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© THE INHIBITION OF MALIGNANT CELL GROWTH BY KETONE BODIES

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Summary. The effect of ketone bodies on the growth, in culture, of transformed lymphoblasts (Raji cells) was investigated. Cell growth was inhibited and this effect was reversible, non-toxic, and proportional to the concentration of D-β-hydroxybutyrate up to 20 mM. The total glucose utilisation and the total lactate production were reduced in proportion to the inhibition of cell proliferation. D-β-hydroxybutyrate was not metabolised by the cells. Other glycolytic inhibitors and chemical analogues of D-β-hydroxybutyrate either did not inhibit or proved to be too toxic for cell growth. D-β-hydroxybutyrate also inhibited the growth of rabbit kidney (RK13), HeLa, mouse melanoma (B16), fibroblast and trypsin-dispersed human thyroid and beef testis cells. Moreover, *in vivo* dietary-induced ketosis reduced the number of B16 melanoma deposits in the lungs of C57BL/6 mice by two-thirds. The significance of these results in the clinical management of cancer cachexia is discussed.

INTRODUCTION

Starvation and cancer are both characterised by weight loss. A model for the cachexia of cancer has been suggested based on the comparison of the metabolic aspects of these two states (Conyers *et al.*, 1979a, 1979b). As the concentration of ketone bodies increases in starvation regulatory mechanisms operate to reduce fatty acid mobilisation, glycolysis, gluconeogenesis and protein catabolism in various tissues (Cahill, 1976; Newsholme, 1976). The rate of weight loss is reduced and the decreased energy fluxes between tissues result in an hypometabolic state (Cahill, 1976). Furthermore, cell proliferative activity is also reduced in starvation (Aldewachi *et al.*, 1975; Stragand and Hagemann, 1977). On the other hand, cancer cachexia is an hypermetabolic state in which ketosis is a rather uncommon phenomenon (Gold, 1974; Conyers *et al.*, 1979a) and in which the cancer continues to grow, even in the presence of marked weight loss and poor dietary intake. Forced feeding regimens have been used to overcome the problem of cachexia but they have been associated with increased metabolic rates and increased cancer growth (Burke and Kark, 1977;

Conyers *et al.*, 1979a). We believe that it would be more appropriate to produce, physiologically, an hypometabolic state in cancer patients by iatrogenically inducing ketosis (Conyers *et al.*, 1979a, 1979b). In this manner, the cachexia of cancer and perhaps the malignant cell growth could be reduced. Since many cancer tissues exhibit high rates of glycolysis (Krebs, 1972), artificially induced ketosis could, theoretically, expose cancer patients to the risks of hypoglycaemia and lactic acidosis. We report here the inhibitory effect of ketone bodies on the metabolism and growth of malignant cells.

Materials and Methods

Laboratory supplies

Sodium, D,L-β-hydroxybutyrate and sodium iodoacetate were obtained from Calbiochem, Sydney; lithium acetoacetate from Sigma, U.S.A.; and D,L-serine, D,L-threonine, *sec*-butanol and *n*-butyric acid from BDH chemicals, U.K. All other chemicals were supplied by Ajax Chemicals, U.K.

The RPMI 1640 medium was purchased from Grand Island Biological Company, U.S.A. and the foetal calf serum from Flow Laboratories, Sydney. Gentamicin was a product of Roussel, Sydney. The Commonwealth Serum Laboratories, Melbourne, were the suppliers of benzylpenicillin and were also the original source of the transformed human lymphoblast cell line (Raji) established from Burkitt's lymphoma (Pulvertaft, 1965), the HeLa 229 cell line and the transformed rabbit kidney (RK13) cell line. The transplanted B16 mouse melanoma cell line and the C57BL/6 mice (Fidler, 1974) were originally obtained from the Walter and Eliza Hall Institute, Melbourne.

The fibroblast cell line was established in our laboratory from trypsin-dispersed, human, neonatal foreskins. In the experiments reported here the fibroblasts were used before the tenth passage in culture. Cells were also obtained from excised human thyroid and bovine testis by mincing the tissues and trypsin-dispersion in the usual manner. Epithelial-like cells were obtained from the thyroid tissue and the testis preparation provided mixed cell types.

Culture conditions

In the experiments reported in Table 1 and in Figs 1 and 2 Raji cells were grown in suspension culture in sealed milk dilution bottles incubated at 37°. The culture medium was RPMI 1640 buffered with 20mM Hepes and supplemented with 10% foetal calf serum, 60μg/ml benzylpenicillin and 40μg/ml gentamicin. Additional $NaHCO_3$ was not used. Cultures were seeded at 1×10^5 cells/ml and cell density was adjusted to this concentration at 3-day intervals with media change. Aliquots were taken daily for cell counts (haemocytometers), cell viability studies (trypan blue exclusion method) and metabolite determinations (continuous flow analysis using immobilized enzymes: Noy, Buckle and Alberti, 1978). When required, cells were harvested directly by centrifugation ($50 \times g$ for 5min at room temperature). Before use all media, including additions, were passed through Millipore filters.

In the study reported in Fig. 3 the conditions were varied as follows: RPMI 1640 medium was further supplemented with $NaHCO_3$; the cells were grown as suspensions or monolayers in 35mm plastic Petri dishes (NUNC, Denmark) in an humidified atmosphere of 5% carbon dioxide in air at 37°; cultures were seeded at the concentrations shown in Fig. 3; cell counts were performed in duplicate on days 0, 3 and 6; and, although media were changed on the third day, cell density was not adjusted.

Preliminary mouse study

The C57BL/6 mice used in this study were all males aged 6 weeks. On day 0 of the experiment the mice were randomly divided into two groups of 18. Each mouse received an 0·1ml injection containing 1×10^5 B16 melanoma cells into its tail vein. For the next 14 days group A mice had free access to water and to 200g/l sucrose in water. Group B mice had free access to water and to undiluted, polyunsaturated, blended, edible, vegetable oil (Provincial Traders, Queensland). On days 5, 8, and 11 both groups were provided with laboratory pellets for 1h. Urines were collected and tested for

ketone bodies (Ketostix, Ames). On day 14 the mice were sacrificed, blood samples taken and pooled (n=3) for metabolite determinations and their lung metastases counted by two independent observers with the aid of a dissecting microscope (Fidler, 1974).

Results are reported as the mean with its standard error and the numbers of determinations are given in parentheses. Statistical significance has been calculated by the t-test for unpaired means.

RESULTS

Effects of ketone bodies on growth and metabolism of Raji cells

The transformed lymphoblast cell line (Raji) was cultured in the presence of varying concentrations of sodium β-hydroxybutyrate. This ketone body is supplied commercially as a mixture of the D- and L-isomers and it is assumed in this paper that the effects attributed to sodium β-hydroxybutyrate are due to the naturally occurring D-form. Cell proliferation was inhibited by D-β-hydroxybutyrate (Fig. 1). Twenty mM D-β-hydroxybutyrate resulted in a 70% reduction in cell numbers without affecting cell viability. At 40 mM D-β-hydroxybutyrate viability was reduced by approximately 40% and there was a net loss of cells. Cell growth inhibition was significant as early as 24 h after treatment commenced with the 20 and 40 mM D-β-hydroxybutyrate ($p<0{\cdot}05$), and was significant between all the various concentrations of the inhibitor on the second and third days ($p<0{\cdot}01$).

The inhibition of cell proliferation was reversible, even for those cells which survived the 40 mM D-β-hydroxybutyrate treatment. This can be seen by comparing the slopes of the curves for each D-β-hydroxybutyrate concentration in Fig. 2. These experiments were carried out in the same manner as those reported in Fig. 1. That is, daily cell counts were performed in triplicate and the cell density was adjusted to 1×10^5/ml at 3-day intervals with media change. For clarity, however, the results of each 3-day interval have been plotted as continuations of the previous 3-day interval. The inhibition of cell proliferation was still reversible after the cell had been subcultured continuously in 20 mM D-β-hydroxybutyrate for 2 months. Moreover, cell viability was unaffected (>99%).

The Hemalog D/90 System (Technicon, U.S.A.) allows one to perform differential cell analyses on the basis of cell size and cytochemical activity (Ganon, 1977). Using the Hemalog criteria of cell volume and peroxidase activity, it was found that exposure of the Raji cells to 20 mM D-β-hydroxybutyrate for 6 days resulted in a second distinct population of cells. Morphological changes, however, were minimal. The most obvious one was the formation of multiple small cytoplasmic vesicles.

The total glucose utilisation and the total lactate production were reduced in proportion to the inhibition of cell proliferation (Fig. 1). The lactate production was marginally, though not significantly, greater than that theoretically expected from the glucose utilisation. These values are, however, calculated by difference and so this observation may only be trivial. Alternatively, lactate may also have been produced by the metabolism of other nutrients in the culture medium.

The effects of glycolytic inhibitors and chemical analogues of D-β-hydroxybutyrate on the culture characteristics of Raji cells are shown in Table 1. Of the two glycolytic inhibitors tested only iodoacetate markedly reduced both cell growth and

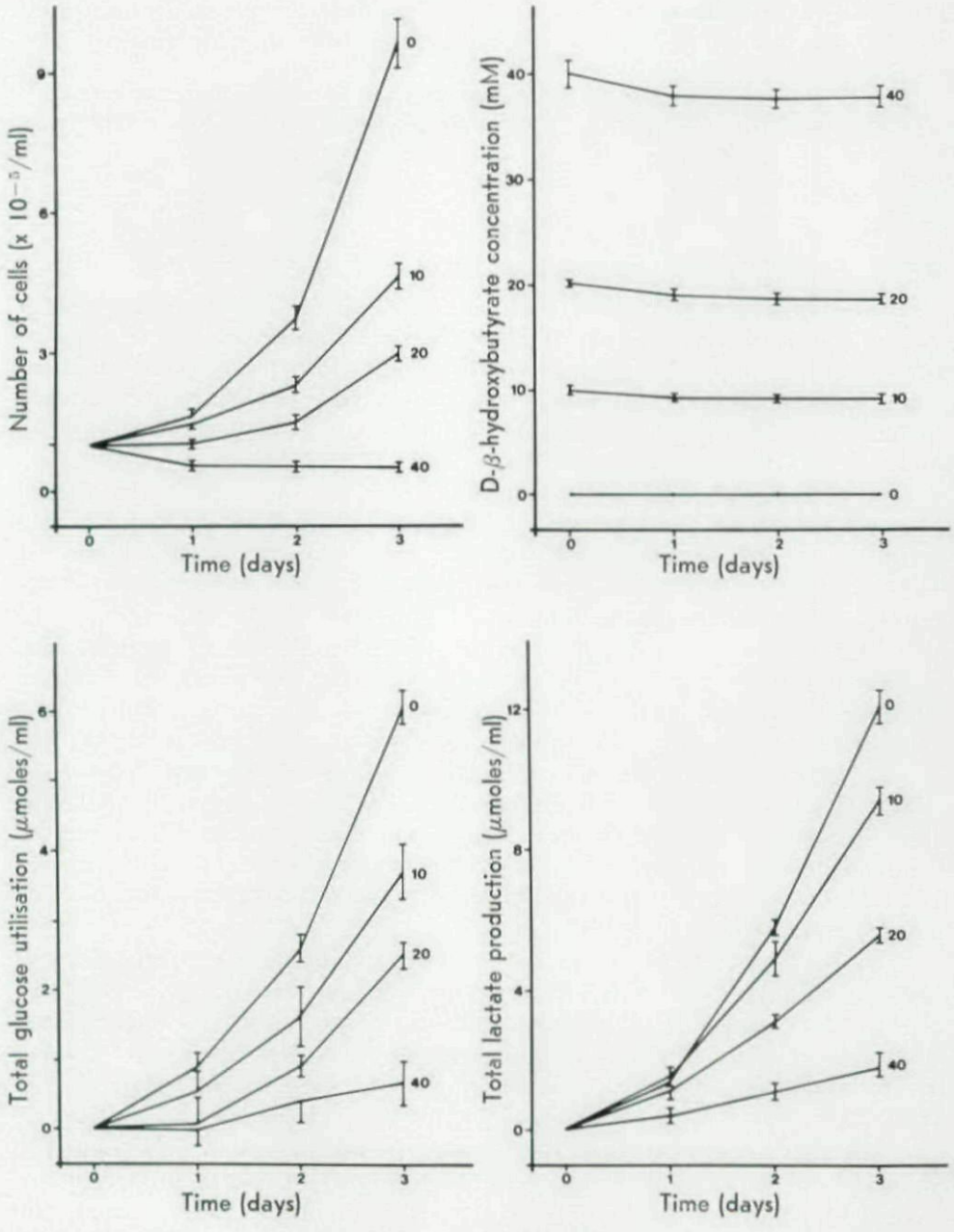

Fig. 1. The effect of D-β-hydroxybutyrate on cell proliferation (upper left graph), glucose utilisation (lower left graph) and lactate production (lower right graph) in Raji cells. The curve labels 0, 10, 20, and 40 (e.g. upper right graph) indicate the concentrations (mM) of D-β-hydroxybutyrate. Each point represents the mean ± the standard error of the mean for 15 determinations in the upper right graph and 9 determinations in the other graphs. The initial concentrations of glucose and lactate were 11·4±0·3mM and 2·48±0·7mM, respectively.

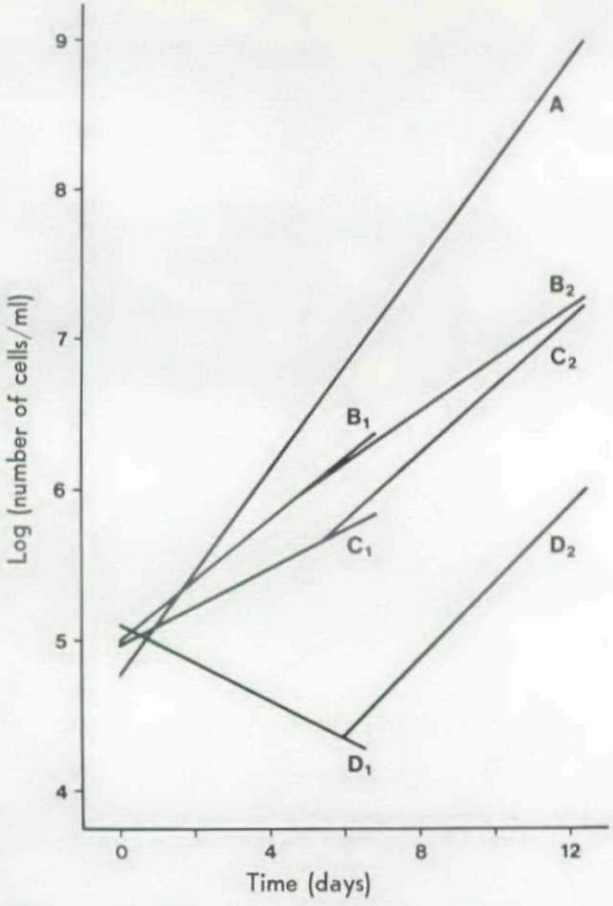

Fig. 2. The reversibility of the effect of D-β-hydroxybutyrate on cell proliferation in Raji cells. The D-β-hydroxybutyrate concentration, slope, standard deviation and correlation coefficient for each curve are as follows (linear regression analysis): A (0mM), $0{\cdot}34 \pm 0{\cdot}01$, $0{\cdot}99$; B_1 (10mM), $0{\cdot}19 \pm 0{\cdot}01$, $0{\cdot}98$; B_2 (20mM), $0{\cdot}18 \pm 0{\cdot}01$, $0{\cdot}97$; C_1 (20mM), $0{\cdot}13 \pm 0{\cdot}01$, $0{\cdot}92$; C_2 (10mM), $0{\cdot}22 \pm 0{\cdot}01$, $0{\cdot}99$; D_1 (40mM), $-0{\cdot}13 \pm 0{\cdot}02$, $0{\cdot}85$; and D_2 (0mM), $0{\cdot}25 \pm 0{\cdot}01$, $0{\cdot}98$. Where indicated, the D-β-hydroxybutyrate concentration was altered at the end of the second 3-day interval.

viability. Fluoride had no significant effect on either parameter. Both inhibitors were present in concentrations commonly used to inhibit glycolytic enzymes. Chemical analogues of the $-CHOH-CHR-COOH$ group either did not inhibit or proved to be too toxic for cell growth. Furthermore, none of these analogues induced any cytochemical or morphological changes. Only acetoacetate, another ketone body, significantly inhibited cell growth without decreasing viability.

In the experiments reported in Table 1 D-β-hydroxybutyrate was only detected in those cultures which initially contained that ketone body. Using the techniques of colourimetry, gas chromatography, N.M.R. and I.R. spectroscopy, it was found that culture media concentrations of salicylate, *n*-butyrate and γ-hydroxyvalerate remained constant. The *n*-butyrate was shown to contain minute traces of isobutyrate

TABLE 1

The effect of glycolytic inhibitors and chemical analogues of D-β-hydroxybutyrate on the culture characteristics of Raji cells.

Addition to culture medium		Final value at 3 days	
Chemical	Initial concentration (mM)	Cell number $\times 10^{-5}$/ml	Viability (%)
Nil	—	6·88±0·68 (6)	>99
Ketone bodies—			
D-β-hydroxybutyrate, Na salt	10	3·53±0·21 (6)	>99
D-β-hydroxybutyrate, Na salt	20	1·95±0·39 (6)*	>99
acetoacetate, Li salt	20	2·94±0·19 (6)*	>99
Glycolytic inhibitors—			
iodoacetate, Na salt	2	0·64±0·10 (3)*	< 5
fluoride, Na salt	10	5·45±0·46 (3)	>99
Chemical analogues—			
n-butyric acid	5	1·17±0·16 (3)*	<15
n-butyric acid	20	0·47±0·05 (6)*	<10
sec-butanol	20	6·13±0·57 (6)	>99
γ-hydroxyvaleric acid	20	4·28±0·51 (6)	>99
succinic acid	20	4·53±0·47 (6)	>99
D, L-serine	20	6·08±0·65 (6)	>99
D, L-threonine	20	6·05±0·75 (6)	>99
salicylate, Na salt	20	0·30±0·04 (6)*	<30

* Significantly different from control, $p<0·01$.

and propanoate. The γ-hydroxyvalerate contained minute traces of acetate. Moreover, the γ-hydroxyvalerate was mainly in the γ-valerolactone form.

Acid production in the cultures was observed by visually noting changes in the colour of the internal indicator, phenol red. Acid production was inversely related to the degree of cell growth inhibition; culture acidity: control > 10 > 20 > 40 mM D-β-hydroxybutyrate (Fig. 1) and γ-hydroxyvalerate >> control ≃ serine ≃ threonine ≃ succinate ≃ *sec*-butanol ≃ fluoride > D-β-hydroxybutyrate > acetoacetate ≃ salicylate ≃ iodoacetate ≃ *n*-butyrate (Table 1). The large acid production associated with γ-hydroxyvalerate may be explained on the basis of the conversion of the γ-valerolactone back to the acid form at neutral pH.

Comparative cell study

In general, transformed cells grew more rapidly than non-transformed cells in RPMI 1640 medium (Fig. 3). HeLa 229 cells, however, only grew at the same rate as fibroblasts and the beef testis cells. In the presence of 20 mM D-β-hydroxybutyrate cell growth was inhibited by 70% for Raji, rabbit kidney and thyroid cells, by 50% for HeLa and beef testis cells and by less than 30% for fibroblasts and mouse melanoma cells.

Preliminary mouse study

Ketone bodies would also appear to have an inhibitory effect on malignant cell

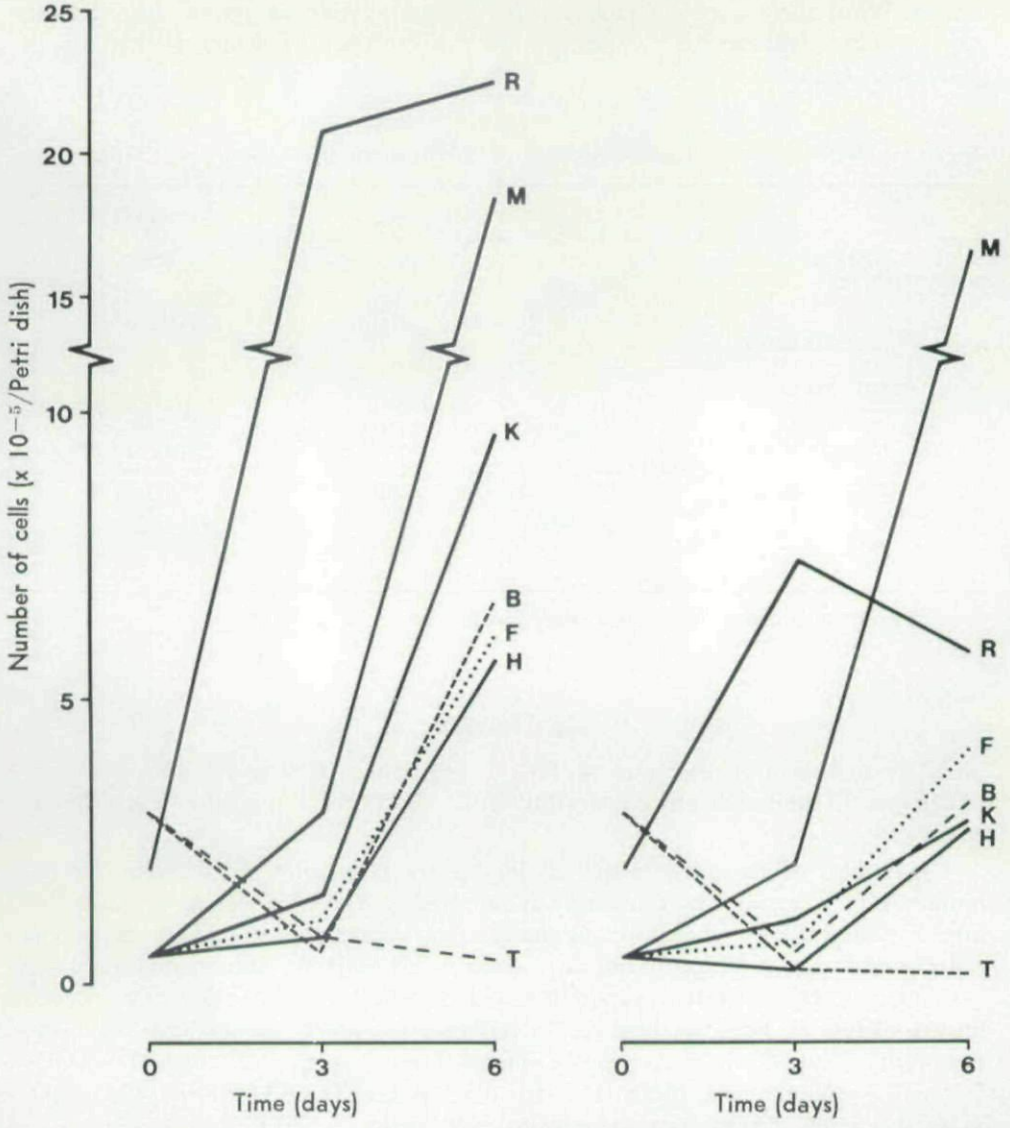

Fig. 3. The effect of D-β-hydroxybutyrate on cell proliferation in transformed (continuous lines) and non-transformed (broken lines) cells; left hand graph, control cells; right hand graph, cells in the presence of 20 mM D-β-hydroxybutyrate. The cell types were: R, Raji cells; M, mouse melanoma cells; K, rabbit kidney cells; H, HeLa cells; B, beef testis cells; F, human fibroblasts; and T, human thyroid cells.

growth *in vivo* (Table 2). At the time of sacrifice the mice fed a high lipid diet (group B) had significantly higher serum D-β-hydroxybutyrate concentrations but only one third the number of lung metastases observed in the sucrose fed mice (group A). Furthermore, during the period of dietary manipulation the urines of group B mice were strongly positive, whereas the urines of group A were negative, for ketone

bodies. While there were no significant differences in body weight or serum lactate concentrations between the two groups, group A mice had markedly elevated serum glucose concentrations.

TABLE 2

The effect of dietary-induced ketosis on the growth of B16 melanoma cells in the lungs of C57BL/6 mice.

	Group A	Group B
Major dietary constituent	sucrose	lipid
Body weight (g)—		
day 0	16·9±0·4 (18)	17·4±0·5 (18)
day 14	14·6±0·5 (18)	14·6±0·4 (18)
Urinary ketones (Ketostix)	—	+++
Serum metabolites (mM)—		
glucose	9·0±0·5 (6)	3·0±0·2 (6)*
lactate	3·6±0·5 (6)	2·5±0·5 (6)
D-β-hydroxybutyrate	0·2±0·1 (6)	8·7±0·5 (6)*
Number of lung metastases	36±9 (18)	12±2 (18)*
range	5–137	1–30

* Significantly different from comparable value in group A, $p<0·01$.

DISCUSSION

The discussion of these findings falls naturally into three parts: the mechanism of cell growth inhibition, the universality of the effect on cells and the clinical implications.

A number of mechanisms present themselves as possible explanations for the inhibition of malignant cell growth by ketone bodies. These include chemical inhibition, hyperosmolarity, chromosomal changes, membrane effects, acidosis and metabolic interactions. A simple chemical reaction seems unlikely, since analogues of the −CHOH−CHR−COOH group either had no effect or were too toxic for cell growth (Table 1). Nor can these results be explained simply on the basis of hyperosmolarity, since 20mM acetoacetate inhibited cell growth more than 20mM D, L-β-hydroxybutyrate (i.e. 10mM D-β-hydroxybutyrate, Table 1). Indeed, the results in Table 1 imply a more specific biological mechanism.

Sodium *n*-butyrate produces reversible changes in morphology, growth rate and enzyme activities of several mammalian cell types in culture (Prasad and Sinha, 1976). The switching of malignant to non-malignant cells in the presence of *n*-butyrate has been linked to the polyacetlyation of histones (Riggs *et al.*, 1977). In those studies the concentration of *n*-butyrate did not exceed 5mM, whereas increasing the concentration of *n*-butyrate to 20mM in our experiments killed the cells. In our hands even 5mM *n*-butyrate proved to be toxic for Raji cells (Table 1).

It is more likely that the ketone bodies decreased cell growth rates by inhibiting the metabolism of glucose, the major nutrient in the medium. The rate limiting step for glycolysis in rat thymus lymphocytes appears to be glucose transport across the

cell membranes (Hume *et al.*, 1978). An interaction between the ketone bodies and the cell membrane is possible, since D-β-hydroxybutyrate has been shown to alter the binding of insulin to cultured lymphocytes (Misbin *et al.*, 1978). Acidosis inhibits tumour growth *in vivo*, and it has been suggested that this occurs through a block in glycolysis at the phosphofructokinase (E.C. 2.7.1.11) level (Anghileri, 1975). It was observed in these experiments, however, that acid production was directly related to cell growth.

Ketone bodies are also fuels in their own right (Cahill, 1976; Newsholme, 1976) and lymphocytes and some tumour cells are known to possess either ketogenic or ketolytic enzymes (Cederbaum and Rubin, 1976; Fenselau, Wallis and Morris, 1976; McGarry and Foster, 1969; Hume *et al.*, 1978). Metabolism of ketone bodies can increase the concentration of citrate which, in turn, can inhibit phosphofructokinase (Newsholme, 1976). Although there was a 6–10% reduction in D-β-hydroxybutyrate concentration over 3 days, it was not significant (Fig. 1). If the transformed lymphoblasts contained the enzyme, D-β-hydroxybutyrate dehydrogenase (E.C. 1.1.1.30), then one might also have expected the concentration of D-β-hydroxybutyrate to fall by one quarter to one third as it equilibrated with acetoacetate. This did not occur and similarly D-β-hydroxybutyrate was not detected in the media containing cells and acetoacetate. Furthermore, while the total glucose utilisation and the total lactate production fell with increasing concentrations of D-β-hydroxybutyrate, the glycolytic flux per individual cell increased (e.g. the glucose utilisation rates per 10^5 cells were in the proportions 1·0:1·3:1·4:2·0 for the control, 10, 20, and 40 mM D-β-hydroxybutyrate cultures, respectively). It must be remembered, however, that these cultures are closed systems and that different cell growth rates result in different nutrient concentrations in the media and different flux rates in the cell.

These results indicate that ketone bodies are not metabolised by the transformed lymphoblasts. Nevertheless, it seems highly probable that ketone bodies interfere with either glucose entry or glucose metabolism, since increasing the glutamine concentration in the culture media from 2 to 7 mM almost abolished the D-β-hydroxybutyrate inhibition of cell growth (Conyers, unpublished data). As a nutrient, glutamine can bypass the regulatory steps in glycolysis. Moreover, the inhibitory effect of ketone bodies on cell growth could not be reproduced by excess quantities of other nutrients such as succinate, serine or threonine or by the glycolytic inhibitor, fluoride (Table 1). While iodoacetate, on the other hand, did inhibit cell growth, it also markedly reduced cell viability. Because iodoacetate inhibits a wide range of sulphydryl-containing enzymes, it can be considered to be a general cell poison whose biological mode of action is unlike that of ketone bodies.

This inhibitory effect of ketone bodies on cell growth is not restricted to Raji cells, as it was observed in 7 different cell types from three different species (Fig. 3). The fact that the effect is not specific for transformed cells would, at first sight, indicate that iatrogenically induced ketosis would be of little value in the management of the cachexia of cancer. *In vivo*, however, one might expect ketosis to act selectively against malignant cells because of the rapid growth of transformed cells (Fig. 3) and the ability of normal cells, in tissues such as brain, heart and skeletal muscle, to use ketone bodies directly as fuels (Newsholme, 1976). This assumption gains strong

support from the results of the preliminary mouse experiment (Table 2). Certainly, the data in Fig. 1 and the findings of the mouse experiment indicate that the induction of ketosis in cancer patients is unlikely to produce hypoglycaemia or lactic acidosis (Conyers *et al.*, 1979b).

Attempts to regulate the metabolism of cancer *in vivo* are not new (Landau *et al.*, 1958; Gold, 1974; Spiers and Wade, 1976). The use of glycolytic poisons as therapeutic agents, however, is so non-specific that serious damage to normal tissues may occur before any significant tumour inhibition is observed (Landau *et al.*, 1958; Gold, 1974). An alternative metabolic approach to the control of tumour glycolysis has been to use hydrazine sulphate or pyridine-2,3-dihydrazide which selectively block gluconeogenesis (Gold, 1974). It is already known that, in leukaemic patients, administration of bacterial glutaminase reduces the blast cell count (Spiers and Wade, 1976), presumably by reducing the amount of circulating glutamine, a known gluconeogenic precursor (Newsholme, 1976). On the other hand, elevations of the metabolic rate can be correlated with the proportion of immature cells in the bloodstream (Gunderson, 1921).

Iatrogenically induced ketosis offers a physiological means of regulating the host glucose metabolism in cancer patients with a consequent reduction in the host metabolic rate and a reversal of the cachectic process (Conyers *et al.*, 1979a, 1979b). The role of the extracellular environment in models of cancer has been discussed elsewhere (Conyers, 1979).

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