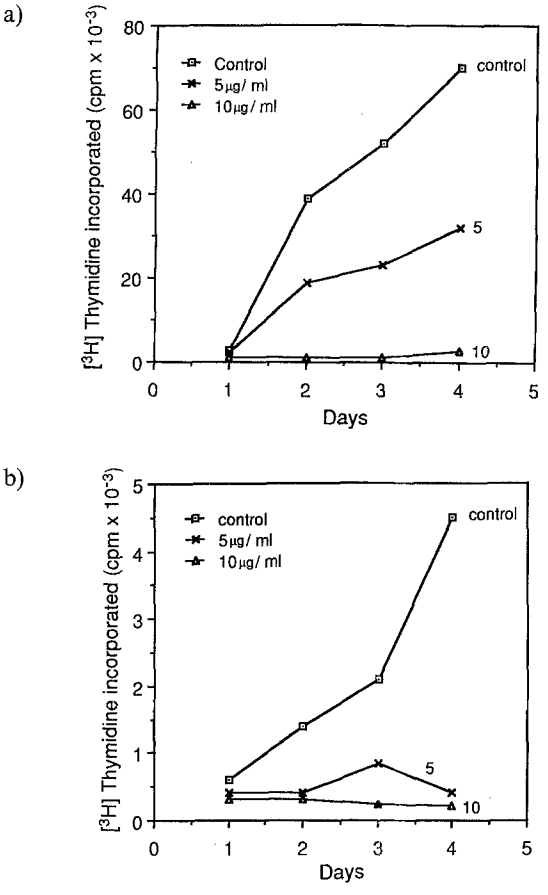

Figure 4. Effect of CAPE on the incorporation of [^{3}H]thymidine into DNA of (*a*) human MCF-7 breast carcinoma and (*b*) human SK-MEL-28 melanoma cells. Cells were maintained in Eagle's minimal essential medium (MEM) with Earle's salts and 10% fetal bovine serum. The cells were seeded in the same medium in tissue culture cluster plates (96 flat bottom wells) at 10^3 cells/well. After 24 h (day 1) cultures were washed and different concentrations of CAPE were added to each well in triplicate. Labeling of cells was accomplished on days 1–4 by incubating with 0.5 mCi [^{3}H]thymidine for 5 h. For further details see Eisinger et al.[6].

of caffeic acid with a lipophilic alcohol may simply facilitate its transport into cells, where it is hydrolyzed. Ester analogs of CAPE may be readily prepared for testing this and other possibilities.

The ready accessibility of analogs and labeled versions of CAPE will simplify further investigations into its mode of action, and may lead to an understanding of the observed differential effects on a molecular structural level. Furthermore, such studies with CAPE and other cytostatic compounds may provide a clearer insight into the molecular events responsible for the dissimilar biological properties exhibited by transformed and normal cells. Because the cytostatic action of CAPE is more dramatic on transformed cells, one may reasonably assume that it is at least partly responsible for the claimed carcinostatic properties of propolis.

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Adenosine-5′-triphosphate levels in experimental CaNT and Fib/t tumours of varying volume and degree of hypoxia

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Summary. The variation of adenosine-5′-triphosphate (ATP) content per unit mass of tumour, versus tumour volume was measured in vivo under normoxic conditions, using CaNT and Fib/t murine tumours grown in CBA and WHT mice respectively. A monotonically decreasing relation was found. Artificially induced tumour hypoxia resulting from 15 min of clamping was accompanied by reduced ATP levels.
Key words. CaNT and Fib/t tumours; degree of hypoxia; tumour volume; ATP.

Many approaches to the improvement of cancer therapy are based on the assumption that the tumour tissue contains viable hypoxic regions that are radioresistant and often chemoresistant too. Such hypoxic regions may therefore be responsible for some failures of treatment[1]. Oxygen diffuses out from the capillaries and is avidly consumed by the active metabolic activity of tumour cells. So oxygen is depleted within a distance of 150–200 µm from the capillary. Hence cells lying more than about 150 µm away from the capillary can exist in hypoxic and anoxic states[2]. Rapid proliferation of tumour cells may disorganize their blood supply to such an extent that sufficient oxygen and nutrients cannot reach all the cells and anaerobic glycolysis is necessary to provide the energy for cell growth and division[3]. These changes re-

sult in mitochondrial dysfunction (lack of oxidative phosphorylation and ATP generation)[4]. During growth, tumours can become more hypoxic and necrotic which results in increased levels of inorganic phosphate and decreased levels of high energy phosphates[1]. If the hypoxic fraction could be determined this would help to predict radiation treatment response. This work attempts to relate mean ATP concentration per unit of tumour mass with tumour volume, as a way of indicating degree of hypoxia.

Male CBA and WHT mice were used. Their ages were between 6 and 8 weeks before any procedures were undertaken and their weight range was 18–23 g. CaNT and Fib/t tumours were grown in CBA and WHT mice respectively. The tumours were maintained by serial passage with inoculation of a tumour cell suspension s.c. into the sternum area of the mice.

Rodent tumours used in the investigation of ATP content versus tumour volume were between 50 and 550 mm^3, as calculated by measurement in three orthogonal directions using Vernier calipers, assuming a spherical shape.

For the investigation of ATP after induction of hypoxia by clamping, only tumour volumes between 150 and 250 mm^3 were used for both strains. The procedure adopted to clamp the tumours was that of Rockwell et al. [5] which retracted the tumour and associated skin away from the body of the mouse. Then a thin string was tied firmly around the skin flap between the host and tumour. The clamp was left in place for 15 min. To prepare samples for ATP determinations, mice were anaesthetized with ether and skin and tissue surrounding the tumour removed. The tumours were cut away and immediately dropped into liquid nitrogen, weighed and pulverized in a mortar with frequent additions of liquid nitrogen. One ml HCl (6% w/v) was added and ground with the tissue, the mixture allowed to become fluid and homogenized in a Potter type glass homogenizer. The homogenate was centrifuged at 17500 × g for 20 min at 4 °C. Then the supernatant was removed and the pH adjusted to 7.5 with 5 M K_2CO_3, followed by centrifugation at 17500 × g for 20 min at 4 °C. The supernatant was used for determination of tumour ATP levels, which was performed according to the enzymatic method of Lamprecht and Trautschold[6]. P values were obtained from a paired one-tailed t-test.

Adenosine-5′-triphosphate levels have been measured in CaNT and Fib/t tumours within the following volume ranges: 50–150 mm^3, 150–250 mm^3, 250–350 mm^3, 350–450 mm^3, and 450–550 mm^3. The entire tumour was removed and then measurements of ATP content were made immediately after sacrifice of the anaesthetized animals. The results are presented in figures 1 and 2. It is clear there is a monotonically decreasing relation between ATP content per unit mass of each tumour type and total tumour volume. This finding is thought to reflect the relation of tumour size versus degree of hypoxia and necrosis[7]. The hypoxic fraction has been observed to augment with increasing tumour size[8].

As tumours increase in volume they derive greater amounts of energy from anaerobic glycolysis. This is because they tend to outgrow their blood supply and higher proportions of their cells become hypoxic. Augmented levels of inorganic phosphate in these tumours result from breakdown of ATP. The high intensity of the distinct sugar phosphate peaks from different human carcinomas examined by in vivo ^{31}P NMR in athymic mice can therefore probably be attributed to accumulation of glycolytic intermediates, such as fructose 1,6-diphosphate, and AMP[9].

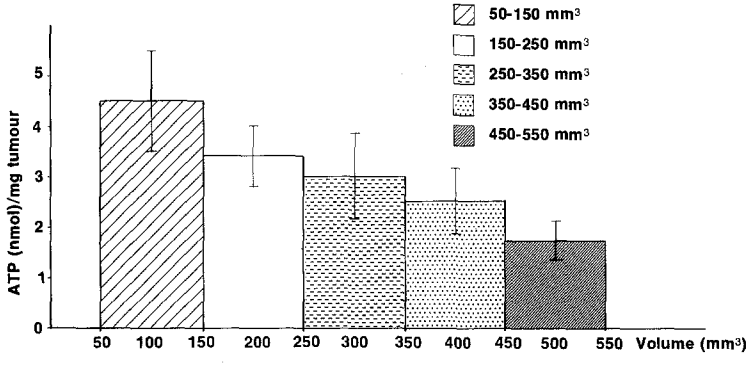

Figure 1. ATP levels (nmol/mg of tumour) in CaNT tumours of different volume ranges. These tumours were grown in the sternal area of CBA mice. The height of each histogram block (ATP level) represents the mean of not less than 7 determinations ± SEM as indicated by the error bars. There is significant difference between ATP levels for each volume range ($p < 0.05$).

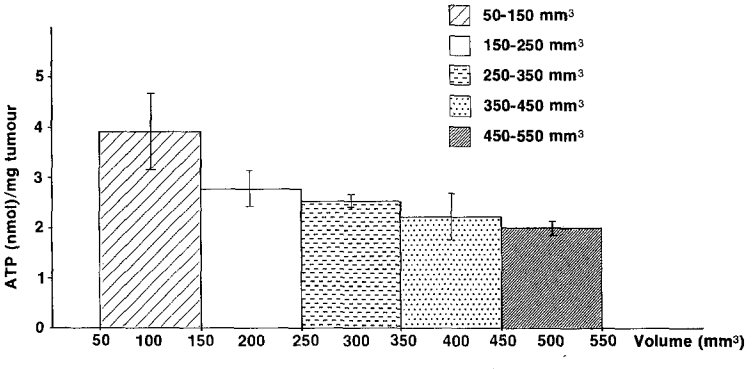

Figure 2. ATP levels (nmol/mg of tumour) in Fib/t tumours of different volume ranges. These tumours were grown in the sternal area of WHT mice. The height of each histogram block (ATP level) represents the mean of not less than 9 determinations ± SEM as indicated by the error bars. There is significant difference between ATP levels for each volume range ($p < 0.01$).

ATP concentrations in normoxic and hypoxic conditions for murine tumours with volumes in the range 150–250 mm^3

Tumour type	Number of determination	Concentration (nmol/mg)
CaNT (normoxic)	21	3.42 ± 0.47*
CaNT (hypoxic)	22	2.57 ± 0.44*
Fib/t (normoxic)	17	2.64 ± 0.39**
Fib/t (hypoxic)	16	1.82 ± 0.24**

Values are means ± SEM. * $p < 0.01$; ** $p < 0.05$.

Adenosine-5′-triphosphate tumour concentrations were also measured 15 min after clamping CaNT and Fib/t tumours. The table presents the ATP concentrations for the normoxic and hypoxic tumours. In CaNT and Fib/t tumours the levels of ATP were observed to decrease significantly below the control value, 15 min after clamping. The decrease of ATP content in CaNT and Fib/t tumours after induction of hypoxia with respect to the controls can also be associated with impaired blood flow of nutrients and diminished oxygen consumption in the tumours. Any disruption such as the reduced oxygen consumption in normally coupled mitochondria would immediately be reflected in a lack of energy balance between the metabolic processes in the intra and extra-mitochondrial compartments of the cells. This leads to interference with ATP synthesis.

The following properties of tumours which decrease with tumour size, can be considered together. These are normally oxygenated fractions and ATP content per unit mass of tumour. Thus there arises the hypothesis that ATP content per unit mass of tumour inversely reflects its necrotic or hypoxic content. This hypothesis is strengthened by the additional data of the table, that ATP content per unit mass of tumour decreases significantly with artificial hypoxia resulting from clamping.

Adenosine-5′-triphosphate has been shown to modify the response to ionizing radiation in several situations. Tikhomirova et al.[10] have demonstrated that ATP protects against radiation damage from high energy protons. They reported that the survival of CBA and CS7B1 hybrid F1 mice treated with ATP and receiving whole body radiation with 9 GeV protons was reported to have been increased from 63 to 80%. Nikolov et al.[11] have shown that ATP administration provides protection for monkeys (*Macaca mulatta*) against a dose of 8.3 Gy gamma irradiation (^{137}Cs). The survival in a control group was only 5% and after ATP administration (two injections of 37 mg kg^{-1} body weight) it was 50%. Nishizawa et al.[12] investigated the effect of ATP deprivation, by 2,4 dinitrophenol (DPN) in murine melanoma cells in culture, on the x-irradiation response of these cells. The survival curves were changed as a result of post irradiation treatment with DPN, with a diminished shoulder (implying less radiation damage repair) and also a decreased extrapolation number (n). Benova et al.[13] conducted experiments concerned with protection of CS7BL male mice from genetic radiation damage in germ-cell genetic structures, using a combination of ATP, aminoethylisothiuronium Br-HBr and serotonin. Such a combination was found to reduce, by a factor of two, the number of cellular metaphases with translocations observed after 3 Gy of x-rays to mouse spermatagonia compared with irradiated mice not receiving the combination. Removal of ATP from the combination led to a significant reduction (59%) in protective effect.

These findings indicate that there is a likely role for ATP in systems which repair radiation damage. Therefore this high energy phosphate compound should be considered as a highly desirable component in selecting combinations of agents intended to protect against radiation injury. Also the endogenous ATP pool increases, as a result of cell metabolism after different types of radiation in vitro and in vivo[14]. All this evidence implies that increased ATP consumption is most probably related to repair of radiation damage in various cellular structures[15].

Tumours are subject to abnormal growth and growth depends on energy metabolism. Evidently some aspects of energy metabolism or of its control are therefore changed in comparison to normal tissues[16]. This work examines the variation of ATP levels with tumour size and may provide a better understanding of processes associated with energy, such as repair after radiation damage, and its relation to different degrees of hypoxia. Hence the potential then exists for using the variation of ATP concentration with tumour size in the development of novel strategies for radiosensitization of radioresistant hypoxic cells. This depends on being able to recognise the degree of hypoxia, and hence radioresistance, versus tumour size. Determining ATP levels in tumour metabolism can complement existing methods[17,18] to examine the degree of tumour hypoxia.

The observation that the effect of radiation and chemotherapeutic treatment of some tumours may be size dependent[7] can possibly now be explained by the variation of ATP content with tumour size. The central role of ATP in tumour metabolism and the implied degree of hypoxia in terms of ATP content per unit of mass with tumour volume, have been described in this work. This may contribute to improved prescriptions for tumour treatment by providing knowledge associated with the degree of hypoxia and hence further understanding of ATP's role in radiosensitisation and repair, which deserve further investigation.

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