitate of protein-tannic acid-heavy metal complex are discussed.

Tannic acid (TA) was recently applied as an additional fixative[2-4] as well as a staining substitute[5-7] for biological specimens in electron microscopy. Simionescus and Simionescus[8] reviewed the action of TA-glutaraldehyde mixture on fresh tissues and stated that these preparations are usually affected by a) the unsatisfactory penetration of cells; b) the formation of interstitial precipitates, and c) the extraction or precipitation of some tissue constituents. The present communication reports the effect of TA on Ehrlich ascites (EA) tumor cells in suspension. The surface morphological changes of EA cells after TA treatment are presented and the possible mechanism of these alterations is discussed.

Material and methods. The Ehrlich ascites tumor cells in suspension were kindly provided by Dr Y.C. Kong of the Department of Biochemistry, the Chinese University of Hong Kong. The tumor is carried in this laboratory by s.c. and i.p. transplantations in WHT/HT (Swiss) mice.

In all the experiments performed, tumor cell pellets were made from peritoneal fluid containing tumor cells and were fixed in 2.5% glutaraldehyde with or without 4 or 8% tannic acid at 20 °C. Some specimens were post-fixed in 1% osmium tetroxide. Routine procedures were followed for dehydration and embedding in Epon 812. Sections were examined with or without uranyl acetate and lead citrate staining and studied with a Philips EM 300 at 60 kV. Thick sections of about 1 µm were cut and stained with paragon[9] for light microscopic observations.

Results. Under the light microscope, the tumor cell pellet showed that it contained mainly EA cells with only a few blood cells and a small amount of cell debris. The tumor cells appeared viable and mitotic figures were frequently seen (figures 1 and 2). In the control group, i.e. without the addition of tannic acid to the fixative, the tumor cells were round and the surface was smooth (figure 1). When tannic acid was added to the fixative, the cell membrane looked very thick and always contained numerous microvilli. Heavy metal deposits were found on the cell membrane as well as in the interstitial spaces (figure 2).

The fine structure of the EA tumor cells have been described by many investigators[10-15]. Again, under the electron microscope, tumor cells in the control group always possessed a smooth surface with only a few microvilli. The cell membrane was well defined. When tannic acid was added to the fixative, the cell membrane appeared to be very dense and irregular. An increase in microvilli and cytoplasmic blebs was an obvious feature. Heavy metal particles were found in the interstitial space and on the cell membrane (figures 3-5). When the specimen was fixed in TA-glutaraldehyde mixture, but without post-fixing in osmium tetroxide, the results were rather similar to that just described. However, the contrast was much less. The cellu-

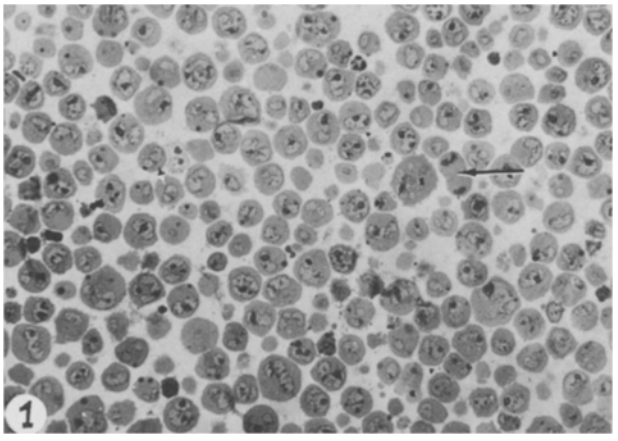


Fig. 1. 1-µm thick plastic section showing EA cells fixed in 2.5% glutaraldehyde. Note that the cell membrane is thin and rather SMOOTH. Mitotic cells are present (arrow). Paragon stain. × 470.

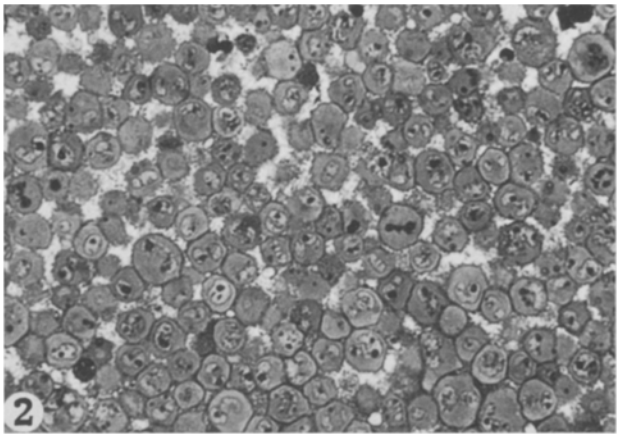


Fig. 2. 1-µm thick plastic section showing EA cells fixed in TA-glutaraldehyde mixture. Note that the cell membrane is thick and heavy metal precipitates are present in the interstitial space. Paragon stain. × 470.

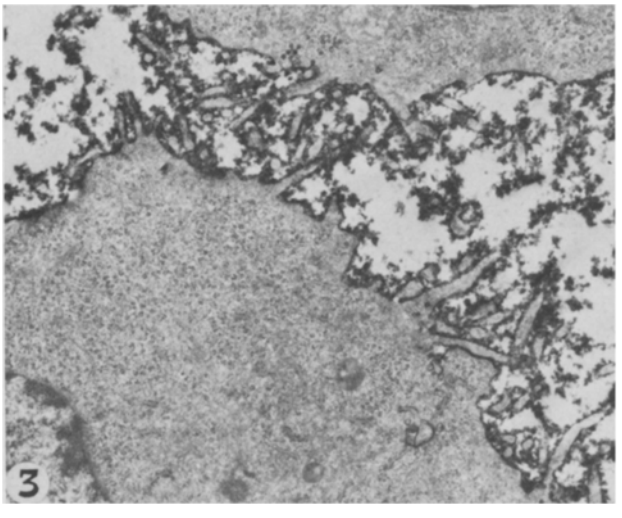


Fig. 3. Electron micrograph showing 2 EA cells with an abundance of microvilli. Heavy metal precipitates are present along the cell membrane and in the interstitial space. × 540.

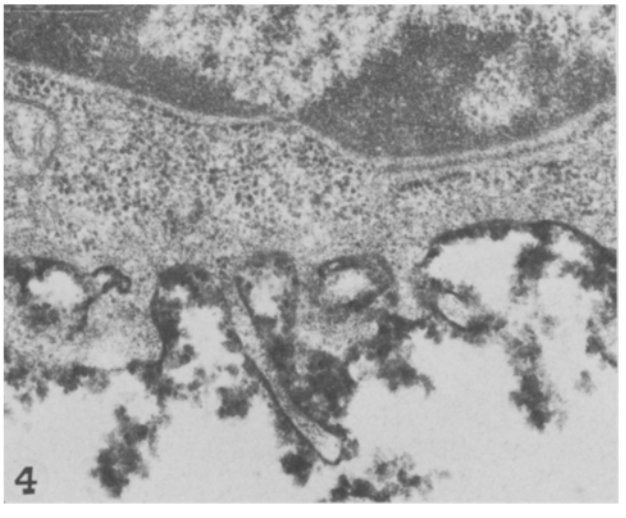


Fig. 4. High power view of an EA cell showing heavy metal particles deposited on the cell membrane. × 610.

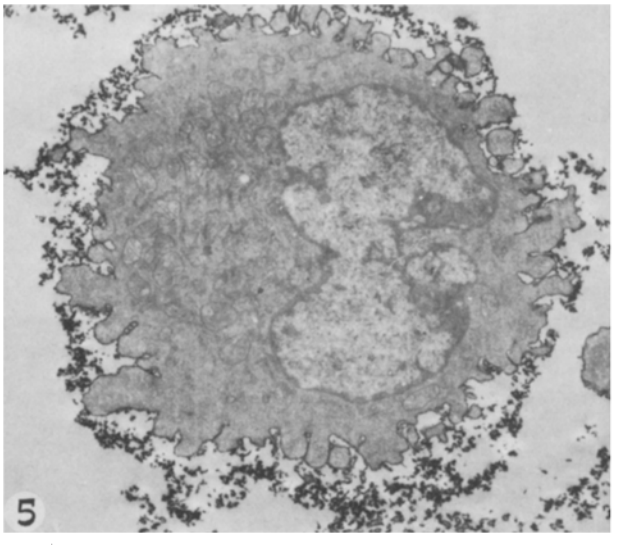


Fig. 5. Electron micrograph showing an EA cell with an abundance of cytoplasmic blebs. × 135.

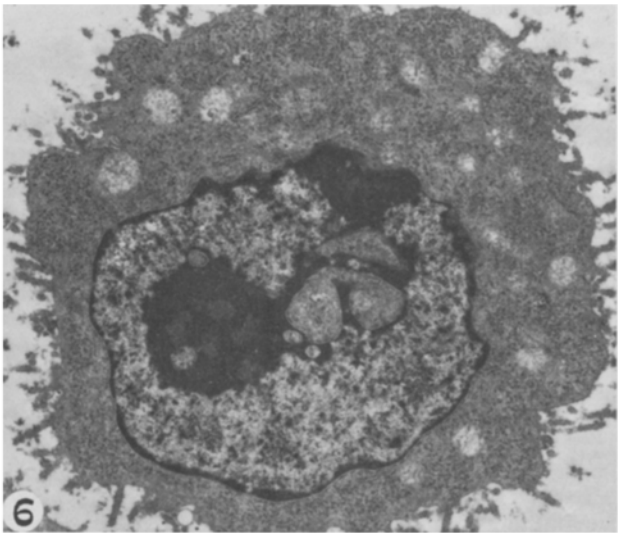


Fig. 6. An EA cell from a specimen fixed in TA-glutaraldehyde without OsO_4 fixation. Note that the heavy metal precipitates have rather low contrast. × 175.

lar elements were well preserved and observation was permissible (figure 6).

Discussion. In his study of the mechanism of precipitation caused by tannic acid fixation, Futaesaku et al.[3] assumes that the precipitate of protein-TA-heavy metal complex stays in the tissue and that TA-fixation gives the high electron density of the region of protein selectivity. Singer and Nicolson[16] propose the fluid mosaic model of membrane structure. According to this model, surface proteins are free to diffuse in a lipid matrix and thus to assume a random or homogenous distribution over the cell surface. If the above hypotheses are correct, then the precipitate of protein-TA-heavy metal deposit on the cell membrane as observed in the present study can probably be used as one of the indicators of the localization of cell membrane protein components.

On the other hand, Futaesaku et al.[3] observed that, on the small intestine, the substance of the cell coat was stained strongly by tannic acid. A similar result was also observed by the author (unpublished result). It is of course possible that the protein-TA-heavy metal complex, as observed by Futaesaku et al. and as seen in the present investigation, only indicates the presence of mucoprotein molecules in the cell coat rather than on the cell membrane itself. It is rather premature at this moment to conclude which of the above possibilities is true. From the present investigation, TA seems to be a nonspecific stain for surface proteins. It may well be that both possibilities are true. The exact mechanism of the precipitate caused by TA fixation needs further investigation.

Our preliminary result also indicates an increase in microvilli and cytoplasmic bleb formation as a result of tannic acid fixation of EA cells in suspension. Tannic acid has also been used to demonstrate the subunit structure of microtubules[17,18]. Cytoskeletal elements are shown interacting indirectly with the plasma membrane or with each other through cross-links or bridging structures[18]. It is likely that, during tannic acid fixation, some of the microtubular system is altered which in turn leads to microvilli and cytoplasmic bleb formation. The precise mechanism by which tannic acid binds proteins and brings about changes in the cell surface configuration remains to be established.

Regardless of the mechanism of protein-TA-heavy metal precipitations on the cell surface, tannic acid obviously acts as a ligand for osmium tetroxide. In view of this, tannic acid can further be used as a ligand for osmium tetroxide in the study of cell surface structures in scanning electron microscopy.

1 Acknowledgment. The author is grateful to Professor F.P. Lisowski for his advice and helpful criticism and to Mr Y.S. Tong for his expert technical assistance. This project was supported by a grant from the Hong Kong University Research Grants Committee.

2 V. Mizuhira and Y. Futaesaku, Proc. Electron Microsc. Am. *29*, 494 (1971).
3 Y. Futaesaku, V. Mizuhira and H. Nakamura, Proc. int. Congr. Histochem. Cytochem. *4*, 155 (1972).
4 R. Rodwald and M.J. Karnovsky, J. Cell Biol. *60*, 423 (1974).
5 L.G. Tilney, J. Bryan, D.J. Bush, K. Fujiwara, M.S. Mooseker, D.B. Murphy and D.H. Snyder, J. Cell Biol. *59*, 267 (1973).
6 F.L. Shienwold and D.E. Kelly, J. Cell Biol. *63*, 313a (1974).
7 P.R. Burton, R.E. Hinkley and G.B. Pierson, J. Cell Biol. *65*, 227 (1975).
8 N. Simionescu and M. Simionescu, J. Cell Biol. *70*, 658 (1976).
9 B.O. Spurlock, B.A. Margeret, M.D. Skinner and A.A. Kattine, Am. J. clin. Path. *46*, 252 (1966).
10 G. Yasuzumi and S. Higashizawa, Gann *47*, 527 (1956).
11 O.A. Myking and Å. Abro, Acta path. microbiol. scand. *82*, 571 (1974).
12 W.R. Adams and A.M. Prince, J. biophys. biochem. Cytol. *3*, 161 (1957).
13 T. Kodama and M. Kodama, J. nat. Cancer Inst. *50*, 707 (1973).
14 E.C. Chew, Abstr. 3rd Asian Cancer Conference, p. 119. Manila 1977.
15 E.C. Chew, Relationship between A-type and C-type particles in Ehrlich ascites tumor cells, submitted.
16 S.J. Singer and G.L. Nicolson, Science *175*, 720 (1972).
17 V. Mizuhira and Y. Futaesaku, Proc. 8th int. Congr. Electron Microsc. Canberra *2*, 340 (1974).
18 M.S. Mooseker and L.G. Tilney, J. Cell Biol. *56*, 13 (1973).

Determination of proliferative compartments in human tumors

H.M. Rabes[1], P. Carl and U. Rattenhuber

Pathologisches Institut und Urologische Klinik und Poliklinik der Universität München, Thalkirchner Strasse 36, D-8 München 2 (Federal Republic of Germany), 28 April 1978

Summary. Extracorporeal normothermic perfusion with radioactive thymidine allows a cytological, histological and structural analysis of proliferative compartments of human kidney carcinomas in whole-tumor autoradiograms.

From experimental animals it is known that tumor growth is a result of antagonistic intratumoral dynamics which have been described in terms of cell kinetics[2], growth fraction[3] and cell loss[4]. In humans, a direct view on the internal growth pattern of solid tumors is difficult to obtain. Tumor biopsis incubated in tritiated thymidine for labelling cells in DNA synthesis[5-7], do not reflect the proliferative structure of the whole tumor. Systemic application to the patient of radioactive DNA precursors and subsequent serial resections from tumor tissue for the determination of cell cycle parameters[8] should, for ethical reasons, be confined to extreme cases. A new method has therefore been developed to study the intratumoral growth pattern in human tumors by means of postoperative perfusion. Kidney carcinomas are favourable objects for this approach. A single renal artery supplies blood to both, kidney and carcinoma. The idea is to perfuse, immediately after operative resection, the tumor-bearing organ through this artery with a physiological medium containing radioactive DNA precursors and thus to label cells in DNA synthesis.

Materials and methods. The apparatus used for kidney tumor perfusion consists of a chamber to incubate the tumor-bearing organ at 37.5 °C in blood compatible with the tumor-patient's blood group, with additions of dextran solution to lower the hematocrit down to about 25-30%, heparin to prevent blood clotting, and $NaHCO_3$ to adjust a physiological pH. The perfusate is circulated by an occlusive roller pump (Stöckert, Munich, FRG) with an electonically regulated pressure and a variable flow rate of up to 425 ml/min into the renal artery. Physiological P_{O2} values are obtained by a membrane oxygenator (5M0321, Travenol Laboratories, Morton Grove, Ill., USA). The venous outflow is recirculated. During the perfusion, tritiated (1.3 μCi/ml, sp. act. 5 C/mmole) or ^{14}C-labelled thymi-