PHYSIOLOGICAL AND NON-PHYSIOLOGICAL INDUCTION OF GASTROINTESTINAL DIFFERENTIATION

BY

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Declaration

I hereby declare that this thesis is my own unaided work. It is being submitted for the Masters degree in Science at the University of Port Elizabeth, Port Elizabeth, South Africa. It has not been submitted before for any degree or examination at any other University.

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S.C.A. Brauns

On this _____day of _____,19_____

TABLE OF CONTENTS

SUMMARY

The human colonic carcinoma cell lines HT-29 and Caco-2 both exhibit structural and functional differentiation under appropriate culture conditions. HT-29 can be induced to differentiate by treatment with short-chain fatty acids or acetoacetate. Caco-2 cells differentiate spontaneously upon contact inhibition. In this study HT-29 cells were treated with 5 mM acetate, propionate, butyrate and acetoacetate (physiological inducers) to assess their effects on the expression of carbonic anhydrase 1, sucrase-isomaltase and alkaline phosphatase which are reported to be markers of gastrointestinal differentiation. The maturation induction observed was compared to that of the spontaneous differentiation observed in Caco-2 cells. Assays were performed over an 18 day period. Results showed a close correlation ($p < 0.05$) between HT-29 and Caco-2 cell on days 4 and 12. These results indicate that differentiation reported in both cell lines is comparable and can be used as a basis for further comparative studies.

In addition, parallel experiments to the above were conducted using a selection of nine rationally designed cyclic dipeptides (CDPs) potential drug entities which were chosen as non-physiological inducers. The results showed that the cyclic dipeptides were able to induce the gastrointestinal phenotype as observed in HT-29 cells treated with physiological inducers. Studies on the effects of energy-related metabolism in HT-29 and Caco-2 cells as induced by physiological and non-physiological inducers indicated that energy metabolism is a significant role player in gastrointestinal differentiation. The results reported show a decrease in ATP concentrations indicating that the cyclic dipeptides, like physiological inducers, affect the energy state of the HT-29 cells and thus may effect the differentiation of these cells. A positive correlation was found between histone phsophorylation and differentiation confirming that histone phsophorylation was partly responsible for the decrease in ATP concentrations. It is suggested that the induction of differentiation in HT-29 cells could be either due to non-specific transcription of genes by activation of a chromatin switch or specific by the activation of signal transduction pathways based on the flux of ATP through the cells. Differential display RT-PCR is probably the most sensitive method that could be used to validate the suggestion of either a nonspecific transcription of genes or a specific differentiation reported for HT-29 cells.

OPSOMMING

Albei die HT-29 en Caco-2 dikderm kanker sellyne vertoon beide strukturele en funksionele differensiëring in die teenwoordigheid van toepaslike kweek omstandighede. Differensiëring kan in HT-29 selle voortgebring word deur middel van kuring met kort-ketting vetsuure of acetoacetate. Spontane differensiëring van Caco-2 selle tydens kontak inhibiese kom voor. HT-29 selle is in hierdie studie met 5mM asynsuursout, propionsuursout en butierisesuursout en acetoacetate (fisiologiese induseermiddels) gekuur om die effek op carbonic anhydrase I, sucrase isomaltase en alkaline phosphatase, wat almal antekenaars van differensiëring in die dikderm is, te meet. Die geinduseerde maturasie wat opgelet was, was met die spontane differensiëring in Caco-2 selle vergelyk. Proewe is oor ‘n 18-dae tydperk uitgevoor. ‘n Noue verband ($p<0.05$) tussen HT-29 en Caco-2 selle op dae 4 en 12 is getoon. Hierdie uitslae toon dat differensiëring wat in albei sellyne berig is, is vergelykbaar en kan dus as die grondslag vir verdere vergelykings studies gebruik word.

Verdere proefnemings is gelyktydig uitgevoer, waar die effek ‘n verskydenheid van nege rasionaal ontwerpde siekliese peptiede getoes is. Die sikliese peptiede is gekies op grond wan hulle nie-fisiologiese induseerder status. Uitslae van hierdie proefnemings het getoon dat die sikliese peptiede wel in staat was om die ingewande fenotipe (wat in HT-29 selle met fisiologiese induseermiddels gekuur is) voor te bring.

Eksperimente oor dir uitwerking van energie verwandte metabolisme in HT-29 en Caco-2 selle, wat deur fisiologiese en nie-fisiologiese induseermiddels voortgebring was, het getoon dat energie metabolisme ‘n belangrike rol in ingewande differensiëring speel. ‘n Vermindering in ATP konsentrasie in die selle is deur hierdie uitslae getoon. Sikliese peptiede, soos die fisiologiese induseermiddels, het dus ‘n uitwerking op die energiebalans van die HT-29 selle en kan dus differensiëring in die selle voortbring. ‘n Positiewe korrelase tussen histoon fofforilasie en differensiëring is ook bevind. Hierdie bevinding is ‘n bevestiging dat histoon foforilasie gedeeltelik verantwoordelik vir dir vermindering van ATP is. Dit is voorgestel dat differensiëring in HT-29 selle voortgebring mag word deur die oorskrywing van nie-spesefieke gene wat deur ‘n “chromatien skakelaar” geaktiveer is of spesefieke gene wat deur die vloeing van ATP deur die selle gedurende seintransduksie geaktiveer word.

Differential display RT-PCR is dus waarskynlik die sensitiefste metode wat gebruik kan word om te bevestig of die transkripsie van gene in HT-29 selle of spesefieke of nie-spesefieke differensiëring ondergaan.

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LIST OF ABBREVIATIONS

cAMP	Cyclic Adenosine Monophosphate
ATP	Adenosine-5'Triphosphate
°C	Degrees Celcius
CA 1	Carbonic Anhydraes 1
μCi	MicroCurie
CDP	Cyclic Dipeptide
CoA	Coenzyme A
CPM	Counts Per Minute
DD	Differential Display
DMEM	Dulbecco's Modification of Eagle's Minimum Essential Medium
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra-acetic acid
F	Phenylalanine
FCS	Foetal Calf Serum
g	Grams
μg	Micrograms
GIT	Gastrointestinal Tract
l	Litre
μl	Microlitre
M	Molar
mg	Milligrams
ml	Millilitre
mM	Millimolar

MW	Molecular Weight
P	Proline
p	Statistical Significance Value
PAGE	Polyacrylamide Gel Electrophoresis
PBSA	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
r	Correlation Coefficient
RNA	Ribonucleic Acid
rpm	Revolutions per minute
SCFA	Short chain Fatty Acid
SDS	Sodium Dodecyl Sulphate
TCA	Tricarboxylic Acid Cycle
Tris	Tris (Hydroxymethyl) Aminomethane
W	Tryptophan
Y	Tyrosine

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: INTRODUCTION

Research into the differentiation induction of gastrointestinal epithelial cells has become prominent in recent years (Huet *et al.* 1987, Gamet *et al.* 1992, Sowden *et al.* 1993, Graz and Cowley, 1997). In the last two decades numerous studies have been conducted using colonic carcinoma cell lines as models to study the progression of cellular differentiation that occurs within the normal gastrointestinal tract (GIT) (Pinto *et al.* 1982, 1983, Augeron and Laboisse, 1984, Darmoul *et al.* 1992, Velcich *et al.* 1995). The cell lines represented in the studies mentioned here have been shown to exhibit enterocyte-like differentiation characteristic of the GIT. The HT-29 colonic carcinoma cell line differentiates by induction with external stimuli (Pinto *et al.* 1982) whereas the Caco-2 colonic carcinoma cell line differentiates spontaneously on contact inhibition (Pinto *et al.* 1983). There is reason, however, to question whether the differentiation reported in these studies is terminal differentiation (in which the phenotype remains unchanged after withdrawal of the inducer) or non-specific differential gene expression in which the lineage-specific genes are the most prominent leading to the expression of a dominant phenotype

Graz and Cowley (1997) have shown that the energy status of HT-29 cells is linked to their differentiation. As maturation induction occurs in these cells an increase in the levels of acetyl-coenzyme A within the cells is effected which in turn leads to the acetylation of histone proteins. The acetylation of the histones causes unwinding of the chromatin. The unwinding of the chromatin is non-specific (Alberts *et al.* 1989). Also the mechanism by which the lineage-specific packaging of DNA occurs is not known.

The challenge posed in the above discussion indicates a need to determine whether differentiation:

1) in HT-29 cells is comparable to spontaneous differentiation observed in Caco-2 cells.

2) is true differentiation or generalised transcription, leading to the expression of a lineage-specific phenotype which represses all other phenotypes.

In the following chapters the term differentiation will be replaced with terms such as maturation induction or differential gene expression to accommodate the challenge posed above. The term differentiation will be used only in specific reference to relevant literature.

1.1 Cellular differentiation

The process of renewing the gastrointestinal tract involves the proliferation, migration, differentiation, and eventual loss or death of gastrointestinal epithelial cells. Epithelial renewal begins in more or less discrete proliferative zones throughout the gastrointestinal tract. These include the oesophagus, stomach, small intestine and large intestine. Within these zones undifferentiated cells divide to provide a constant source of new cells, some of which then migrate toward the gut lumen into which they are eventually extruded. As cells migrate, they normally lose the capacity to divide and become differentiated to carry out the specialised functions of that portion of the gastrointestinal tract wherein they arise (Eastwood, 1977).

The pathway of cell differentiation is induced by a primary external stimulus which results in the modification of gene expression, thereby leading to a functionally and/or morphologically altered cell (Balinsky, 1981). Differentiation is considered extremely stable as upon removal of the primary inducing stimulus, the cell persists in its modified (differentiated) state (De Robertis and De Robertis, 1987).

Differentiation of intestinal epithelial cells is similar in the small and large intestines. The cell cycle is less uniform in colonic cells as a proportion of the cells may remain in a prolonged *G1* period, thus giving the colon its unique growth pattern (Williamson and Chir,

1978). Cells in the terminally differentiated state may be defined as being arrested in the *G0* phase of the cell cycle and are characterised by having a low transition probability from *G0* to *G1/S* (Pardee, et al, 1978).

In vivo, evidence for the permanence of the differentiated phenotype has been advanced by Prescott and Flexer (1986). These authors state that the approximately 100 different cell types found in the adult human body can roughly be divided into two groups on the basis of their proliferative capacity (Prescott and Flexer, 1986). The cells of the first group, including nerve cells, red blood cells, skeletal muscle and heart muscle cells, can be considered to be in the terminally differentiated state and do not divide (Alberts *et al.* 1989). In contrast, the cells of the second group, which comprise the remaining ninety six cell types, undergo cellular programming and may be induced to divide even after they have changed functionally, e.g. as in the repair of muscle damage. This division may be suspended indefinitely and only induced by cell damage (Nurse, 1987) or the application of physiological inducers (Prescott and Flexer, 1986). Both groups may however begin uncontrolled division with the onset of neoplasia (Vile, 1990).

During the process of cell division, cells within the proliferative zone undergo a sequence of phases called the cell renewal cycle (Figure 1.1). After mitosis (*M* phase), proliferating daughter cells enter the first portion of the interphase, the postmitotic-presynthetic gap, or *G1* phase. DNA replication then takes place during the DNA synthesis (*S*) phase, followed by another interval, the postsynthetic-premitotic gap, or *G2* phase before mitosis is repeated. Cells which have undergone mitosis do not necessarily continue the cyclic process of cell division. Some cells may exit *G1* and enter a quiescent stage of the cell cycle called the *G0*

phase, during which DNA synthesis and mitosis are temporarily suspended, but the potential for cell proliferation remains (Eastwood, 1977).

The events of the cell cycle are accompanied by great metabolic activity. Although DNA synthesis occurs only during a discrete period, synthesis of RNA and protein is active throughout the cycle except during the M phase, when RNA synthesis stops and protein synthesis decreases. During *G1* phase, the replication of new DNA is anticipated by the synthesis of enzymes invloved in nucleic acid metabolism. Indeed proliferating cells have characteristic patterns of nucleic acid metabolic enzymes which change as the cells differentiate and migrate out of the proliferative zone. Thymidine kinase activity, for instance, is high in the proliferating crypt cells of human and rat small and large intestine, but activity diminishes as the cells migrate up the crypts onto the villi (Eastwood, 1977).

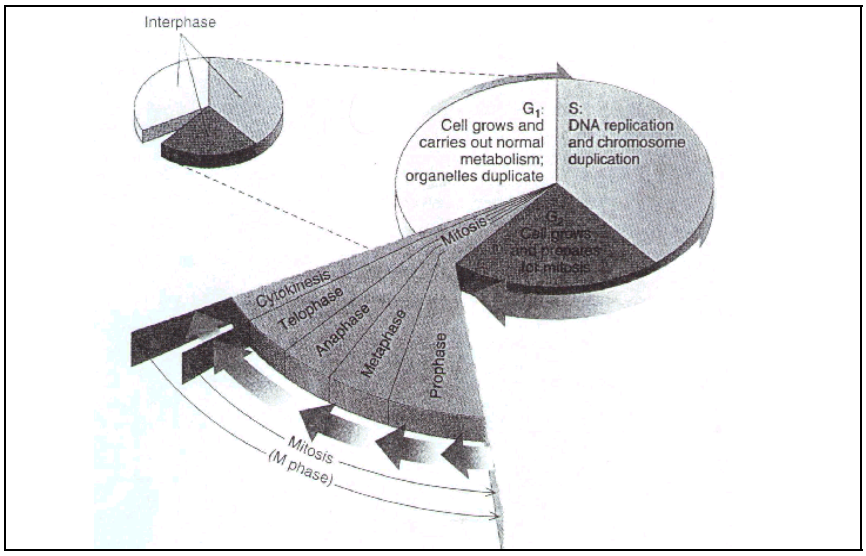


Figure 1.1: The eukaryotic cell renewal cycle. This diagram of the cell cycle indicates the stages through which the cell passes from one division to the next. The cell cycle can be divided into two stages: M phase and interphase. *M* phase includes the successive stages of mitosis and cytokinesis. Interphase is divided into *G1*, *S*, and *G2* phases - *S* phase being equivalent to the period of DNA synthesis. Taken from Karp (1996).

Different cellular processes such as cell growth, DNA replication, and mitosis, all must be coordinated during cell cycle progression. This coordination is accomplished by cell cycle progression being controlled at two major points. The first of these is the restriction (R) point found late in the *G1* phase. The R point is the point at which the cells become committed beyond the point of return. The second control point is found between the *G2* and M phases and prevents the initiation of mitosis until DNA replication is completed (Cooper, 1997).

Studies conducted to elucidate the factors involved in the cell cycle regulation have revealed that the cell cycle of all eukaryotes is controlled by a conserved set of protein kinases, which are responsible for triggering the major cell cycle transitions (Cooper, 1997). The first of these is MPF (maturation promoting factor), a dimer of Cdc2 and cyclin B. Further research has established that both Cdc2 and cyclin B are members of large families of related proteins, different members of these families controlling progression through distinct phases of the cell cycle (Cooper, 1997). Progression through the G1 restriction point is controlled by complexes of Cdk2, Cdk4, and Cdk6 with D-type cyclins. Cdk2/cyclin E complexes function later in *G1* and are required for the *G1* to *S* transition. Cdk2/cyclin A complexes are then required for progression through *S* phase, and Cdc2/cyclin B complexes drive the *G2* to *M* transition (Cooper, 1997).

It is accepted that the differentiated state of a cell is a function of the activity of a specific set of expressed genes to the exclusion of gene products active in differentiated cells of other types (Wolpert, 1982). Differentiation is regulated by eight possible mechanisms at the molecular level. According to De Robertis and De Robertis these are: (1) non-reversible

changes in the methylation of the DNA leading to changes in gene expression; (2) transcriptional control by the action of repressors, activators or transducers; (3) post-transcriptional control by differential RNA processing; (4) translational control affected both by post-transcriptional modifications of mRNA and ribosome binding factors; (5) post-translational modifications of proteins including glycosylation and methylation; (6) gene amplification at the genome level (not shown in humans); (7) gene transpositions; and (8) genomic rearrangement which results in only tissue specific genes being accessible to transcription enzymes (Holliday and Pugh, 1975).

In all cells except those of cancers and early embryos, there is a coordination between cell growth and discrete steps of cell cycle that ultimately lead to mitosis and cytokinesis. This coordination occurs at the R point where the cells take stock of parameters influencing cell division. If there is a loss of coordination of cell proliferation, the cells will undergo continuous, uncontrolled division leading to tumorigenesis. This last factor is both necessary and fundamental to tumorigenesis. Neoplastic cells are characterised by a loss of regulation between cell proliferation and cell differentiation. Thus, a neoplastic population may not necessarily display a high rate of cell proliferation, but rather a continuous cell proliferation with all cells having the same chance of replicating. The uncontrolled cell division which results is continuous but not necessarily faster than the cell division occurring in the healthy organ (Alberts *et al.* 1989).

The rate of proliferation is controlled by the passage through the *G1* phase while the success of the proliferative event is dependent upon two processes, namely nuclear division (karyokinesis) and cytoplasmic division (cytokinesis). Both of these processes occur during

and at the end of the M phase. Neoplasia can arise during the nuclear division process, due to incorrect duplication or incorrect separation of the cells' chromosomes (Alderson, 1982).

1.2 Cellular Programming

Cellular programming is defined as "a transient change in the functional state of the cell, characterised by a reversible change in gene expression" (Tsanev, 1975). Cellular programming is not mutually exclusive to proliferation, since the cells revert back to their original state upon removal of the inducer (of programming) and may thus divide (Tsanev, 1975). With respect to the cell cycle, cells which have undergone a change in cell programming, have been "resting" in the *G1Q* phase rather than having been arrested in the *G0* phase as occurs with terminally differentiated cells (Alberts *et al.* 1989).

Cellular programming may be induced by programmed gene rearrangements altering gene expression or by reversible changes in the methylation patterns of the DNA (Kolata, 1985). Programmed gene rearrangements are part of the normal developmental program of an organism. These programmed rearrangements can be grouped into the categories of amplification and DNA rearrangements that alter gene expression (Borst, 1986).

1.3 Induction of Differentiation in the Gastrointestinal Tract

In vitro differentiation can result from the action of soluble - physiological and soluble - non-physiological compounds (Freshney, 1987). This is in opposition to Lash (1968) who argued that (i) in vitro differentiation is artificial due to the nature of the in vitro conditions, (ii) "dedifferentiation" may not represent actual differentiation mechanisms and (iii) the

differentiation process may be at the biochemical level and not visible morphologically (Balinsky, 1981).

1.3.1 Physiological Inducers of Differentiation:

1.3.1.1 Short-chain fatty acids (SCFAs)

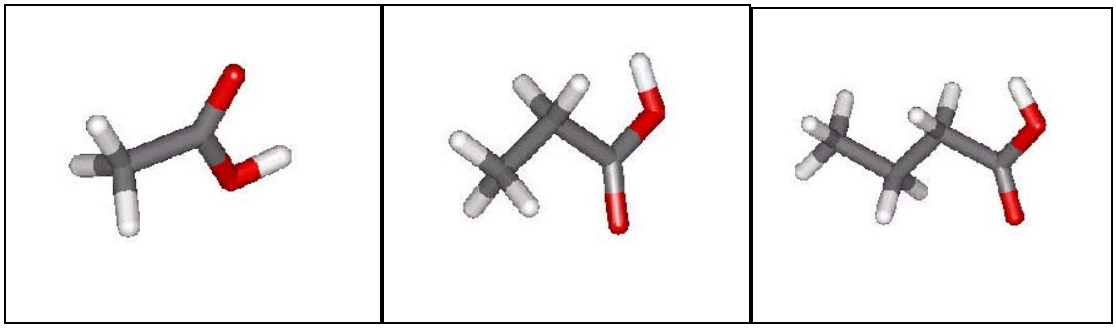
Short-chain fatty acids refer to carboxylic acids with chain length of up to 6 carbon atoms. They are weak acids with pK-values of about 4.8 and are water-soluble at the concentrations at which they are found in the human body (McFarlane *et al.* 1992). Over the past decade evidence has rapidly accumulated which indicates that malabsorbed polysaccharides (resistant starch and dietary fibre) entering the colon are fermented by the bacteria present in the gut to SCFAs (Cummings and Englyst, 1983). As a result, SCFAs are the predominant anions in the luminal fluid contributing 100-150mM total solute to the luminal contents (Rowe *et al.* 1994).

The major fermentable substrates entering the colon are starch and dietary fibre. However, to a lesser extent, malabsorbed sugars such as arabinose, xylose, mannose, and rhamnose, and mucous secreted by the GIT also are fermented to SCFAs (Royall *et al.* 1990). Macfarlane *et al.* (1992) studied the colonic contents obtained from two human sudden death victims within 3 hours of death. Fermentation products including various SCFAs, lactate and ethanol were obtained from different regions of the colon. The SCFAs obtained were acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, isocaproate and caproate. Acetate, propionate and butyrate were the principal organic anions found in the colonic contents (Macfarlane *et al.* 1992). Acetic, propionic and butyric acid have proved to be the most important SCFAs compared to valeric, caproic and all the isoforms of the of the

aforementioned SCFAs, being the most prominent in the gut and also the most-studied of the SCFAs. They are present in the gut at a respective molar ratio of 60:20:20 (Royall *et al.* 1990). The chemical properties of these SCFAs are shown in Table 1. The three-dimensional structures of the SCFAs are shown in Figure 1.2.

Table1.1: Chemical properties of the important SCFAs. Adapted from the Merck Index (1987).

Fatty acid	**Chemical name**	**Number of carbon atoms**	**MW**	**Chemical formula**
Acetate	Acetic acid	2	60.5	CH_3COOH
Propionate	Propionic acid	3	74.08	$CH_3CH2COOH$
n-Butyrate	Butanoic acid	4	88.1	$CH_3(CH)2COOH$



A **B** **C**

Figure 1.2: The three-dimensional structures of A - acetic acid, B - propionic acid and C - butyric acid. Grey = Carbon chains, red = Oxygen, white = hydrogen.

1.3.1.2 Ketones

Acetyl CoA formed during fatty acid oxidation enters the TCA cycle only if fat and carbohydrate degradation are appropriately balanced. In the liver, entry of acetyl CoA into the TCA cycle depends on the availability of oxaloacetate for the formation of citrate, but the concentration of oxaloacetate is lowered if carbohydrate is unavailable or improperly utilised. Under such conditions, acetyl CoA is diverted to the formation of acetoacetate (Figure 1.3) and D-3-hydroxybutyrate which are sometimes referred to as ketone bodies. The major site of production of these ketone bodies is the liver. Until a few years ago these ketones were regarded as degradation products of little physiological value. However, studies have revealed that these derivatives of acetyl CoA are important molecules in energy metabolism (Stryer,1988).

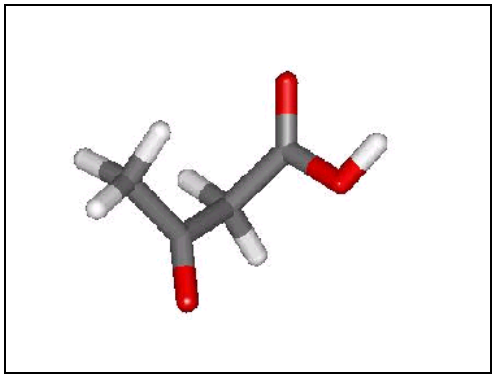


Figure 1.3: The three-dimensional structure of acetoacetate. Grey = Carbon chains, red = Oxygen and white = Hydrogen.

Studies by Henning and Hird (1972) have shown that tissue from the caecum and the proximal colon of wild rabbits was found to form ketone bodies in the presence of butyrate.

Most of the ketogenic activity and oxygen consumption was found to be associated with the mucosa. The gut tissues which showed high ketogenic activity during incubations in vitro were from regions whose contents are characterised by high concentrations of SCFAs. The metabolic conversion of butyrate into ketone bodies is a feature which is common to the epithelia of all fermentative organs so far studied (Henning and Hird, 1972). A consequence of the pathway of acetoacetate synthesis is liberation of CoA , and this may be one of its important functions. Tissues such as liver and caecal epithelium are presented with high concentrations of fatty acids, which could result in overesterification of CoA. Under these conditions the citrate synthase reaction would be the only source of free CoA. Since high concentrations of fatty acids may cause inhibition of the citrate synthase reaction the pathway of ketogenesis would become an important extra system for the maintenance of a steady state concentration of free CoA. The conversion of butyrate to ketone bodies may also be important in maintaining a constant supply of energy for the mucosal cells in a manner that has been suggested for the liver (Henning and Hird, 1972).

1.3.1.3 Effects of SCFAs and ketones on somatic cells

SCFAs have been shown to have a variety of effects on the function and morphology of the colonic epithelium. They are important anions in the stimulation of colonic fluid and sodium absorption. They also affect mucosal energy metabolism, colonic mucosal blood flow, ileal motility, caecal mucin secretion, colonocyte cellular differentiation and mucosal cell proliferation. Furthermore, butyrate specifically has been shown to have numerous effects involving differentiation and alteration of malignant to more benign phenotypes of various cultured tumor cell types (Scheppach, 1994). Darzynkiewiz *et al.* (have shown that

in the presence of butyrate, murine leukemic L1210 cells cease proliferation and become arrested in the *G1A* component of the *G1* phase of the cell cycle.

SCFAs are the predominant energy source for the colonic epithelium (Roediger, 1980), contributing 400-1000 kJ of energy which represents 5-10% of an average persons daily energy requirements (Royall *et al.* 1990). In isolated human colonocytes Roediger (1980) showed that more than 70% of oxygen consumed in the ascending and descending colon was due to butyrate oxidation.

The results of studies by Graz and Cowley (1997) on the investigation of the effects of SCFAs as an energy source in HT-29 cells suggested that the differentiation observed is related to the most readily available source of carbon administered to the cells. By tracing the flux of ^{14}C from acetate, propionate and butyrate through the cells, it appeared that the SCFAs were stored as acetoacetate within the first 24 hours after application to the cells. Graz and Cowley (1997) conclude that HT-29 cells utilise SCFAs in preference to glucose, metabolise these to ketones, thereby raising the energy state and effecting the observed morphological changes in the cells.

SCFAs stimulate Na^+ absorption by activating apical Na^+/H^+ exchange in colonocytes. Allosteric activation of Na^+/H^+ exchange by intracellular H^+ allows cells to regulate H^+ efflux rates based on the cellular acid load. Intracellular acidification of colonocytes is believed to be the primary mechanism by which SCFAs stimulate Na^+/H^+ exchange. Cytosolic acidification is predicted following cellular uptake of SCFAs both via nonionic diffusion and via colonic $SCFA^-/HCO_3^-$ exchange. However there are several observations

which are difficult to incorporate into this model of SCFA-stimulated Na^+ absorption. Rowe *et al.* (1994) have addressed certain fundamental limitations in the currently accepted model of SCFA-stimulated Na^+ absorption. Their results suggest that propionate activates Na^+/H^+ exchange via changes in pH and that transepithelial gradients of this SCFA can cause preferential activation of apical Na^+/H^+ exchange (Rowe *et al.* 1994). By stimulating sodium and water absorption, SCFAs act as an anti-diarrhoeal agent. Antibiotics have been shown to cause diarrhoea and, simultaneously, reduce faecal SCFA concentrations and SCFA production rates *in vitro*. Many cases of lactose intolerance are clinically inapparent because bacterial lactose fermentation reduces the osmotic load and generates SCFA, which are subsequently absorbed. Thus, SCFA absorption plays an important role in colonic carbohydrate and sodium salvage (Scheppach, 1994).

Butyrate has been shown to have a variety of effects on different types of cancers. It has been found to be an anti proliferative and differentiating agent in various cancer cell lines *in vitro* (Kruh *et al.* 1991). Hague *et al.* (1995) have shown that butyrate induces apoptosis in 3 colorectal tumor cell lines. In the extension of their study to three adenoma and four carcinoma cell lines, Hague *et al.* (1995) have shown butyrate as well as acetate and propionate induce apoptosis at physiological concentrations, but of the three SCFAs, butyrate was the most effective.

The products of proto-oncogenes have been suggested to play a central role in the regulation of proliferation and differentiation by functioning at distinct steps in intracellular growth cascades. For example, the c-*sis* gene encodes a polypeptide similar to the beta-chain of platelet-derived growth factor, and the c-*fms*, c-*neu*, and c-*erb* oncogenes encode growth

factor receptor-like membrane proteins. The products of *ras* genes resemble the G proteins that modulate adenylate cyclase and other mebrane-associated enzymes and ion channels, whereas c-*myc* and c-*fos* are DNA-binding proteins that have been postulated to function as intracellular mediators of growth factor signals (Olson *et al.* 1987). In various cell types butyrate alters the activity and/or the expression of several genes related to cell growth (c-*myc*, c-*fos, P53* and *cdc*2) (Gamet *et al.* 1992).

Sodium butyrate induces phenotypic alterations in a variety of solid tumor cell lines such as Hela cells, breast cancer cells colorectal carcinoma and retinoblastoma. The anti-tumor effects of butyrate that include growth inhibition and decrease in tumorogenicity are accompanied by changes in enzyme activities, receptor content and histone structure. Butyrate has been used to select stably differentiated clones of the HT-29 cell line that resemble goblet cells and produce mucin (Velcich and Augenlicht, 1993). Butyrate has been shown to induce *MUC3* gene expression in HT-29 cells (Velcich *et al.* 1995). The hyperacetylation of histones by butyrate has previously been shown in friend erythroleukemic cells (Nordenberg *et al.* 1987). Butyrate elicits this response by its inhibition of the enzyme histone deacetylase. The hyperacetylation of the inner histones causes unwinding of the chromatin resulting in less dense chromatin which results in an increased DNase 1 sensitivity of associated DNA sequences (Graz and Cowley 1997). Graz and Cowley (1997) demonstrated a similar response in HT-29 carcinoma cells treated with acetoacetate.

Butyrate markedly inhibits Bl6 mouse melanoma and Hela cell growth and alters the morphological appearance of the cells (Nordenberg *et al.* 1987). Changes in morphology have also been reported in HT-29 cells treated with various SCFAs including acetate,

propionate and butyrate. Changes in morphology of up to 32% of the 9-day cell population and 92% of 18-day cell population to dendritic, secretory or giant cell morphotypes are induced by the applied SCFAs (Graz and Cowely, 1997).

Gope and Gope (1993) have shown that treatment of HT-29 cells with sodium butyrate caused a decrease in the level of retinoblastoma (RB1) gene expression on day seven of butyrate treatment but a gradual decrease in the level of expression of *p53* gene. Butyrate has also shown to have caused reversal in the phosphorylation status of the retinoblastoma protein (*pRb*) (Gope and Gope, 1993).

1.3.2 Non-Physiological Inducers of Differentiation

Cyclic dipeptides (Figure 1.3) have been chosen as non-physiological inducers for this study owing to their recent popularity in the field of rational drug design and the fact that they have been shown to exhibit maturation induction activity in vitro (Milne *et al.* 1998). Traditional methods of drug design, such as systematic screening of large series of synthetic and natural substances, are increasingly being supplemented by methods exploiting the increasing knowledge of macromolecular targets and physical principles of drug-receptor interactions. The latter approach is termed rational drug design or "structure-based ligand design" and serves to accelerate the development of a biologically active ligand towards the status of a drug useful in humans, animals and phytochemistry. Rational drug design allows for increased specificity of a drug with a low side *effect* profile, rendering it an ideal therapeutic agent. Due to their simplicity and limited conformational freedom, cyclic dipeptides are readily used as model compounds in studies aimed at the development and

refinement of criteria for conformational insights into larger peptides (Anteunis, 1978, Bovey, 1974).

1.3.2.1 Cyclic Peptides

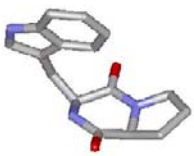
Conformationally restricted peptides present simple and valuable models to gain conformational insight into larger peptides and proteins and their three-dimensional structure-bioactivity relationships (Anteunis, 1978). Cyclic peptides through short-range cyclizations have during the past two decades attracted considerable attention because of their limited conformational freedom and higher probability of conformational homogeneity when compared with their linear analogs (Toniolo, 1990). Proline is an important *imino* acid of many proteins and neuropeptides and imposes certain conformational restraints on these biomolecules. In addition, the conformational aspects of the pyrrolidine ring system are of particular interest as they reveal different modes of puckering of the five-membered ring system (Chacko *et al.* 1983). Secondly, proline is the only residue which leads to an N-alkylamide bond when incorporated into a peptide via natural biochemical pathways. Numerous peptides with important biological activity (e.g. didemnin and cyclosporin) contain N-methyl amino acids. Thirdly, the *cis-trans* isomerism of the N-alkylamide bond involving the amino group of proline has been implicated in the biological activity of peptides. Brandl & Deber (1986) have proposed that *cis-trans* isomerism of proline residues might play a role in transduction of transmembrane proteins.

The inclusion/incorporation of essential aromatic amino acids offers a model system of limited complexity for studying the influence of solvents and solvent mixtures on intramolecular interactions during the excited lifetime of the chromophore.

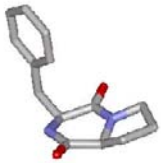
Tryptophan contains an indole ring (a benzene ring fused to a pyrrole ring) which is found in many pharmacologically active compounds, including hallucinogens and other drugs which have mental or emotional effects e.g. Lysergic acid diethylamide (LSD), Dimethyltryptamine (DMT), Psilocybin, Harmaline and Strychnine. *Bromocriptine, Ergotamine, Indapamide, Indomethacin, Sumatriptan* and *Ondansetron* are examples of drugs containing an indole ring which are available on the market today.

Although cyclic dipeptides (2,5 diketopiperazines, DKP's or 2,5 dioxypiperazines, DOP's) have been known since the beginning of this century (Fischer, 1906), it is only during the past years that they have attracted considerable interest (Sammes, 1975). They do not exist as zwitterions and the simple members of this group of peptides are often neutral compounds. They are easily formed from unprotected linear dipeptides in basic conditions especially if the necessary head-to-tail folding is not prevented by stearic constraints (Fischer, 1906). Such compounds appear of interest in studies on the thermodynamic behaviour of non-ionic compounds in water because they share the capability of establishing hydrogen bonds with the solvent (via the two *cis* amide groups in the DKP-ring) and of giving rise to hydrophobic interactions (to an extent determined by the R substituents) (Crescenzi *et al.* 1973). The structural conformations of the cyclic dipeptides used in this study are shown in Figure 1.3.

Many derivatives of these compounds with the DKP-ring show antiviral properties for example, the gliotoxins (Ottenheijm *et al.* 1976), and others, such as bicyclomycin, are powerful antibiotics (Bodansky *et al.* 1973) and anti-tumor agents (Jensen *et al.* 1973).

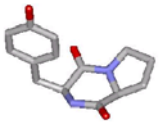


Cyclo(Pro-Trp)



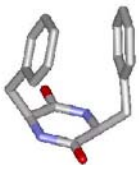
Cyclo(Phe-Pro)

Cyclo(Pro-Pro)

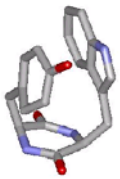


Cyclo(Tyr-Pro)

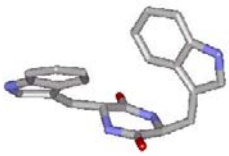
Cyclo(Trp-Pro)



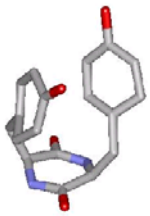
Cyclo(Phe-Phe)



Cyclo(Tyr-Trp)



Cyclo(Trp-Trp)



Cyclo(Tyr-Tyr)

Figure 1.4: Three-dimensional structures of the nine cyclic dipeptides used in the present study. Trp - tryptophan, Tyr - tyrosine, Pro - proline, Phe - phenylalanine.

= Carbon chains = Oxygen = Nitrogen

Dioxopiperazines are used by nature to hold small peptide links together, as in the growth factor rhodotorulic acid (Atkins & Neilands, 1968). The biological implications of DKP's are further demonstrated by the fact that spontaneous formation from higher linear peptides containing *imino* acid residues may occur (Goodman *et al.* 1962). Some DKP's are metabolic intermediates or provoke the destruction of the secondary globular protein structure (Crescenzi *et al.* 1973).

1.4 HT-29 and the Caco-2 colonic carcinoma cell lines

Numerous biochemical and morphological studies have shown the intestinal epithelium to be an attractive model for the study of basic questions (cell renewal, cell death, mechanisms of differentiation, pathogenesis of disease) in cell biology. However, such studies have been impeded by its complex structure, its cellular heterogeneity, and its tight adhesion to underlying nonepithelial tissues. Cultured cells from such an epithelium could provide a useful tool for studying cellular metabolism, cell differentiation, and the formation and maintenance of cell surface polarity. It has not been possible yet to establish epithelial cell lines displaying the structural and functional properties of differentiated epithelial cells using normal adult or foetal rat intestine. The few cell lines derived from the intestinal mucosa did not exhibit the morphological features of differentiated phenotypes. In 1972, Fogh isolated and cultured cells from human adenocarcinomas. It was shown that one of these, the HT-29 line, was able to undergo differentiation and express an enterocyte-like phenotype when grown in glucose-free medium (Pinto *et al.* 1982; Zweibaum *et al.* 1985).

Pinto *et al.* (1982) showed that the replacement of glucose by galactose in the culture medium resulted in a typical although partial structural and functional enterocytic differentiation of the colon carcinoma cell line HT-29. In an attempt to extend their approach to other human colon cancer cell lines, they observed that one of them, Caco-2, spontaneously exhibited enterocyte-like differentiation patterns (Pinto *et al.* 1983). Both cell lines have subsequently been used extensively as models for the study of the structural and functional characteristics of the gastrointestinal tract.

In the absence of glucose, the HT-29 cells display morphological changes with polarization of the cultured cells being one of the first changes indicating differentiation. Defined brush borders appear on the apical surface of the polarized cells, while tight junctions are found on the lateral walls between adjacent cells. The cells joined by tight junctions, form a permeability barrier closely resembling the simple epithelium found in the intestine (Pinto *et al.* 1982). Increased activity of brush border-associated enzymes such as alkaline phosphatase, aminopeptidase, dipeptidyl peptidase IV, and the disaccharidases is found. Sucrase-isomaltase, often used as a marker for differentiated enterocytes, was shown to be expressed at apical brush borders of a subpopulation (30-50%) of differentiated cells obtained in sugar-free medium (Zweibaum *et al.* 1985).

The HT-29 cell line has been suggested to be pluripotent (Huet *et al.* 1987) as it induced tumors with a heterogenous enterocyte-like pattern of differentiation as well as a large number of mucus-secreting colonies under nutritional conditions described by Pinto *et al.* (1982). Clones derived from undifferentiated HT-29 cells remain capable of differentiation into two cell types when grown without glucose. This indicates that some cells of the

parent HT-29 line are multipotent and may be functionally comparable to uncommitted stem cells in intestinal crypts. Subclones have been obtained that differentiate into monolayers consisting of a single cell type. These subclones finally provide an epithelial culture system in which the differentiation and function of a specific intestinal cell type can be reproducibly studied *in vitro*. One subclone, HT-29-18C1, has been shown by biochemical, immunologic, and morphologic methods to express functional polarity and brush border organisation typical of absorptive cells in human foetal colon, a stage at which colonic cells express hydrolases that are restricted to small intestine after birth. These approaches also resulted in a subclone, designated HT-29-18N2, with the morphological features of goblet cells and containing some mucin reactivity (Huet *et al.* 1987).

In all the cancer cell lines used as models for the study of conditions allowing full expression of differentiation characters, the action of the various inducers is reversible. When a differentiated phenotype is induced in a culture, the transfer of the cells into an inducer-free medium allows the reconstitution of the population of self-renewing cells from the uninduced cells. Moreover, when rechallenged with the inducers, these self-renewing cells express unchanged sensitivity to the inducer (Augeron and Laboisse, 1984). This indicates that the cells are not permanently affected by these treatments. However, studies by Augeron and Laboisse (1984), indicated that following a treatment with sodium butyrate (NaBT), "a potent inducer of intestinal differentiation", morphologically altered cell populations emerged in the HT-29 human colonic cancer cell line. They reported that these altered cells represent in fact differentiated cell populations and showed that these differentiated phenotypes are permanent and stable in standard medium (Augeron and Laboisse, 1984).

The polar-planar compound hexamethyline bisacetamide (HMBA) can inhibit HT-29 cell growth and induce a more benign phenotype. Shroy *et al.* (1994) performed parallel studies using NaBT and HMBA. Both 5 mM HMBA and 5 mM NaBT were found to be potent inhibitors of monolayer growth. Despite these parallel effects on growth and in vitro markers of a benign phenotype, effects on intestinal differentiation were discordant. NaBT induced significant increases in membrane-associated alkaline phosphatase activity, cytosolic mucin content, and ultrastructural evidence of intestinal differentiation. HMBA not only failed to induce markers of intestinal differentiation, but attenuated NaBT effects when used in combination. These data suggest that growth and intestinal differentiation are independently regulated in HT-29 cells (Shroy *et al.* 1994).

Results reported by Pinto *et al.* (1983) show that monolayers of the Caco-2 cell line exhibit structural and functional differentiation patterns characteristic of mature enterocytes. This is particularly the case in post-confluent cultures, when all the layer is covered by brush border mirovilli which are functionally differentiated, as shown by their immunoreactivity to anti-brush border enzyme antibodies, and by the high levels of alkaline phosphatase sucrase-isomaltase, and to a lesser extent, aminopeptidase activities. These activities are much greater than those found in confluent cultures of the undifferentiated cell line HT-29 (Pinto *et al.* 1983).

The structural and functional differentiation of the brush border microvilli of Caco-2 cells is associated with a polarization of the epithelial monolayer in post-confluent cultures. This structural polarity is apparent from the position of the microvilli and from the presence of tight junctions which are specific features of polarized epithelia. The occurrence of domes strongly suggests that the monolayer of confluent Caco-2 cells is also functionally polarized.

These domes start to appear only when confluence has been reached. This would suggest that the functional differentiation of the cells corresponds to a maturation process, closely resembling the situation that is found in the normal intestine (Pinto *et al.* 1983).

Caco-2 cells have become a useful model in the study of the structural and functional properties of the normal gastrointestinal tract, whereas the HT-29 cell line provides a useful model for the investigation of the processes involving the differentiation of the gastrointestinal tract. The reason for Caco-2 cells, which originate from the colon, differentiating into enterocytes is not clear, nor is it known why Caco-2 should be the only cell line of colorectal origin to have the property of differentiating spontaneously in culture. One possible explanation is that Caco-2 cells represent yet one more example of the similarity that exists between cancer and foetal cells (Pinto *et al.* 1983)

1.5 "Unitarian Theory of Epithelial Cell Origin" or selection of committed subpopulations?

The origin of the differentiated populations induced in HT-29 cells is subject to two possible explanations:

The first explanation is presented by Lesuffleur, *et al.* (1990) who suggest that differentiation found in HT-29 cells in not due to the direct action of the applied agent, but rather due to the selection of a previously committed subpopulation of the cells. The argument of Lesuffleur *et al.* (1990) is very similar to the "selection model of neoplastic growth" of Nowell (Leith and Dexter, 1986), which is based on genetic instability of neoplastic cells

(having arisen from a single transformed cell) and selection of favoured variant clones forming the cellular subtypes in heterogeneous neoplasms.

Lesuffleur, *et al.* (1990) state that the selection of the differentiated subpopulations is preceded by a high mortality rate. This is supported by the results found in all of the SCFA-treated cultures except in the acetate-treated culture where the incidence of morphologically altered cells is preceded by a high proliferative rate. However, Lesuffleur, *et al.*'s (1990) study showed that selection occurs during an exponential growth phase, which is supported by the results of the acetate-treated cultures, but not by the other SCFA-treated cultures. Furthermore, proliferation of differentiated cells in neoplasias has been documented by Leith and Dexter (1986). Tarella *et al.* (1982) equate clonal selection with genetic heterogeneity. Neoplasms usually exhibit a high percentage of genetic heterogeneity. One would expect the cell lines produced from cancer to exhibit similar genetic heterogeneity. This was supported by the differences in chromosome number found in the individual SCFA-treated cultures found in this study.

The second explanation takes into consideration the "Unitarian Theory of epithelial cell origin" of Cheng and Leblond (1974) which states that the cells in the GIT all originate from a single stem cell. This theory is very similar to the "stem cell differentiation model of neoplastic growth" of Newby (Leith and Dexter, 1986). This model is predicated on the basis of the differentiation of stem cells in neoplastic tissue (having arisen from a single transformed cell), giving rise to the cellular subtypes in a heterogeneous cancer.

The Unitarian Theory by Cheng and Leblond (1974) explains the differentiation of the four cell types in the GIT to be from multipotent stem cells in the Crypts of Lieberk hn, rather than from four different types of precursor cells. This theory has been supported by the results of Augeron and Laboisse (1984), Laboisse *et al.* (1986) and Huet *et al.* (1987) who postulated that individual cells could act as precursors to all of the cell types found after differentiation induction.

Three different cell types were found at the light microscopical level suggesting that three rather than two subpopulations are present in undifferentiated HT-29 cells. This however is contradictory to the findings of Lesuffleur *et al.* (1990) who only found two cell types in their study. Laboisse *et al.* (1986) only found two cell types in their cultures but were able to show by repeated cloning that these two cell types were produced from a single multipotential stem cell. Furthermore, if undifferentiated HT-29 cultures contained committed subpopulations the selection of these subpopulations should give rise to differentiated cells with very similar characteristics. The argument presented by Augeron and Laboisse (1984) suggested that the differentiation seen was due to clonal selection. Furthermore, if the cells arose from distinct subpopulations, the changes in gene expression as assessed indirectly by enzyme assays and membrane protein patterns and directly by DNA methylation, should have been very similar. Finally, if clonal selection was the major force acting on the SCFA and acetoacetate-treated cultures, the growth of the cultures should follow the classical sigmoidal curve and the cell numbers should not remain static for the first 10 days of culture. Thus there should have been a marked increase in the percentage of morphologically altered cells, if these were specifically being selected for.

The above three arguments with respect to the unitarian theory are indirect, yet show that the induction of differentiation in HT-29 cells cannot be simply explained using the "selection theory" only. This is finally corroborated by the 5 % differentiated cells found in any untreated HT-29 culture (Trugnan, *et al.* 1987). These cells could arise by localized metabolic stress being placed upon previously committed cells or they could arise spontaneously by random differentiation. The differentiation in both cases would be directed, albeit aberrantly, by the genetic programming of the tissue from which the original tumour arose (Leith and Dexter, 1986). The static cell populations induced by most of the SCFAs and acetoacetate (Augeron and Laboisse, 1984; Graz and Cowley, 1997) suggest that a differentiation event rather than clonal selection was occurring, which can be explained by the fact that proliferation and differentiation are mutually exclusive, which is not the case in a selection event.

1.6 Aims

The aim of the proposed study was to develop a model to describe the mechanism of action of the expression of a gastrointestinal phenotype in differentiated HT-29 cells.

In order to fulfil this aim, the following sub-aims had to be met:

1) To determine the most reproducible markers of colonic differentiation that can be used for both HT-29 and Caco-2 in order to assay both cell lines at similar stages of differentiation.
2) To develop rapid, sensitive, miniaturized methods to screen for maturation induction potential in gastrointestinal cells.

3) To determine the point in time at which both cell lines are at similar stages in the differentiation process to allow direct comparison between spontaneous and induced differentiation.

4) To induce the differential phenotype by physiological (ie. SCFAs) and non-physiological (ie. cyclic dipeptides) compounds.

5) To determine one of the potential metabolic switches leading to differentiation induction.

CHAPTER 2: DIFFERENTIATION MARKERS

2.1 Biochemical markers

HT-29 cells originate from a colonic carcinoma. Thus it is to be expected that the markers chosen to indicate differentiation of HT-29 cells would be those of colonic differentiation. This has been shown not to be the case with regards to the choice of the markers assayed in literature. As pointed out by Schroy *et al.* (1994), one of the inherent problems in studying colon carcinoma cell differentiation is the lack of definitive markers of terminally differentiated colonic epithelial cells. Most *in vitro* models of colon carcinoma cell differentiation have been defined almost exclusively on the basis of so-called markers of intestinal differentiation rather than colonic differentiation.

A survey of the literature (Table 2.1) pertaining to conventional differentiation markers studied in HT-29 cell differentiation was conducted in order to guide the choice of markers to be assayed in the current study. The data was plotted on a histogram to show selection of the markers of choice (Figure 2.1). Of all the markers cited in literature carbonic anhydrase 1 (CA1) was the only marker specifically referred to as a marker of colonic differentiation (Sowden, 1993). Other markers were referred to either as small intestinal differentiation markers or simply as intestinal differentiation markers. Inclusion of CA1 as one of the markers to be assayed was thus indicated. Alkaline phosphatase and sucrase-isomaltase were also selected as markers to be assayed because of their relative prominence in literature as well as their use as criteria for differentiation (Pinto *et al.* 1983).

The afformentioned discussion poses a challenge: if HT-29 is a colonic carcinoma cell line, why is it that the differentiation markers studied are those of small intestinal differentiation?

Table 2.1: Literature survey of differentiation markers used in the assessment of HT-29 cell differentiation

Author	**Differentiation Marker**
Pinto *et al.* (1982)	Sucrase-Isomaltase, Alkaline Phosphatase
Pinto *et al.* (1983)	Sucrase-Isomaltase, Alkaline Phosphatase Aminopeptidase, Ultrastructure
Augeron and Laboisse (1984)	Ultrastructure
Wice *et al.* (1985)	Sucrase-Isomaltase, Alkaline Phosphatase Aminopeptidase
Chantret *et al.* (1987)	Sucrase-Isomaltase
Friedman *et al.* (1987)	Carcinoembryonic antigen
Huet *et al.* (1987)	Sucrase-Isomaltase, Aminopeptidase, Ultrastructure
Le Bivic and Arsanto (1987)	Alkaline Phosphatase
Trugnan *et al.* (1987)	Sucrase-Isomaltase, Aminopeptidase Dipeptidyl peptidase IV
Godefroy *et al.* (1988)	Histocompatability antigens, Sucrase-Isomaltase
Le Bivic and Reggio (1988)	Ultrastructure
Lesuffleur *et al.* (1990)	Villin
Reynier *et al.* (1991)	Alkaline Phosphatase, Aminopeptidase Cardiolipin
Darmoul *et al.* (1992)	Dipeptidyl peptidase IV
Goodwin *et al.* (1992)	Mucin protein 3, Ultrastructure
Gummer (1992)	Sucrase-Isomaltase, Alkaline Phosphatase Aminopeptidase, Carcinoembryonic antigen
Kellof *et al.* (1992)	PCNA, Ki67 (Proliferation biomarkers)

Table 2.1 (Continued): Literature survey of differentiation markers used in the assessment of HT-29 cell differentiation

Author	Differentiation Marker
Garcia de Herreros *et al.* (1993)	Mucin protein 3, Carcinoembryonic antigen, Dipeptidyl peptidase IV, Villin
Gope and Gope (1993)	*p53*
Lesuffleur *et al.* (1993)	Mucin protein 3, Villin, Dipeptidyl peptidase IV
Sowden *et al.* (1993)	Carbonic anhydrase
Velcich and Augenlicht (1993)	Mucin protein 2
Velcich *et al.* (1995)	Carcinoembryonic antigen, Alkaline Phosphatase, Mucin protein 3
Cargnello *et al.* (1996)	Calretinin
Chazaud *et al.* (1996)	Ultrastructure
Heerdt *et al.* (1996)	Alkaline Phosphatase, Mucin protein 2
Merchant *et al.* (1996)	*p53*
Graz *et al.* (1997)	Carbonic anhydrase, Cell cycle progression, Sucrase-Isomaltase

Considering the embryonic development of the GIT, it is understood that each separate zone (the stomach, esophagus, small intestine, and large intestine) develop from cells that are predisposed or committed to differentiate into cells characteristic of the respective zones (Balinski, 1981). According to Balinski (1981), in higher vertebrates the fourth phase of ontogenetic development (gastrulation) involves the development of two or more layers of cells (germinal layers) from a single layer of cells, the blastoderm. The germinal layers are complex rudiments from which the organs of the animal's body are derived. The external

layer is called the ectoderm then just next to it is the mesoderm, and the innermost layer is called the endoderm from which the alimentary canal and digestive glands are formed.

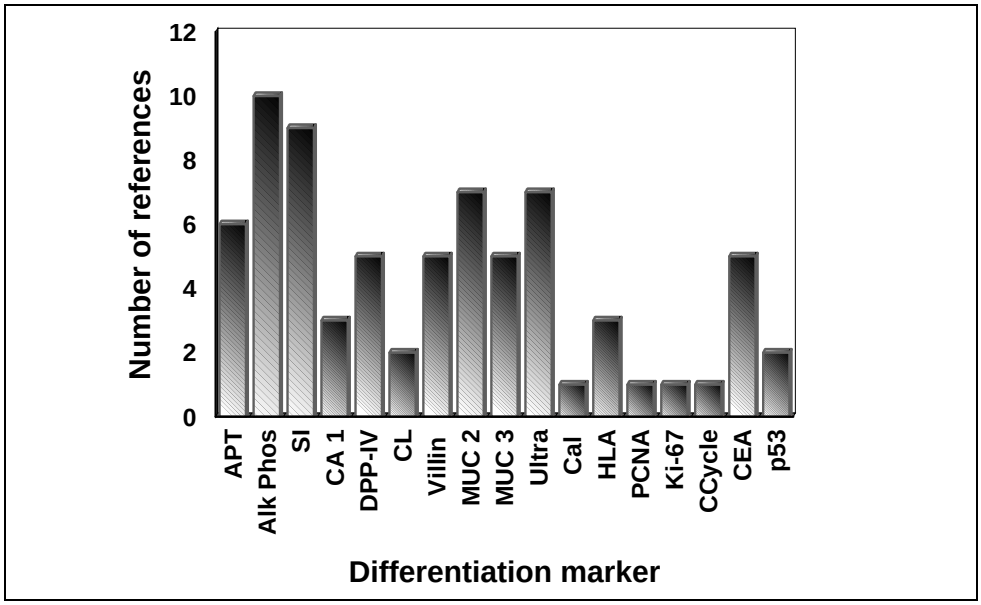


Figure 2.1: Representation of the relative occurrence in literature of various markers of enterocytic differentiation studied in HT-29 cell differentiation. APT - aminopeptidase, Alk Phos - alkaline phosphatase, SI - sucrase-isomaltaese, CA 1 - carbonic anhydrase 1, DPP IV - dipeptidyl petidase IV, CL - cardiolipin, MUC 2 - mucin protein 2, MUC 3 - mucin protein 3, Ultra - Ultrastructure, Cal - calretinin, HLA - Histocompatability antigens, PCNA, Ki67 - proliferation biomarkers, Ccycle - cell cycle progression, CEA - Carcinoembryonic antigen

During it's early stages of development, the alimentary canal is subdivided into a foregut, midgut and hingut. It is the midgut which, under normal conditions, differentiates into the stomach and intestine. Balinski (1981) further describes that in the earlier stages (72 hours) of gestation the fate of the endodermal cells "destined to participate in the formation of various organ rudiments" is a function of the position that each cell or group of cells occupies in the embryo as a whole (Balinski, 1981).

In experiments carried out to substantiate the previous statement it was shown that on the second day of incubation the differentiation of endodermal explants corresponds to their

prospective significance. Furthermore, the small and large intestine are shown to be derived from discrete parts of the endodermal gut which implies that the cells of these parts of the GIT are predetermined to perform the functions characteristic of the small and large intestine respectively (Balinski, 1981). It is therefore unlikely for colonic cells to be present in parts of the small intestine and vice versa.

Within the small intestine (Figure 2.2) itself there is distinction between the types of cells which arise, these then performing specific functions. The columnar epithelium of the small intestine contains absorptive cells, goblet cells, enteroendocrine cells and Paneth cells. The absorptive cells synthesize brush border enzymes which include the disaccharidases, petidases, nucleosidases and phosphatases, all of which facilitate digestion at surface of the epithelial cells that line the villi. The goblet cells are mucus-secreting cells and the enteroendocrine cells are the source of the enterogastrones. The Paneth cells help to fortify the small intestine's defenses against certain bacteria by releasing lysozyme.

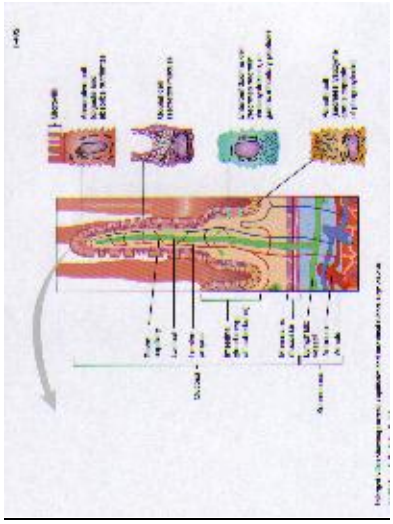


Figure 2.2: The mucosa of the small intestine showing the representative cell types. Taken from Totora and Grabowski (1992).

The wall of the large intestine (Figure 2.3), on the other hand, is simple compared to the small intestine. There are no villi or permanent circular folds in the mucosa. The mucosa consists of simple columnar epithelium which contains mostly absorption and goblet cells. In contrast to the small intestine the absorptive cells of the large intestine function primarily in water absorption.

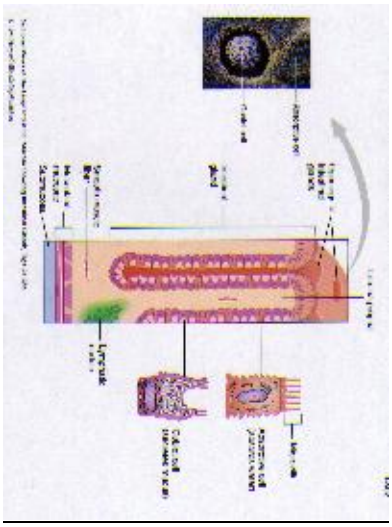


Figure 2.3: The mucosa of the large intestine. Taken from Totora and Grabowski (1992).

Taking the above discussion into consideration, the significance of the HT-29 cell line as a model for studying colonic differentiation can be questioned. The differentiation induced in HT-29 cells, produces cells with gene products that are accepted as markers of small intestinal differentiation rather than that of colonic differentiation. This further emphasises the need to investigate whether the maturation induction observed in the HT-29 cell line represents true differentiation or a generalized induction of transcription.

2.2 Markers of energy metabolism

Figure 2.4 proposes a possible energy-related metabolic pathway for induction of differential phenotype in gastrointestinal cells exposed to compounds capable of affecting their glucose metabolism. The glucose metabolism of neoplastic cells is impaired. Instead of pyruvate molecules, that are produced by glycolysis, entering the TCA cycle (Warburg, 1956), glucose is rapidly converted to lactic acid (Denis *et al.* 1984). According to Warburg (1956) the low

energy yield produced by the glucose to lactic acid reaction, results in the loss of cellular differentiation found in most neoplastic tissues.

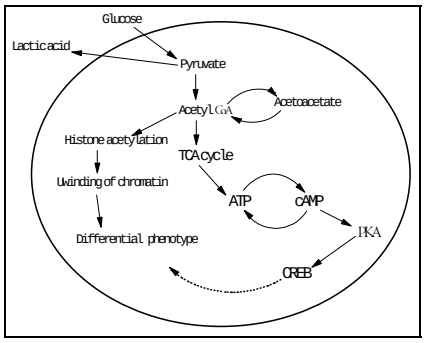


Figure 2.4: Proposed metabolic pathways (excluding the relevant enzymes) involved in the induction of differential phenotype by SCFAs and acetoacetate or cyclic dipeptides in HT-29 cells. The figure is explained in the text.

As with other neoplastic cells, HT-29 cells have an impaired glycolytic pathway (Royall *et al.* 1990). Undifferentiated HT-29 cells show increased glucose absorption with a concomitant increase in pyruvate production and lactic acid secretion (Rousset, 1986). This impaired glucose metabolism in HT-29 cells appears to be linked to the loss of cellular differentiation in these cells (Graz and Cowley, 1997). The modulation of the glucose flux in Caco-2 cells has been linked to their state of enterocytic differentiation. In Caco-2 cells there is a decrease in glucose absorption with a concomitant decrease in lactic acid secretion (Rousset, 1986).

Determining the uptake of glucose from the growth medium, pyruvate production within the cells and lactic acid secretion into the medium would thus enable the investigation of whether the physiological or non-physiological inducers induce enterocytic differentiation of the cells and subsequently modulate the glucose flux. It is, however, important to note that

the treatment with inducers does not repair the metabolism, but simply obviates the need for glycolysis by providing an alternative energy source of acetyl-CoA.

A recent study has shown that the energy state in HT-29 cells is linked to their differentiation (Graz and Cowley, 1997). HT-29 cells utilize SCFAs or acetoacetate in preference to glucose as a carbon or energy source (Graz and Cowley, 1997). The results of this study show that HT-29 cells convert SCFAs to acetoacetate which is then stored in the cells thereby increasing their total energy state, which in turn effects the reported terminal differentiation of these cells. Thus in addition to the biochemical markers mentioned in 2.1, we propose that energy-related metabolic markers (Figure 2.4) could also be used to assess the state of differentiation in the colonic carcinoma cell lines.

The molecule acetyl-coenzyme A (acetyl-CoA) plays a central role in the onset of the cellular differentiation discussed above. For example, acetate can act as a direct energy source by complexing with coenzyme A and entering the tricarboxylic acid (TCA) cycle as acetyl-CoA (Lehninger *et al.* 1993). Other SCFAs with even-numbered carbon backbones have to be metabolized by beta-oxidation to acetate before they could be used as an energy source by the cells, a process that occurs in the mitochondrial matrix irrespective of the phyisiological state of the cells (Roediger, 1990). The metabolism of SCFAs with odd numbered carbon backbones has still to be elucidated even though it is known that compounds such as propionate are able to induce ketogenesis in HT-29 cells (Graz and Cowley, 1997). The acetyl-CoA can be used either in the TCA cycle to produce ATP and reduced cofactors with the carbon from the SCFAs being excreted as carbon dioxide or, two acetyl-CoA molecules can complex to form acetoacetate, as seen in the high percentage of carbon fluxed from the

SCFAs into acetoacetate (Henning and Hird, 1972). The third possibilty is that acetate from acetyl-CoA may be used to acetylate proteins (Lenhinger *et al.* 1993).

Entry of acetyl-CoA into the TCA cycle would alter the levels of ATP which may be quantified. Changes in ATP levels would affect the cAMP signal transduction pathway which could result in the possible phosphorylation of the cAMP responsive element-binding protein (CREB) via a PKA (protein kinase A)-mediated pathway. It is important to note that protein kinases, such as PKA do not function in isolation within the cell. For instance, the phosphorylation events effected by protein kinases may be reversed by protein phosphatases (Cooper, 1997).

In many animal cells, increases in cAMP activate the transcription of specific target genes which affect proliferation and differentiation and contain a regulatory sequence called the cAMP responsive element (CRE). In this case the signal is carried from the cytoplasm to the nucleus by the catalytic subunit of PKA. Within the nucleus PKA phosphorylates CREB leading to the activation of cAMP-inducible genes (Cooper, 1997). Within a given cell lineage cAMP/PKA will activate a specific group of target genes which will differ in specificity from genes activated via the same pathway in cells of a different lineage (Alberts, 1989). The de-condensation of the chromatin referred to earlier can only activate the transcription of lineage-specific genes irrespective of whether these genes are regulated by CREB (Aukena, 1997). CREB activates transcription through several domains that bind basal transcription factors and activates cAMP-responsive transcription through a separate region called the kinase inducible domain. CREB binds to DNA on the CRE at the palindromic octant TGACGTCA as a dimer or as a monomer, but only the dimer form is

transcriptionally active. The CREB sequence appears to be divided into 3 regions: 1) a DNA-binding domain consisting primarily of basic amino acids, 2) a trans-activation region containing a cluster of phosphorylation sites, and 3) a 'leucine-zipper' dimerization domain (Montminy *et al.* 1990a).

The trans-activation region contains potential phosphorylation sites for protein kinase A (PKA), protien kinase C (PKC) and casein kinase II. When these sites are phosphorylated they may activate transcription by providing an acidic surface which can interact with target proteins in RNA polymerase II transcription complex. Montminy *et al.* (1990a) showed that phosphorylation of CREB by PKA at Ser-133, within the kinase motif, is critical for transcription activation. In addition to cAMP-dependant transcription, CREB also stimulates cellular genes in response to calcium entry. The possible phosphorylation of CREB may be examined by immunochemical techniques to determine the influence of the physiological and non-physiological inducers at this level of transcription.

Montminy *et al.* (1990b) have shown that CREB is the only known transcription factor that is regulated by PKA. CREB-mediated expression has been suggested to be lineage-specific provided that the genes are available for transcription (Aukena, 1997; Montminy *et al.* 1990b). Thus, the determination of biochemical marker activity in cells treated with inducers in combination with an inhibitor of PKA could indicate whether the activation of a differential phenotype is specific via a cAMP-dependent pathway or non-specific via the unwinding of the chromatin (chromatin de-condensation). The differential phenotype in Figure 2.4 refers to the expression of biochemical markers representing a phenotype which

represses the expression of all other transcribed genes, hence becoming the dominant phenotype.

Of the cyclic dipeptides (CDPs) chosen for this study some contain amino acids which are ketogenic (Salway, 1998). For example, the catabolism of tryptophan (Figure 2.5) involves the formation of acetoacetate. The acetoacetate formed can be converted to acetyl-CoA which can either enter the TCA cycle or be used for the acetylation of histone proteins. The effects of the CDPs and SCFAs on the cells can then be investigated by measuring the flux of certain intermediates in the TCA cycle (eg. isocitrate dehydrogenase) or by measuring the acetylation of the histones.

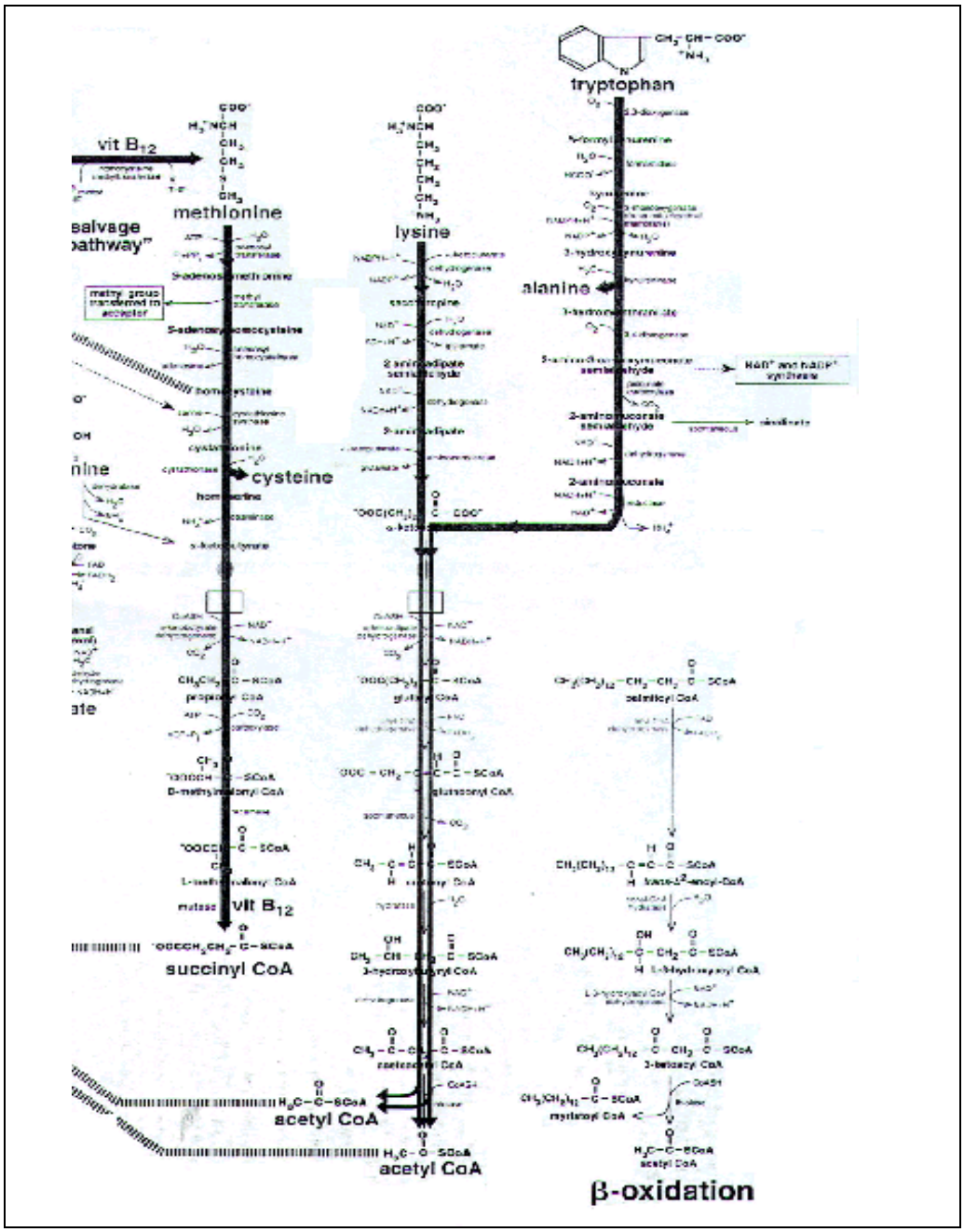


Figure 2.5: Catabolism of tryptophan illustrating the ketogenic potential of cyclic dipeptides containing tryptophan. Taken from Salway (1998).

In summary then, the known effects of SCFAs on energy metabolism in HT-29 cells are the decrease in ATP, glucose and lactic acid production, leading to an increase in the expression of biochemical markers of eneterocytic differentiation. This response is brought about by

the beta-oxidation of SCFAs with even numbered carbon backbones to acetyl-CoA which may enter the TCA cycle or be metabolized by alternative pathways as described ealier. The effects of SCFAs on CREB, pyruvate levels, the TCA cycle and histone acetylation are not known.

The possible mechanisms by which cyclic dipeptides may affect energy metabolism in HT-29 and Caco-2 cells are:

1) Catabolism of CDPs such as tryptophan and proline to acetoacetate (see Figure 2.5)

2) Catabolism of CDPs into amino acids which may enter the TCA cycle (see Figure 2.6)

3) Receptor-mediated cAMP secondary signalling which is not part of this study as it is not directly related to the energy state of the cell.

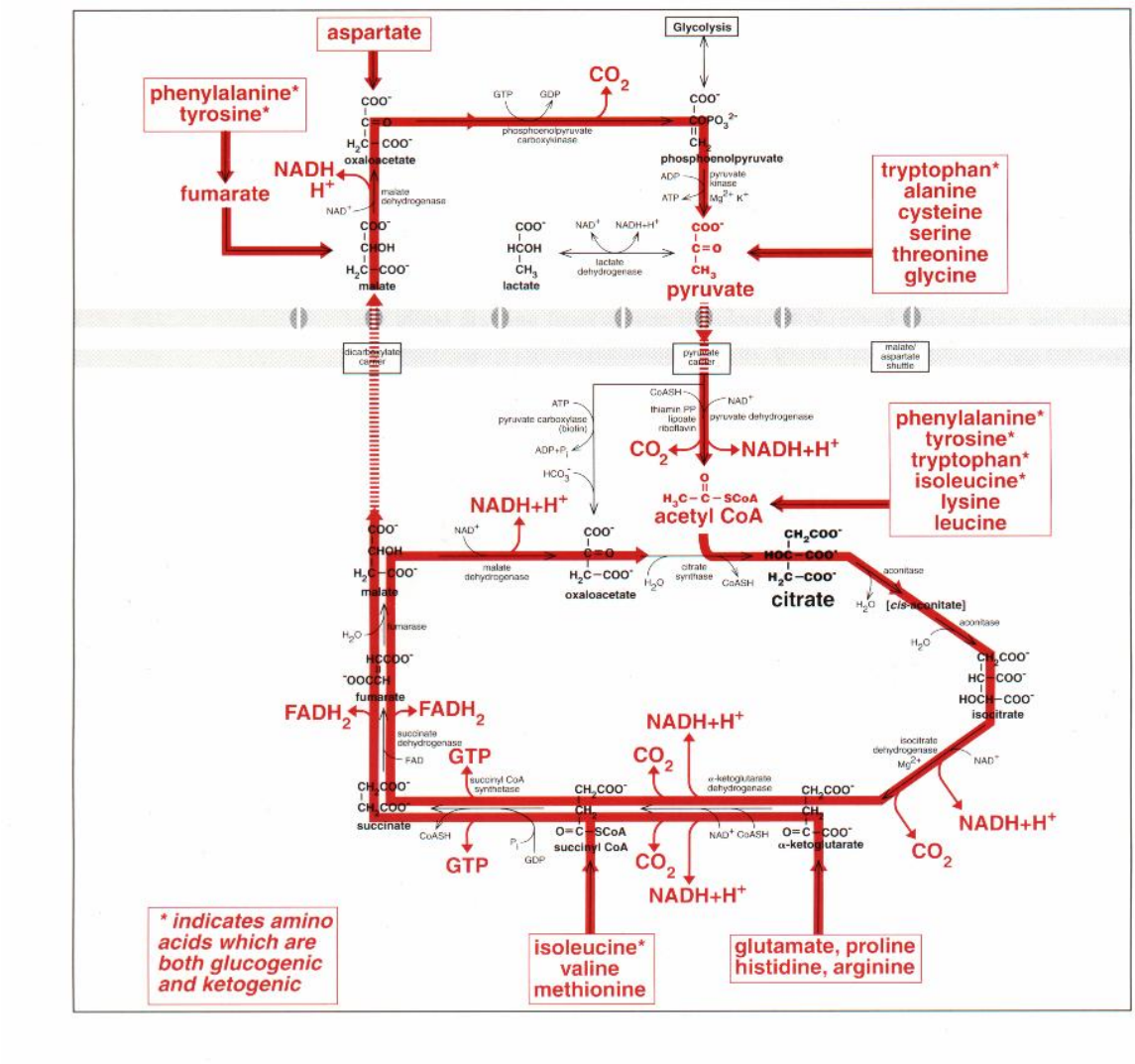


Figure 2.6: Catabolism of ketogenic and non-ketogenic amino acids showing their entry into the TCA cycle. Taken from Salway (1998).

CHAPTER 3: BIOCHEMICAL DETECTION OF THE INDUCED GASTROINTESTINAL TRACT PHENOTYPE

In this chapter aims 2, 3 and 4 listed in 1.6 were addressed. These aims involve:

a) the development of rapid, sensitive, miniaturized methods to screen for maturation induction potential in gastrointestinal cells *in vitro*.

b) determining the point in time where both cell lines are at similar stages in the differentiation process to allow direct comparison between spontaneous and induced differentiation.

c) Induction of the differential phenotype by physiological (ie. SCFAs and acetoacetate) and non-physiological (ie. cyclic dipeptides) compounds.

3.1 Materials and methods

The preparation of all solutions and reagents utilized is described in Appendix A.

3.1.1 Cell culture

HT-29 (donated by Miss P. Sommer, Department of Anatomical Science, Medical School, University of Witwatersrand) and Caco-2 cells (obtained from Highveld Biologicals, Kelvin, SA) were routinely maintained in 25 cm^2 culture flasks (Corning Incorporated, Corning, New York) in Dulbecco's modification of Eagles minimum essential medium (DMEM) (Highveld Biologicals) supplemented with 10% heat inactivated foetal calf serum (FCS) (Delta, R.S.A). Cells were subcultured 1:3 at 75% confluence. For experimental purposes the

cells were subcultured at 75% confluence and seeded into sterile 96 - well plates (Corning Incorporated) at a concentration of 50 000 cells per well.

3.1.2 Physiological and non-physiological inducers

Treatment of HT-29 cells with physiological and non-physiological inducers of differentiation was initiated 48 hours following their seeding into multi-well plates to allow for recovery from trypsin treatment. This was referred to as day 0. For the duration of the culture multi-well plates, the media with appropriate treatments was replaced as was necessary. The culture plates were incubated in sealed, moist containers to prevent evaporation of the growth medium during the culture period. The abbreviations used to describe the treatments administered to the cells are listd in Tables 3.1 and 3.2.

Table 3.1: Abbreviations for non-physiological inducer treatments administered to HT-29 and Caco-2 cells

Treatment	**Abbreviation for HT-29**	**Abbreviation for Caco-2**
Cyclo(Pro-Trp)	HT-29(PW)	Caco-2(PW)
Cyclo(Trp-Pro)	HT-29(WP)	Caco-2(WP)
Cyclo(Trp-Trp)	HT-29(WW)	Caco-2(WW)
Cyclo(Pro-Pro)	HT-29(PP)	Caco-2(PP)
Cyclo(Phe-Phe)	HT-29(FF)	Caco-2(FF)
Cyclo(Phe-Pro)	HT-29(FP)	Caco-2(FP)
Cyclo(Tyr-Tyr)	HT-29(YY)	Caco-2(YY)
Cyclo(Tyr-Pro)	HT-29(YP)	Caco-2(YP)

Table 3.2: Abbreviations for physiological inducer treatments administered to HT-29 cells

Cell Line	Treatment	Abbreviation
HT-29	Acetate	HT-29a
	Propionate	HT-29p
	Butyrate	HT-29b
	Acetoacetate	HT-29aa

3.1.3 Preparation of media

The SCFAs, and acetoacetate were dissolved in DMEM, filter-sterilized through a 0.2μm syringe filter and 200μl was added to the cells at a concentration of 5 mM which is an approximation of the physiological level. The CDPs were dissolved in 1ml of glycerol and then dissolved in DMEM, filter- sterilized through a 0.2μm syringe filter and 200μl of each compound was added to the cells at a final concentration of 125μg/ml.

3.1.4 Sample days

The HT-29 cells were exposed to the SCFAs for a total of 21 days. The sample days chosen were 1, 4, 9, 12, 18 and 21. Controls were included in which HT-29 cells were grown in DMEM supplemented with 10 % FCS without the addition of SCFAs. Day 1 was chosen in the HT-29 cells as this was felt to be most indicative of the immediate response of the cells to treatment with differentiation inducers. Day 4 was deemed to be important as the tendency for proliferation to cease in HT-29 cells treated with inducer has been shown (Graz and Cowley, 1997). Day 9 was selected in the HT-29 cells as these cells are reported to be well-adjusted to the culture conditions during treatment with inducers during this time frame

(Graz and Cowley, 1997). Terminal differentiation has been demonstrated in HT-29 cells after a 12 day treatment with butyrate (Augeron and Laboisse, 1984), thereby indicating selection of day 12 as a sample day. Day 18 was used by Graz and Cowley (1997) to show permanence of differentiation. Day 21 was chosen since this is the maximum period of successful culture of HT-29 cells. Caco-2 cells reach differentiation 3 days after confluence (Pinto *et al*, 1983) and were thus sampled on days 3, 9, 12, 18 and 21 for direct comparison to HT-29 cells. From the results of the studies with physiological inducers day 3 for Caco-2 and day 4 for HT-29 and day 12 for both cell lines were chosen as sample days for studies with non-physiological inducers.

3.1.5 Differentiation marker assays

All differentiation marker assays were carried out as microtiter plate assays which were standardised using commercially available enzymes. Preparation of the solutions utelized was done according to the methods described in Appendix A.

3.1.5.1 Sucrase-isomaltase

Sucrase-isomaltase was assayed according to a modification of Vassuer (1989). On each sample day the spent medium was removed and the cells in 96-well plates were washed twice with phosphate buffered saline (PBSA) containing EDTA (0.2g/l). At room temperature 25µl of buffer (0.1M sodium maleate buffer, pH 6.0) and 25µl of the appropriate substrate were added to each well. The reaction mixture was designed to have a substrate concentration of 28mM. For the assay of isomaltase (Sigma, St Louis) the substrate was palatinose (Sigma, St Louis), due to the expense of isomaltose. Sucrose (Saarchem, Krugersdorp, R.S.A) was used as substrate for the assay of sucrase activity. The hydrolysis

was allowed to proceed for 30min at 37°C before addition of 200µl Tris-glucose oxidase reagent (TGO). The absorbance was read at 492nm using a microtiter plate reader (Labsystems Multiskan MS, Finland) after a two hour incubation at 37°C. For all assays two controls were run simultaneously. A reagent blank contained buffer instead of enzyme. The second control was used to allow for glucose contamination from the enzyme or substrate solutions themselves. In this control the substrate solution was added after TGO reagent is added to the enzyme solution. In all assays one unit is defined as the amount of enzyme hydrolysing one mmole substrate per minute under assay conditions. Since the method involves the assay of glucose, the product of hydrolysis, the micrograms of glucose obtained from a glucose standard curve (Figure3.1) is indicative of the amount of substrate hydrolysed.

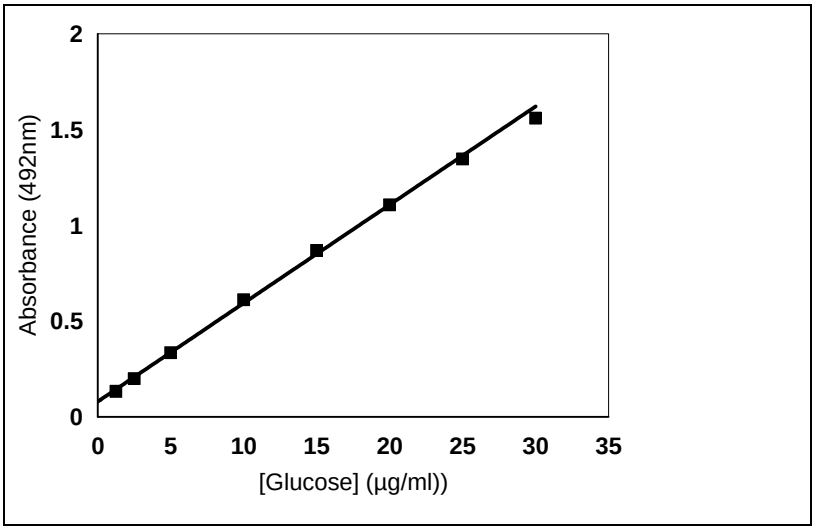


Figure 3.1: Glucose standard curve ($r^2 = 0.9991$). Prepared as described in the text.

Standard curves were constructed using the highest grade of enzymes available. For the purpose of standardizing the assays 25µl of the commercial enzyme (Sigma, St Louis) was used in the reaction mixture. As no intestinal glycosidases are currently available on the market microbial sources for similar enzymes had to be used to determine the linearity of the assay. Baker's yeast isomaltase and invertase were substituted for isomaltase and sucrase respectively. For isomaltase, linearity was established in low microgram quantities whereas invertase had a linear range in the low nanogram region (Figure 3.2).

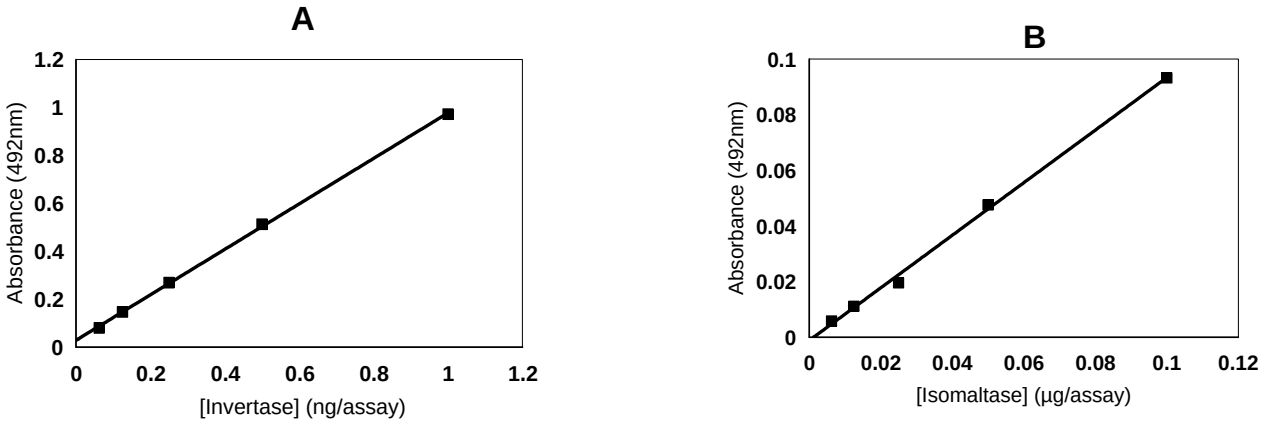


Figure 3.2: Standard curves for A, Invertase ($r^2 = 1$) and B, Isomaltase ($r^2=0.999$). Prepared as described in text.

3.1.5.2 Carbonic Anhydrase

When CO_2 is dissolved in water it is slowly hydrated to carbonic acid which spontaneously ionises according to the following equation:

$$CO_2 + H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow H^+ + HCO_3^- \qquad (1)$$

Hydration occurs in the pH range 6.5 to 10.0, whereas the reverse dehydration takes place in the pH range 5.5 to 7.5. This reverse reaction is catalysed by many oxy-acid buffers, including phosphate, cacodylate, Veronal, chromate, borate, selenite, and also by the enzyme carbonic anhydrase which catalyses both phases of the reaction equally (Waygood, 1956).

When a solution of CO_2 is mixed with an alkaline buffer, the pH drops rapidly, accompanied by the hydration of CO_2. The decrease in time taken for the buffer containing enzyme to drop to a specified pH is used as measure of catalytic rate. The lower pH value is determined by a sharp change in the colour of the of an appropriate indicator when the capacity of the solution changes markedly.

The principle above is that of a colorimetric method. The employed in this study was that of the CO_2-Veronal indicator method with a number of modifications to the method to suit the requirements of a discontinuous microtiter plate assay:

Bromothymol blue is added to Veronal buffer (equal volumes of 0.022M Veronal and 0.022M sodium salt of Veronal giving a pH of 7.95) in a ratio of 200µl indicator to 3ml buffer. In each assay 150µl buffer is added to 100µl deionised water and 15µl of enzyme (Carbonic anhydrase- Boeringer Manheim GmbH, Germany). In the case of the test samples, the cells in the 96-well plates were washed twice with PBSA and 15µl deionised water was added to the cells in place of enzyme solution. The microtiter plate containing the reaction mixtures was placed on ice in a sealed container for 15min. An inlet was then made in the sealed container to flush CO_2 through the system for 1min, thus saturating the atmosphere of the container with CO_2. The drop in pH produced a colour change due to

the increase in CO_2. The absorbance was then read immediately at 412nm against a blank containing the reaction mixture described above with 15µl deionised water in place of enzyme.

A standard curve (Figure 3.3) was constructed using commercial carbonic anhydrase 1. The enzyme activity was determined directly from the standard curve.

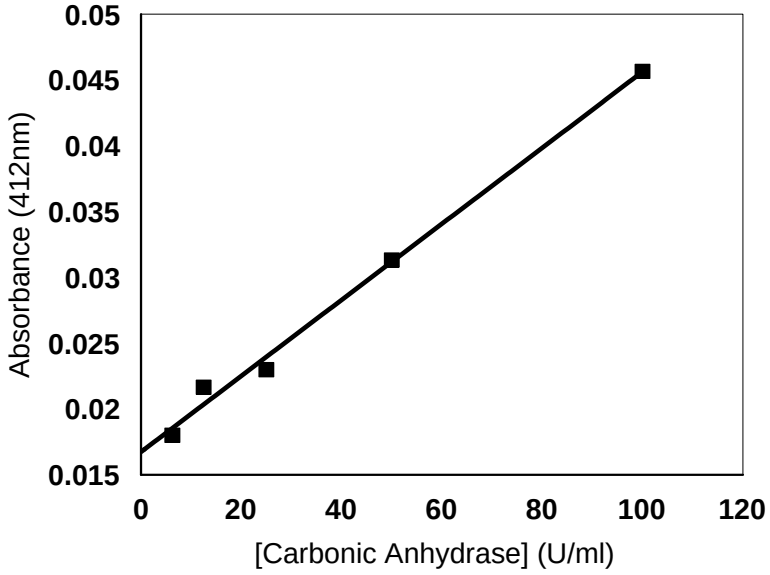


Figure 3.3: Carbonic anhydrase standard curve ($r^2 = 0.996$). Prepared as described in text.

3.1.5.3 Alkaline phosphatase

Alkaline phosphatase catalyses the hydrolysis of numerous phosphate esters of primary and secondary alcohols, sugar alcohols, cyclic alcohols, phenols and amines. The enzyme hydrolyses inorganic pyrophosphate. For the determination of activity, p-nitrophenyl phosphate (Merck, Darmstad,Germany) is used as substrate which is converted to p-nitrophenol, a coloured product, the absorbance of which can be measured at 412nm

(Bergmeyer, 1974). The method used in this study is that described by Bergmeyer (1974) for alkaline phosphatase from calf intestine, with some minor modifications to accommodate a microtiter plate assay:

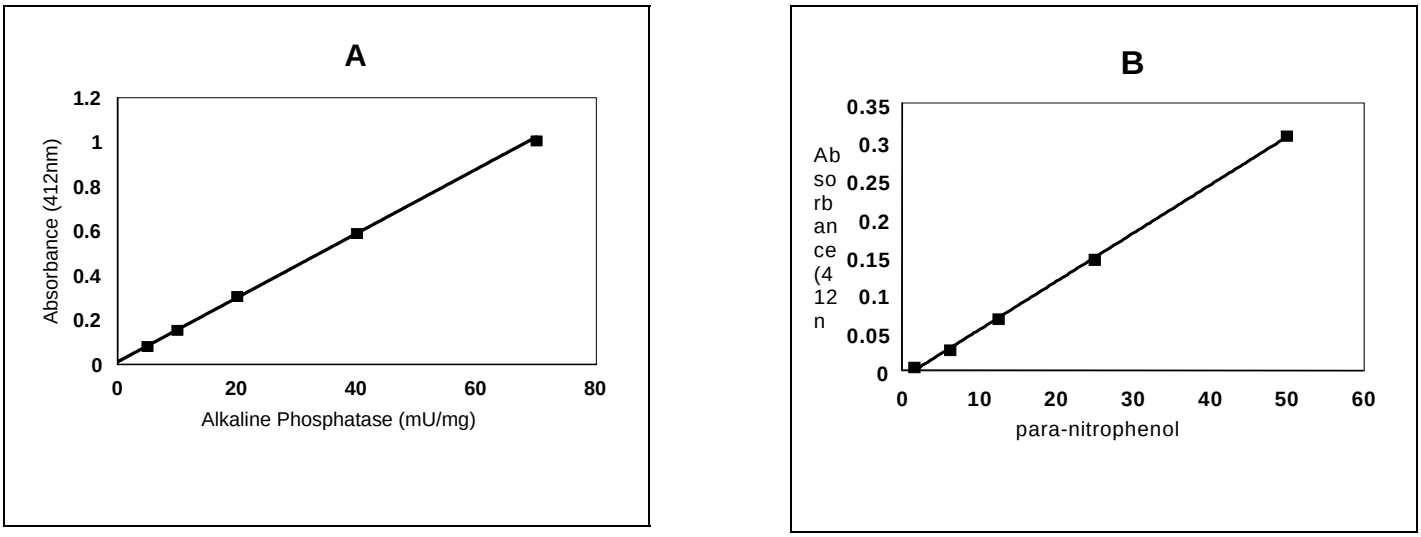


Figure 3.4: Standard curves for A, Aalkaline phosphatase ($r^2 = 1$) and B, *p*-nitrophenol ($r^2 = 0.999$). Prepared as described in text

A standard curve (Figure 3.4 A) was constructed using commercial alkaline phosphatase (Merck, Darmstadt, Germany). The reaction mixture in this case contained 26µl enzyme solution in 1mM MgCl2, with 200µl buffer and 40µl substrate. Enzyme activity was calculated as µmole/l *p*-nitrophenol produced per minute. The amount of *p*-nitrophenol was obtained from a *p*-nitrophenol standard curve (Figure3.4 B).

On each sample day the spent medium was removed and the cells were washed twice with deionised water. At room temperature 26µl 1mM MgCl2 and 200µl glycine buffer (0.1M, pH 10.5, 1mM MgCl2, 0.1mM ZnCl2 was added to each well containing cells. At time zero 40µl of p-nitrophenol phosphate (0.03M) was added to the reaction mixture and the

absorbance was read at 412nm after 5min at 25°C against a blank which contained the above reaction mixture excluding cells.

3.1.6 Cell Numbers

Cell counts were performed for both HT-29 and Caco-2 on comparative wells for the duration of the culture period (21 days). This was done to monitor the cell growth and to accommodate for cell loss, particularly after day 11 for HT-29 (Graz and Cowley, 1997). The cell counts were done in quadruplicate using an Improved Brite-Line Neubauer haemacytometer (Superior, Germany) and inverted phase contrast microscope (Nikon, Japan). A total count was done using 0.4% trypan blue (Gurr micoscopy, Germany) in a 1:1 ratio with the cell suspension. Cell counts done in order to relate enzyme activites to cell numbers.

3.1.7 Activity Calculations

The final activities of the enzymes are expressed as:

$$\text{Activity/Cell number} \times 10^5 \text{cells} \qquad (2)$$

"Cell number" in (2) is calculated from:

$$A/B \times C \qquad (3)$$

where "A" is the initial seeding number for the cell count, "B" is the initial seeding number for each experiment, and "C" is the number of cells counted on each day.

3.1.8 Statistical analysis

All r2 values were obtained using Lotus 123 software. All statistical analysis was performed using Prizm software.

3.2 Results

Figures 3.5 - 3.8 present the maturation induction profiles of HT-29 cells treated with physiological inducers as compared to that of the spontaneously-differentiated Caco-2 cells. Data for both cell lines are plotted on the same set of axes for each biochemical marker. The data for maturation induction effected by the non-physiological inducers is plotted as histograms (Figures 3.9 - 3.12) with both cell lines on the same set of axes for each biochemical marker. All data points represent the mean of triplicate experiments. Enzyme activities are expressed as activity/100 000 cells as described in 3.1.6. Enzyme activity was thereby related to cell numbers in order to enable comparison between treatments and cell lines and to account for any cell loss which may have occurred during culture. Figure 3.5 shows a close correlation of the alkaline phosphatase activity on days 4, 9, 12 and 18 between the HT-29 and Caco-2 cells. An even closer correlation between the carbonic anhydrase I activities of the two cell lines on days 4, 9 and 12 is shown in Figure 3.6. Statistical analysis showed that the closest correlation for the state of differentiation was found between day 3 for Caco-2 and day 4 for HT-29 and day 12 for both cell lines. Subsequently the sample days showing closest correlation were chosen to assess the affects of the non-physiological inducers on the differentiation of HT-29 and Caco-2 cells.

3.2.1 Physiological Inducers

The results are shown on the next page.

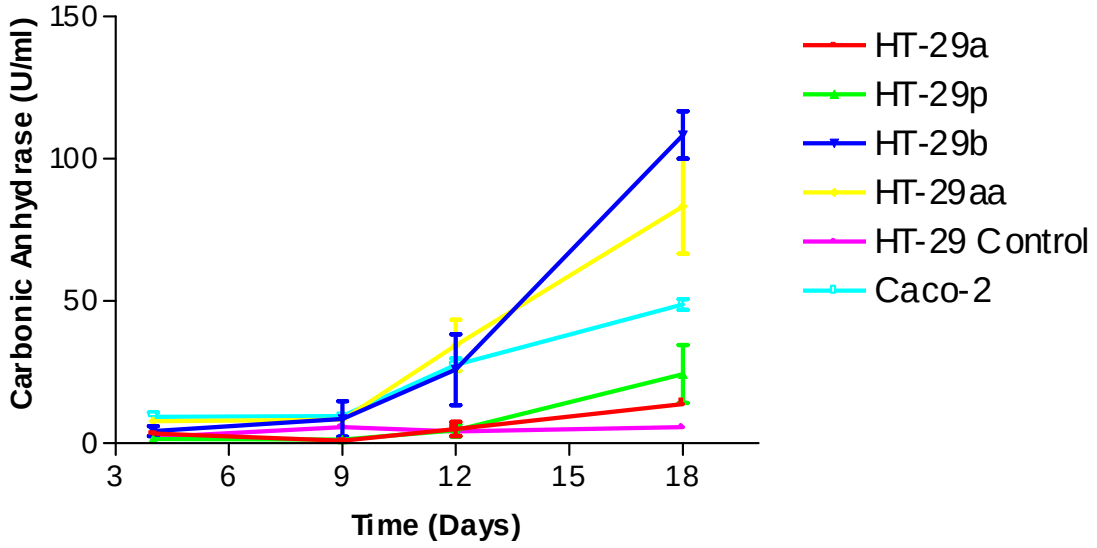


Figure 3.5: Maturation induction profiles showing the comparison between HT-29 cells and the spontaneously differentiating Caco-2 cells with respect to carbonic anhydrase activity. Each data point represents the mean of triplicate experiments.

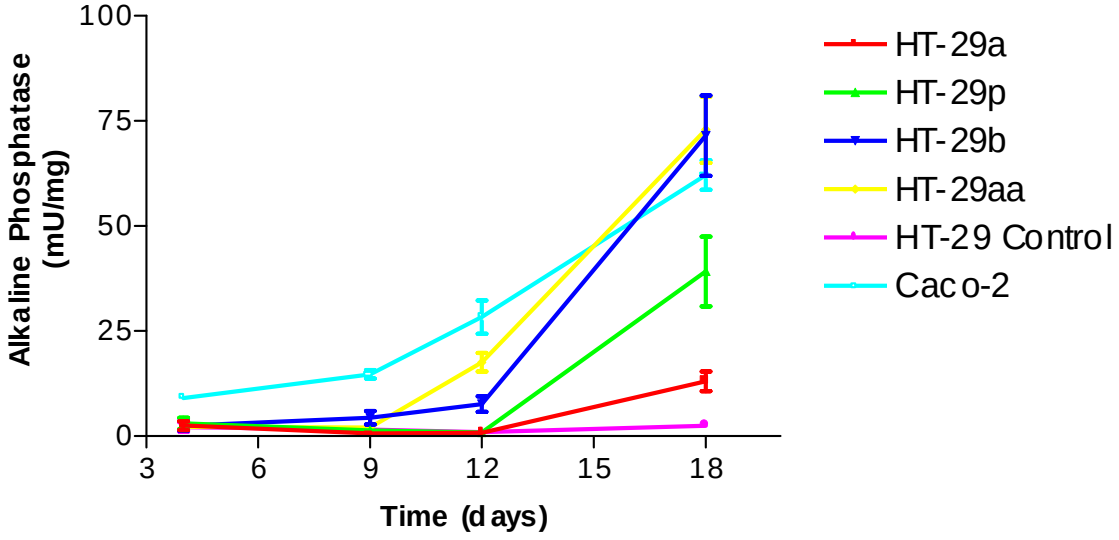


Figure 3.6: Maturation induction profiles showing the comparison between HT-29 cells and the spontaneously differentiating Caco-2 cells with respect to alkaline phosphatase activity. Each data point represents the mean of triplicate experiments.

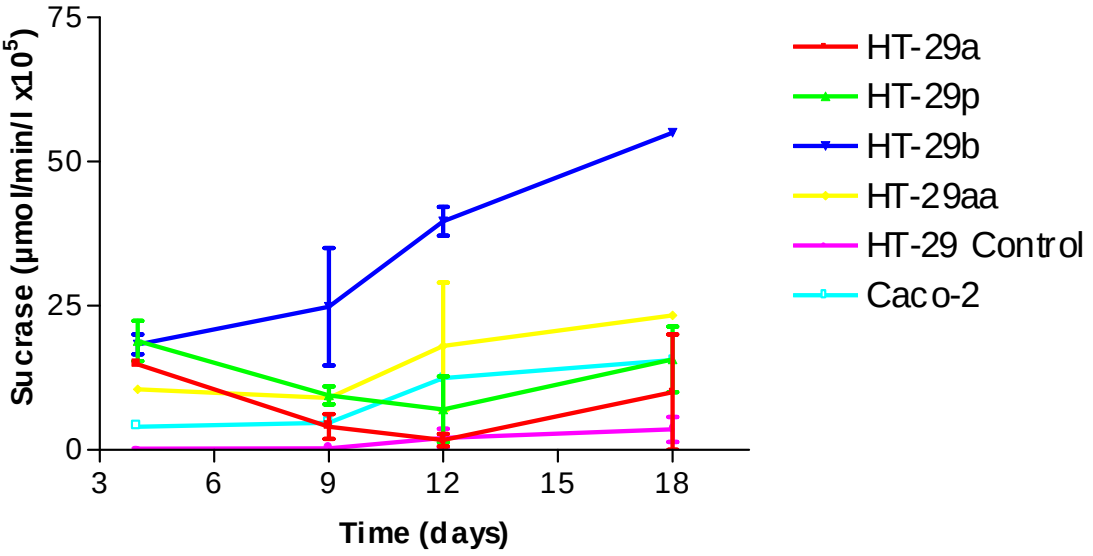


Figure 3.7: Maturation induction profiles showing the comparison between HT-29 cells and the spontaneously differentiation of Caco-2 cells with respect to sucrase activity. Each data point represents the mean of triplicate experiments.

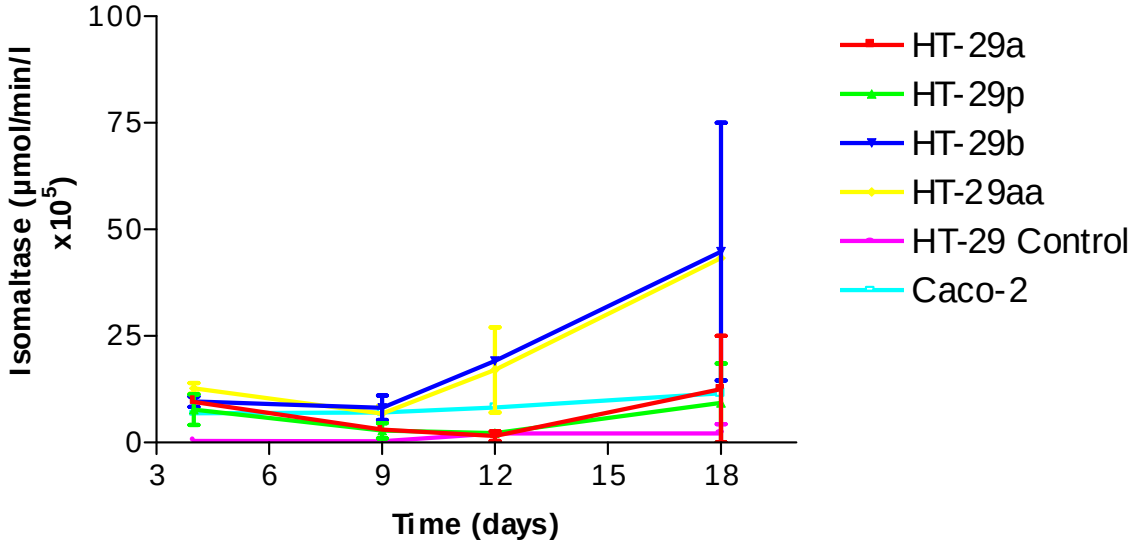


Figure 3.8: Maturation induction profiles showing the comparison between HT-29 cells and the spontaneously differentiating Caco-2 cells with respect to isomaltase activity. Each data point represents the mean of triplicate experiments.

3.2.2 Non-physiological Inducers

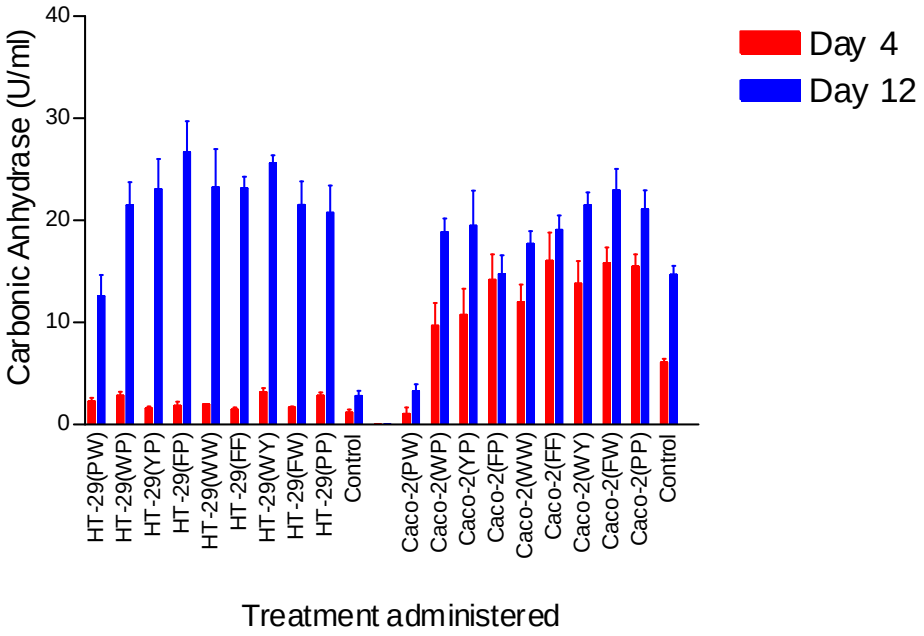


Figure 3.9: Expression of Carbonic anhydrase 1 enzyme in HT-29 and Caco-2 cells in response to non-physiological inducers. Results compare expression levels on day 4 to that on day 12. Each data point represents the mean of triplicate experimants

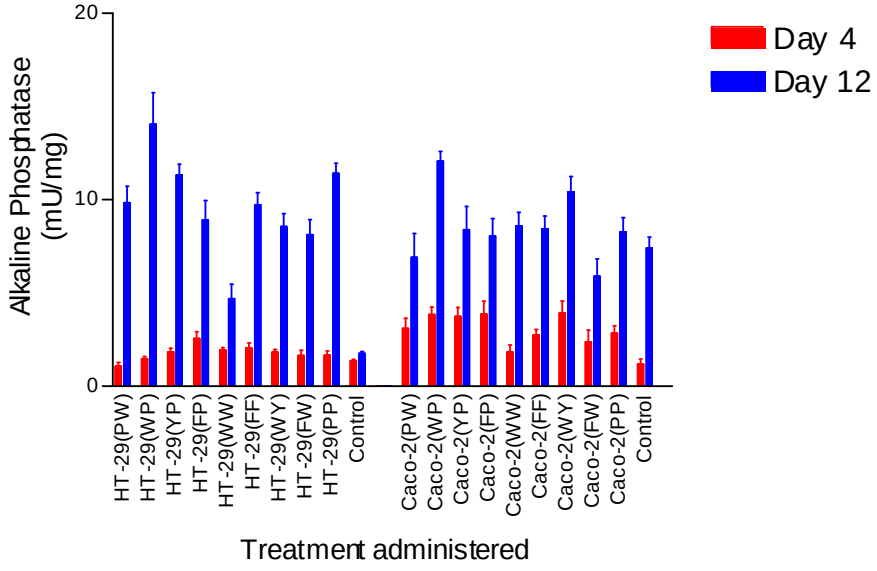


Figure 3.10: Expression of alkaline phosphatase in HT-29 and Caco-2 cells in response to non-physiological inducers. Results compare expression levels on day 4 to that on day 12. Each data point represents the mean of triplicate experimants

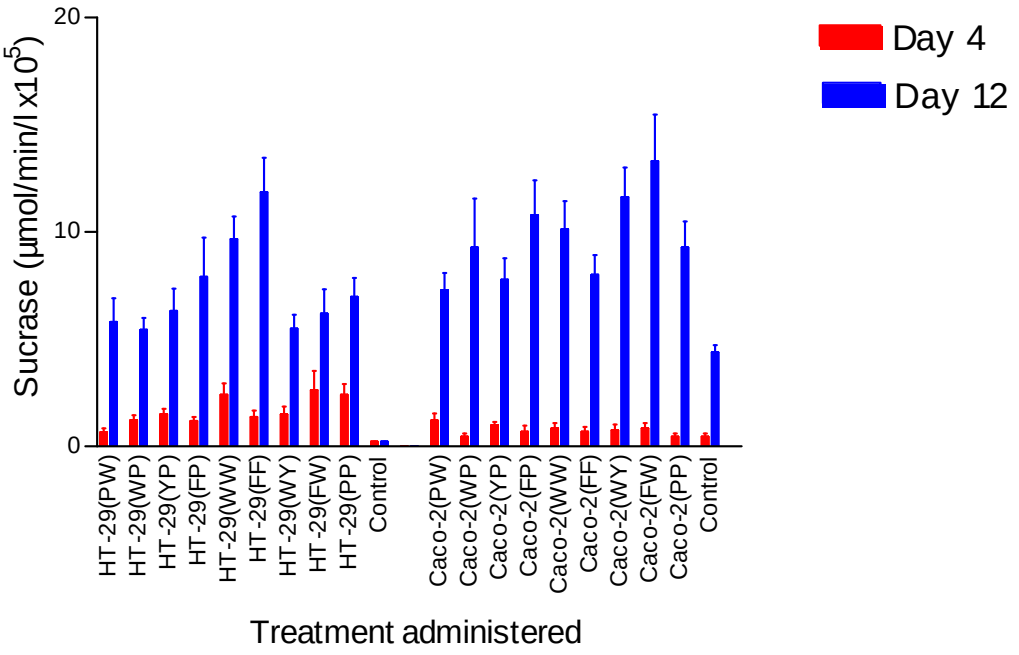


Figure 3.11: Expression of sucrase in HT-29 and Caco-2 cells in response to non-physiological inducers. Results compare expression levels on day 4 to that on day 12. Each data point represents the mean of triplicate experimants.

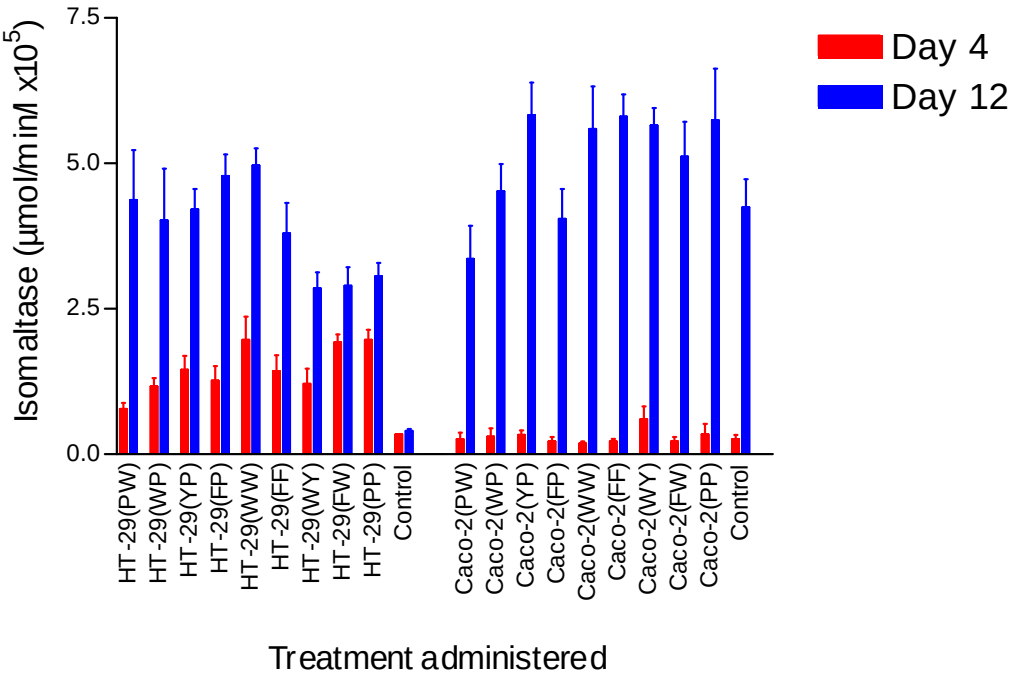


Figure3.12: Expression of isomaltase in HT-29 and Caco-2 cells in response to non-physiological inducers. Results compare expression levels on day 4 to that on day 12. Each data point represents the mean of triplicate experimants.

3.3 Discussion

The modified carbonic anhydrase assay was standardized successfully. The standard curve (Figure 3.3) generated for measuring CA 1 activity in the 50 units/mg to 150 units/mg range was linear with a correlation coefficient of 0.998. The assay was used successfully to determine CA 1 levels in both HT-29 and Caco-2 cells previously done by fluorescent antibody tracing. The modified sucrase-isomaltse assay was also standardized successfully. Standard curves were constructed for measuring isomaltase in the 0.00625 μg to 0.1 μg range and for measuring invertse (equivalent to sucrase) in the 0.0625 ng to 1.0 ng range. Both standard curves (Figure 3.2) were linear with correlation coefficients of 0.999 for isomaltase and 1 for invertase. The assay was used successfully to determine sucrase-isomaltase levels in HT-29 and Caco-2 cells. Both the modified assays described here are simple and rapid methods as compared to the earlier methods described in literature (Waygood, 1956; Vassuer, 1989), and the use of 96-well microplates permits the analysis of a large number of samples to be carried out simultaneously.

From the results in this and other studies it is evident that SCFAs (Augeron and Laboisse, 1984; Gamet *et al.* 1992) and acetoacetate (Graz and Cowley, 1997) induce a differential phenotype in HT-29 cells. Butyrate and acetoacetate are seen to be more potent inducers than acetate and propionate. This observation is illustrated by the marked increase in Sucrase-Isomaltase, Alkaline Phosphatase and Carbonic Anhydrase 1 activity from day 12 to day 18 for HT-29b and HT-29aa cells. These results concur with those reported in the literature (Gamet *et al.* 1992, Graz and Cowley, 1997).

The results obtained here also show that induced differentiation in HT-29 cells is comparable to spontaneous differentiation in Caco-2 cells. For each differentiation marker profile the overall activity measurements were seen to be similar for HT-29 and Caco-2 cells. More specifically, carbonic anhydrase activity shows very close correlation ($p < 0.05$) between HT-29 and Caco- 2 cells on days 4, 9 and 12. This result is of particular significance since carbonic anhydrase I is the only definitive marker of colonic differentiation (Sowden *et al.* 1993). Alkaline phosphatase activity shows close correlation between the two cell lines on days 4, 9, 12, and 18.

Results from the present study have also shown that cyclic dipeptides (Table 3.1), representing non-physiological inducers, are able to induce the gastrointestinal phenotype described earlier in HT-29 cells. The results obtained are similar to those obtained for the physiological inducers showing a marked increase in biochemical marker activity from day 4 to day 12. It is not expected that non-physiological inducers would elicit a similar response to that of the physiological inducers. An explanation for this result may be the fact that three of the amino acids represented among the nine cyclic dipeptides studied are ketogenic and thus are able to enter the TCA cycle as explained in Chapter 2. The CDPs, such as cyclo(Pro-Pro) which do not contain ketogenic amino acids may induce differentiation by entering the TCA cycle via an alternative pathway as illustrated in Figure 2.6.

There is an apparent lack of effect of the CDP's on the differentiation in Caco-2 cells (Figures 3.9 to 3.12). However, a few of the CDP's including cyclo(Trp-Pro) and cyclo(Trp-Tyr) show a significant ($p < 0.05$) increase in induction of the differential phenotype as compared to control cells, which may be explained by a possible cumulative effect of CDP-

induced and spontaneous differentiation. The spontaneous differentiation observed in Caco-2 cells is mediated by the *src* gene product which is specific via *erk* (Brooks and Hart, 1992). Sustained activation of *erk* can lead to differentiation (Marshall, 1995) which is a separate signal transduction pathway to the PKA-dependent pathway. Therefore there is a possible, if speculative, cumulative effect in spontaneously differentiating Caco-2 cells treated with CDP's.

CHAPTER 4: ASSESSMENT OF POTENTIAL ENERGY-RELATED METABOLIC MARKERS OF THE INDUCED GASTROINTESTINAL PHENOTYPE

Graz and Cowley (1997) have shown that the energy-state of HT-29 cells is linked to their differentiation. Upon induction by physiological inducers (ie. SCFAs) HT-29 cells undergo differential gene expression in conjunction with a raise in the total energy state of the cells which ultimately effects the observed morphological and functional changes in the HT-29 cells. The changes referred to here are characteristic of enterocytic differentiation as occurs in the normal gastrointestinal tract. Results from chapter 3 have shown that non-physiological inducers (ie. the cyclic dipeptides), in addition to the SCFAs and acetoacetate, are able to induce the gastrointestinal phenotype described above. Furthermore the spontaneous differential gene expression occurring in the Caco-2 colonic carcinoma cell line is not negatively affected by treatment of the cells with the non-physiological inducers.

The current chapter focuses on the investigation of the effects of the cyclic dipeptides on energy metabolism in HT-29 and Caco-2 cells with the aim of identifying possible energy-related metabolic markers of the observed differential phenotype. In addition the experiments were aimed at determining whether the induction the differential phenotype is a result of a non-specific (generalized) transcription of genes as opposed to true differentiation.

4.1 Methods

The preparation of reagents and solutions utilized is described in Appendix A.

4.1.1 Cell Culture

Cell culture was performed according to the methods described in Chapter 3.

4.1.2 Preparation of media

The cyclic dipeptides cyclo(Trp-Trp), cyclo(Trp-Pro), cyclo(Trp-Tyr), cyclo(Pro-Trp), cyclo(Phe-Phe), cyclo(Phe-Pro), cyclo(Tyr-Pro), cyclo(Phe-Trp), cyclo(Pro-Pro)) were each dissolved in 1ml of glycerol and then dissolved in DMEM, filter-sterilized through a 0.2μm syringe filter. Each compound was added to the cells at a concentration of 125μg/ml on the appropriate assay day.

4.1.3 Assessment of energy-related metabolism in HT-29 and Caco-2 cells

At confluence HT-29 and Caco-2 cells were treated with 125μg/ml of the appropriate inducers. The cells were incubated in 25 at 37°C for 24 hours after which the media was collected and analysed for the uptake of glucose from the growth medium and the release of acetoacetate (ketogenesis) and lactic acid into the growth medium. The cell mass was determined and divided equally into three samples for the determination of ATP levels, isocitrate dehydrogenase (ICD) activity and pyruvate levels within the cells. ATP, isocitrate dehydrogenase and pyruvate levels were determined with the aid of diagnostic kits obtained from Sigma (St Louis). The tests were performed according to the manufacturer's instructions (Sigma). The test for the presence of ketones was performed using Ketodiastix (Bayer Diagnostics, Hampshire, UK).

4.1.4 Immunocytochemical detection of CREB phosphorylated at Ser-133

On days 4 and 12 HT-29 cells and Caco-2 cells were washed with 1mM PBS (pH 7.4) to remove any traces of media in the flasks. The cells were fixed to the flasks by incubating the cells in 1mM PBS (pH 7.4) containing 3% paraformaldehyde (BDH Chmeiclas, Poole, England) for 20mins at 4°C. The cells were washed three times with 1ml TBST. 1ml of blocking buffer was then added to each flask. Then cells were incubated in the blocking buffer for 1hr at room temperature. Once the blocking buffer was removed, the bottom of the flask was cut, producing small squares of the flask bottoms, to which the cells were fixed. The primary antibody (Phospho-specific CREB antibody, New England Biolabs, Bervely), was added to the cells for 24hrs at 4°C. The cells were washed three times with TBST, after which the secondary antibody (antiRabbit IgG-AP conjugate, Sigma, U.S.A.) was added as substrate, and three minutes was allowed for the reaction to occur. The presence of a blue precipitate indicated a positive result. The percentage activity of CREB was graded by visually determining the amount of phosphorylated CREB present in the sample.

4.1.5 Histone modification: Histone Phosophorylation and Acetylation studies

At confluence HT-29 and Caco-2 cells were incubated with 5µl 2mCi/ml 32P-orthophosphate (ICN Pharmaceutical. Irvine, California) together with inducers in DMEM; and 5µl 0.25mCi 14C-acetae (NEN, Boston, U.S.A.) together with 5ml of the aforementioned 5mM inducers.

4.1.5.1 Extraction of nuclei

After 24 hours in the presence of inducers and the radionuclide, nuclei extractions were done according to De Groot (1982) as follows: On the assay days, the cells were centrifuged at 750rpm for 10min. The pellets were washed twice in $Ca2^{+}$, $Mg2^{+}$- free saline water (CMFSW), containing 0.01% EDTA (BDH Chemicals Ltd., Poole, England). The pellet was re-suspended in 1ml 0.5M sucrose (NT Laboratories, Excom, Johannesburg), 10mM Tris (Merck, Germany), pH7,5, to swell the cells. The cells were dissociated by the loose plunge of a Dounce homogenizer, after which the concentration was adjusted to 1:20 relative to packed cell volume. The cells were lysed with an equal volume of buffer containing 20mM NaCl (NT Laboratories, Excom, Johannesburg), 10mM MgCl2 (Saarchem, Chamdor, England), 20mM Tris, pH7,5; 1% TritonX-100 (BDH Chemicals Ltd., Poole, England) with mixing by vortexing. The nuclei is now re-suspended in 0,5ml of buffer containing 0.25M sucrose, 10mM NaCl, 5mM MgCl2, pH7,5. The suspension was once again centrifuged at 1000rpm for 10min.

4.1.5.2 Histone extraction

Histones were extracted according to Wiekowski and DePamphilis (1993). The nuclear pellet was re-suspended in 200µl nuclear disruption buffer using a vortex device. The nuclei were recovered by centrifuge at 12000 rpm for 10min at 4°C. The pellet was re-suspended in 200µl chromatin was buffer and centrifuged to remove any proteins that were soluble in 140mM salt. The pellet was re-suspended in 50µl nuclear disruption buffer, which was then sonicated for seven seconds using a sonicator bath (50-60 Hz) (Bandelin Sonorex NK51). The histones were extracted into acid for 1hr by adding 5,5µl 3.6N H_2SO_4 (BDH Chemicals,

Poole England) to each sample. Cell debris were then removed by centrifugation at 12000rpm at 4°C for 30mins. The supernatant which contained the histones was recovered. Histone proteins were precipitated by adding 10 volumes of acetone (Associated Chemical Enterprises, Glenvista, South Africa). The reaction was incubated overnight at –20oc to allow for the precipitation of the histones. The precipitate was recovered by centrifugation at 12000rpm at 4°C for 30min. The supernatant was discarded and the pellet dried under vacuum for 10mins. The pellet was re-suspended in 500µl deionised water. The Folin-Lowry protein assay was used to determine the amount of protein in each sample. Liquid scintillation analysis was used to assay the radioactive product.

4.1.5.3 Folin-Lowry protein determination

Protein content of samples were determined in order to calculate the amount of phosphorylation or acetylation per milligram of protein. This was then used to calculate the CPM per mg of protein to allow for the comparison of results. Protein determination was performed by adding 0.23ml of the alkaline solution to 0.05ml of the test solution. The sample was mixed thoroughly and allowed to stand for 10min before 0.02ml of the diluted Folin-Ciocalteau reagent was added rapidly with immediate mixing. After 30min the extinction was read against the blank at 600nm using a Labsystem Multiskan MS (Multiskan Transmit Program Rev. 1.3. (1995)) and the protein content of the samples was determined from the standard curve.

4.1.5.4 Liquid scintillation analysis

Polyethylene vials and lids were washed in 1% PCC-Plus extra strength detergent (Pierce, U.S.A.) in water for 6hrs, after which they were rinsed in running tap water for 30mins. The vials were placed into a 0.1N HCl (Merck, Darmstadt, Germany) solution for 6hrs, after which they were rinsed with two changes of distilled water for 30mins. The vial were dried in a hot air oven at 60°C for 48hrs, after which they were stored until used. 10ml radiolabelled histone extracts were added to 3ml of the scintillation cocktail (Ultlima Gold XR, Packard) in each vial. The histones were gently mixed into the scintillation cocktail, after which they were equilibrated in the dark for 4hrs, allowing the phosphorescence induced in the scintillant by sunlight, to decay before counting. The vials, as well as standardized controls, were counted for 5mins in a Liquid Scintillation Counter (Tri Carb 2300 TR Liquid Scintillation Analyzer, Packard). CPM (counts per minute) were recorded to allow the determination of the acetylation and phosphorylation of the samples in relation to the control sample.

4.1.6 Protein kinase inhibition studies

At confluence HT-29 and Caco-2 cells were sub-cultured from 25cm^2 flasks (Corning) into 6-well plates (Corning) at a concentration of 200 000 cells per ml. On day 0 the cells were treated with the appropriate inducers and 10μg/ml of a protein kinase A inhibitor obtained from Sigma. On day 4 the levels of carbonic anhydrase 1 and alkaline phosphatase were determined using the assay methods for the biochemical markers as described in Chapter 3.

4.2 Results and discussion

A link has been shown between energy state and differentiation in HT-29 cells. This would suggest that metabolites linked to the production of ATP, and signal transduction pathways utilizing ATP could be used as markers of the reported differentiation in HT-29 cells. HT-29 cells have an impaired glycolytic pathway (Royall *et al.* 1990), with a low energy yield and a high glucose uptake and are undifferentiated in their native form (Pinto *et al.* 1982, Augeron and Laboisse, 1984). HT-29 cells treated with CDPs display decreased glucose utilization with a concomitant decrease in pyruvate and lactate levels (Table 4.1). A similar result was obtained for the physiological inducers (SCFAs), in particular butyrate and acetoacetate which exhibited a more pronounced effect than acetate and propionate. These results correlate with those obtained by Graz and Cowley (1997) for the effects of SCFAs on the differentiation of HT-29 cells. The CDPs showed no significant ($p > 0.05$) effect on Caco-2 cells.

The low levels of pyruvate in HT-29 cells suggest that the TCA cycle may be affected and that a separate source of acetyl-CoA is required. The results obtained for isocitrate dehydrogenase (ICD) activity (Figure 4.1) within cells treated with CDPs were inconclusive with no significant ($p > 0.05$) difference from that obtained for control cells. Thus ICD may be excluded as a potential marker. Further studies investigating the effects of CDPs and SCFAs on metabolites of the TCA cycle as potential markers of differentiation need to be conducted. Those CDPs proven to be ketogenic (Table 4.1) may increase the total energy state of the cell, which would effect the terminal differentiation as the cells use the available acetoacetate in preference to glucose as an energy source as reported by Graz and Cowley (1997). The acetoacetate provides acetate that can act as an energy source by complexing

with CoA and entering the TCA cycle as acetyl-CoA (Tsanev, 1975). Acetyl-CoA can be used by the TCA cycle to produce either ATP or acetoacetate (McGarry and Foster, 1980). It is hypothesized that acetoacetate increases the probability of differential gene expression in HT-29 cells, obviating the need for higher cellular ATP concentration (Graz and Cowley, 1997). The result obtained for the SCFAs show no significant difference compared to those of the CDPs. The results for Caco-2 cells show that the CDPs have no significant ($p > 0.05$) effect on the ICD levels of these cells.

Table 4.1: **Effect of cyclic dipeptides on selected energy-related metabolites in HT-29 cells.**

Treatment 24 h HT-29	Lactate Production (mmol/l)[a]	Glucose Utilization (%)[b]	Ketogenesis[d]	Pyruvate Production ((mmol/l)/gram cells)[c]
cyclo(Pro-Trp)	11.0 ± 0.3	46.7± 0	++	29.0 ± 11.4
cyclo(Trp-Pro)	8.3± 1.8	54.3± 14.4	+++	37.4 ± 16.8
cyclo(Tyr-Pro)	9.5 ± 2.1	34.3± 12.5	++	29.1 ± 6.8
cyclo(Phe-Pro)	9.4 ±0.2	27.9± 17.3	++	9.9 ± 5.6
cyclo(Trp-Trp)	9.0 ± 1.0	19.8± 18.8	++	31.7 ± 9.8
cyclo(Phe-Phe)	9.1 ± 0.08	31.7± 4.9	+	41.1 ± 24.9
cyclo(Trp-Tyr)	8.4± 0.5	23.3± 0.6	+	33.8 ± 9.9
cyclo(Phe-Trp)	8.1± 0	25.0± 2.7	+	11.7 ± 2.0
Cyclo(Pro-Pro)	10.7± 0	30.3± 20.3	++	24.0 ± 10.0
Control	10.9 ± 0	59.9± 3.0	+	47.3 ± 29.9

[a] Lactate concentration in culture medium.
[b] Percentage of available glucose utilized.
[c] Pyruvate concentration produced within the cell.
[d] Ketone levels represented as (+) 0.5mmol/l, (++) 1.5mmol/l), (+++) 4mmol/l)

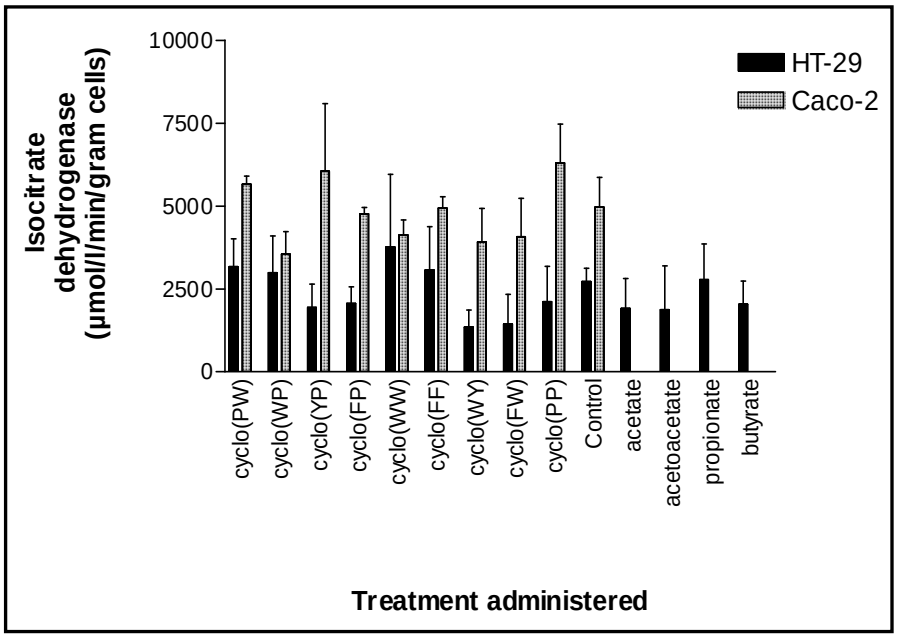


Figure 4.1: Isocitrate dehydrogenase (ICD) activity in HT-29 cells treated with CDPs; SCFAs and acetoacetate, and Caco-2 cells treated with CDPs. P = Proline, W = Tryptophan, Y = Tyrosine, F = Phenylalanine. Cyclo refers to the cyclic conformation of the dipeptide.

An increase in ATP concentrations would be expected for undifferentiated HT-29 cells (Graz and Cowley, 1997). In cultures treated with CDPs, however, a decrease in ATP concentrations was observed for all CDPs except for cyclo(Trp-Trp) and cyclo(Trp-Pro) (Figure 4.2). From the observed increase in the energy requirement as indicated by the decrease in ATP concentrations it is proposed that the ATP may be utilized for two metabolic pathways: either to be the phosphate donor during the phosphorylation of histone proteins or the production of cAMP. Results for the SCFA-treated cells indicate that there is no significant ($p > 0.05$) difference between the effects of physiological and non-physiological inducers on the ATP concentrations or energy requirements of HT-29 cells. For Caco-2 cells the CDPs exhibit no significant ($p > 0.05$) effects with respect to ATP concentrations.

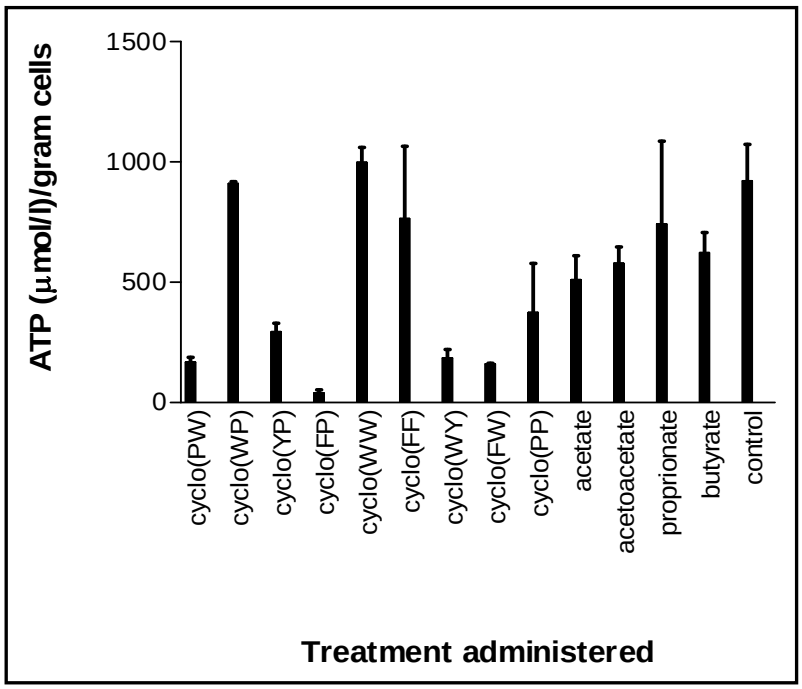


Figure 4.2: ATP concentrations observed in cells treated with physiological and non-physiological inducers. P = Proline, W = Tryptophan, Y = Tyrosine, F = Phenylalanine. Cyclo refers to the cyclic conformation of the dipeptide.

Histone phosphorylation could be indicative of differentiation, but is not and hence reflects non-specific differential gene expression. The results obtained for histone phosphorylation studies (Figure 4.3) indicate a correlation with differentiation which could from a statistical point of view be significant. The results confirmed that histone phosphorylation was partly responsible for the decrease in ATP concentrations. This suggests that CDPs cause an increase in the unwinding of the chromatin from around the nucleosome, making genes more accessible for transcription, providing evidence that the CDPs may activate the chromatin switch (Bodnar and Bradley, 1996). From the results obtained for histone acetylation (Figure 4.4) it is suggested that HT-29(FP) and HT-29(WW) activate the chromatin switch via histone acetylation rather than histone phosphorylation.

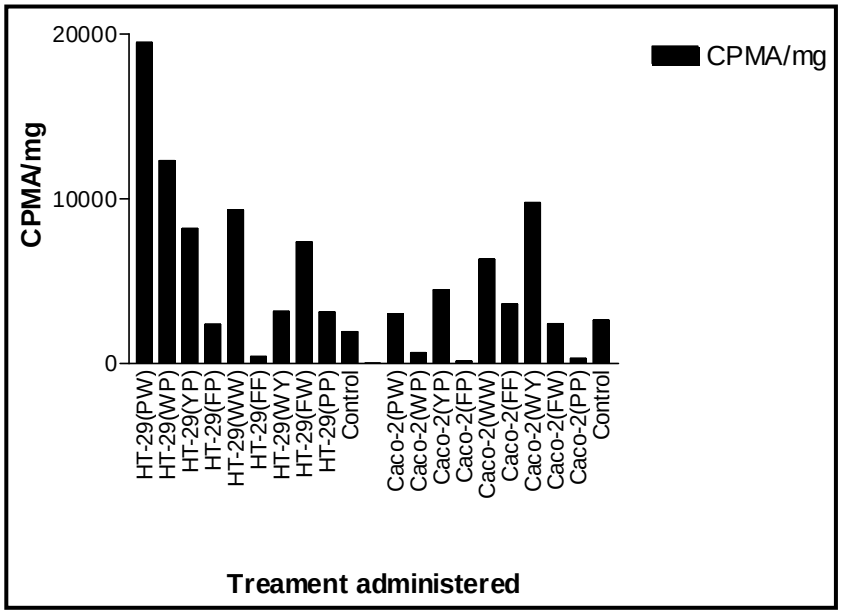


Figure 4.3: Levels of histone phosphorylation as determined in HT-29 and Caco-2 cells treated with non-physiological inducers. P = Proline, W = Tryptophan, Y = Tyrosine, F = Phenylalanine. Cyclo refers to the cyclic conformation of the dipeptide.

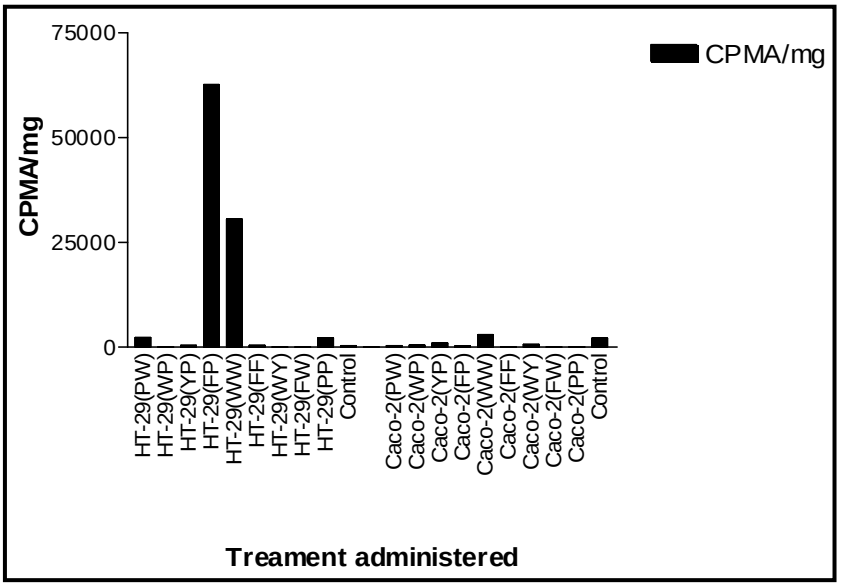


Figure 4.4: Levels of histone acetylation as determined in HT-29 and Caco-2 cells treated with non-physiological inducers. P = Proline, W = Tryptophan, Y = Tyrosine, F = Phenylalanine. Cyclo refers to the cyclic conformation of the dipeptide.

ATP may also be converted to cAMP, and may thus effect the transcription of cAMP-inducible genes through action of the cAMP responsive element binding protein (CREB). The results (Figure 4.5) obtained for the immunocytochemical detection of CREB indicate

that the CDPs may induce the differential phenotype as observed in HT-29 cells on induction by physiological inducers. This result was supported by the increased expression of CA 1 as a biochemical marker of the differential phenotype in response to treatment with all CDPs used (Figures 3.9). A negative correlation ($r^2 = 0.732$; $p < 0.05$) was found between ATP levels and CREB phosphorylation in CDP-treated cells. These results suggest that CREB shows potential to be used as a marker of lineage-dependent induction of the differential phenotype. Inhibition of the cAMP dependent kinase, PKA, resulted in a marked inhibition of CA 1 activity (Figure 4.6) as opposed to the effect on alkaline phosphatase activity (Figure 4.7). This result indicates that CREB is involved in the activation of the colonic differentiation markers (e.g. CA 1) whilst it has less effect on the expression of non-specific markers such as alkaline phosphatase which is expressed in most tissues in the body (Herz *et al.* 1973).

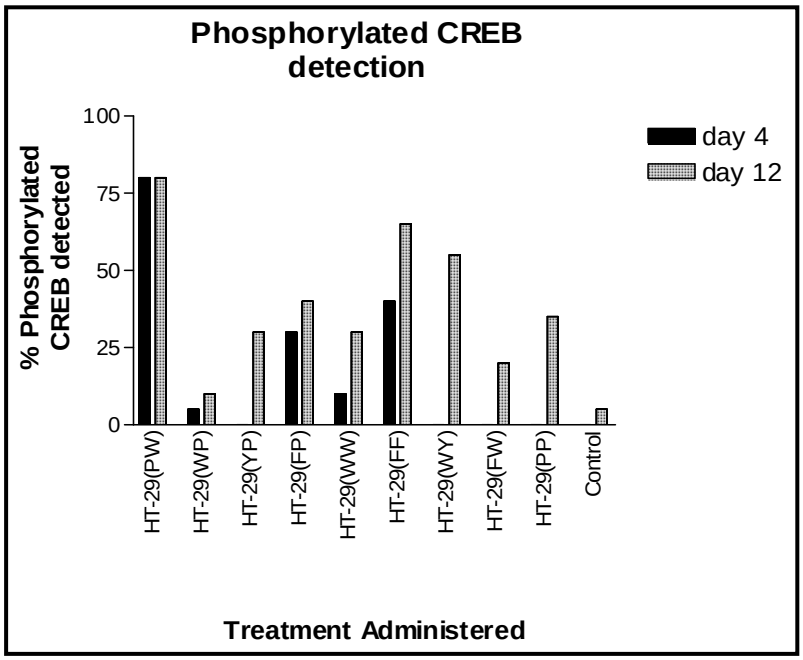


Figure 4.5: Percentage of immunocytochemically detected CREB phosphorylation in HT-29 cells on days 4 and 12 following treatment with non-physiological inducers. P = Proline, W = Tryptophan, Y = Tyrosine, F = Phenylalanine. Cyclo refers to the cyclic conformation of the dipeptide.

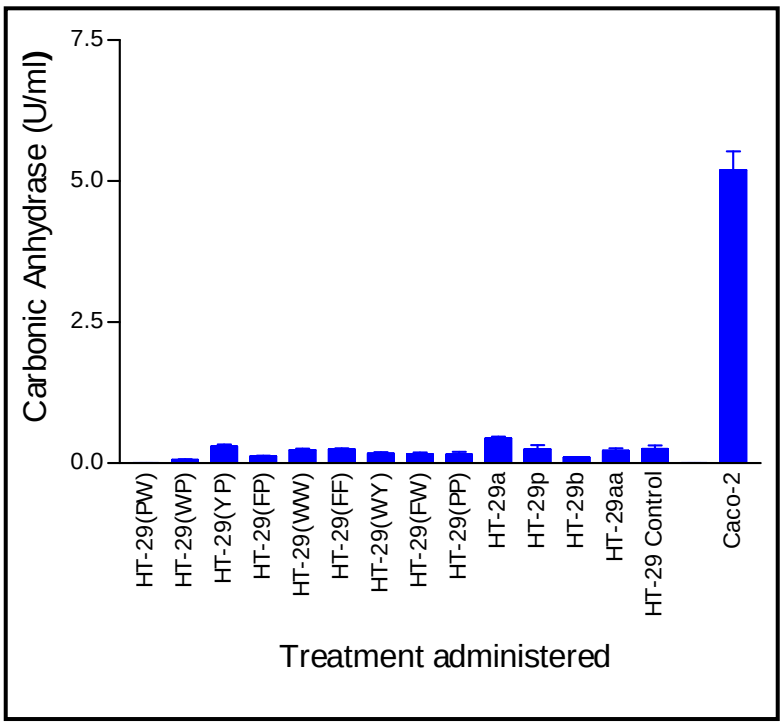


Figure 4.6: Carbonic anhydrase 1 expression in HT-29 and Caco-2 cells showing the effects of PKA inhibition. P = Proline, W = Tryptophan, Y = Tyrosine, F = Phenylalanine. Cyclo refers to the cyclic conformation of the dipeptide.

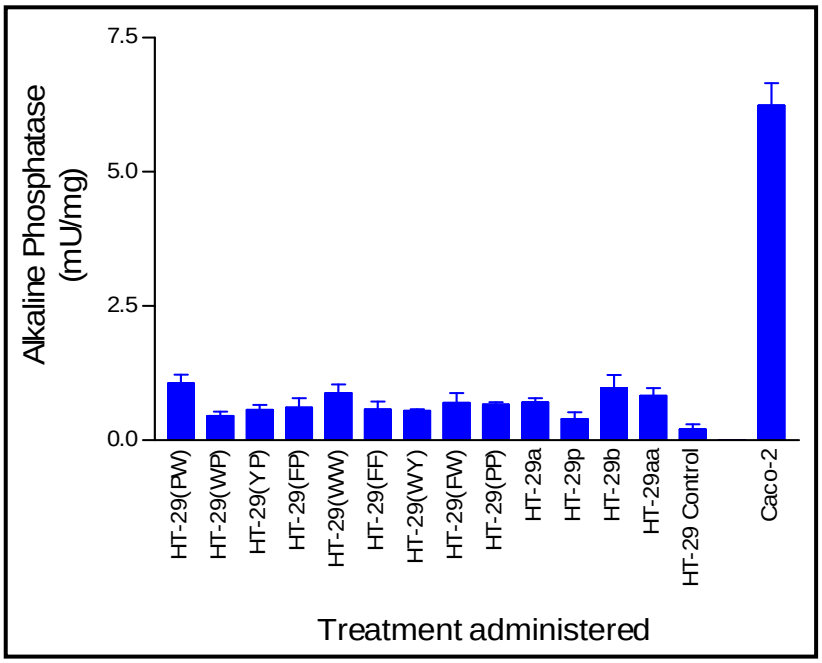


Figure 4.7: Alkaline phosphatase expression in HT-29 and Caco-2 cells showing the effects of PKA inhibition. P = Proline, W = Tryptophan, Y = Tyrosine, F = Phenylalanine. Cyclo refers to the cyclic conformation of the dipeptide.

Results obtained for the ATP levels, pyruvate levels, lactate production, the levels of histone acetylation, histone phosphorylation, levels of CREB phosphorylation, and differentiation marker (CA 1) levels (including PKA-inhibited levels) were compared statistically using Prism software using Pearson-Rank correlations. These comparisons showed an insignificant negative correlation ($r^2 = 0.532$) between ATP levels and pyruvate production which is to be expected since pyruvate is bypassed as an entry point into the TCA cycle. A further insignificant negative correlation ($r^2 = 0.76$) was observed between ATP levels and lactate secretion into the growth medium, which was also to be expected since lactate is no longer shuttled from pyruvate as is the case in undifferentiated HT-29 cells. There was a positive correlation ($r^2 = 0.962$; $p < 0.05$) between ATP levels and histone phosphorylation which supports the hypothesis that the inorganic pyrophosphate groups released on cAMP production from ATP can be used to phosphorylate the histones. An insignificant negative correlation was observed ($r^2 = 0.748$; $p = 0.09$) for ATP levels and histone acetylation. A positive correlation was expected between ATP levels and histone acetylation since this process is ATP-driven. A negative correlation ($r^2 = 0.732$; $p < 0.05$) was found between ATP levels and CREB phosphorylation. The production of ATP would be expected to increases during the differentiative process because of the energy demand of increased metabolic activity during the differentiative process. However this increase is not evident as ATP will be used for protein phosphorylation (such as in CREB), the cAMP cascade, histone phosphorylation (as shown before), and histone acetylation which is an energy-requiring process. The decrease in ATP production which was found corresponds to the decrease in energy charge reported in literature for HT-29 differentiation (Graz and Cowley, 1997). Increases in the levels of cAMP will activate the PKA-dependent phosphorylation of CREB (Cooper, 1997).

There is a direct link between the energy state of a cell and its state or level of differentiation, but what the link is and how it may result in or be the result of differentiation is not known. Energy is needed for the increased metabolic activity required for differentiation. Therefore a increased energy state may indicate a differentiative process (Graz and Cowley, 1997), but cannot be used as a marker of differentiation, because any changes in culture conditions would result in an increased metabolic activity resulting in an increased use of ATP. A positive correlation ($r^2 = 0.865$; $p < 0.05$) between CA 1 expression and CREB phosphorylation, and a negative correlation ($r^2 = 0.762$; $p < 0.05$) between PKA-inhibited levels of CA 1 and CREB phosphorylation were found. This suggests that CA 1 expression is dependent on the phosphorylation of CREB.

Several possible conclusions can be drawn from the results in this study: 1) Carbonic anhydrase I can be used as a lineage specific marker as its expression is inhibited upon inhibition of PKA. Conversely this indicates that alkaline phosphatase which has been used extensively as a marker of intestinal differentiation, is in fact a non-specific marker and should be excluded as a differentiation marker; 2) ATP concentrations may be used as an indicator of the induced differential phenotype; 3) The induction of differentiation in HT-29 cells reported in literature as indicated by the expression of a gastrointestinal phenotype, could be either due to non-specific transcription of genes by activation of the chromatin switch or specific by the activation of signal transduction pathways based on the flux of ATP through the cells. 4) The CDPs are capable of affecting metabolic pathways in the cells, which are linked to the phosphorylation of proteins, which indicates the need for further investigation of these potential drug entities.

Energy metabolism can be seen as a significant role player in gastrointestinal differentiation (Graz and Cowley, 1997) and the metabolites involved indicate potential to be used as markers of differentiation. However, these energy-related metabolic markers would not be lineage-specific. Considering the results for Caco-2 cells the energy-related metabolites are not conclusive as markers for natural differentiation as observed in Caco-2 cells.

CHAPTER 5: ATTEMPT AT OPTIMIZATION OF DIFFERENTIAL DISPLAY TO VALIDATE THE PROPOSED NONSPECIFIC DIFFERENTIAL GENE EXPRESSION IN HT-29 AND CACO-2 CELLS

The observations from the results in chapter 4 suggest that the induced phenotype in HT-29 cells is the result of a nonspecific (generalized) transcription of genes rather than true differentiation. However, the biochemical and molecular markers studied here cannot be used to validate this postulate. The induced phenotype observed in HT-29 cells is expected to be a result of differential gene expression at some point, possibly very early, in the signal transduction pathway which regulates the early transcription events. Hence the differential display technique described by Liang and Pardee (1992) would be a suitable choice to validate the proposal of nonspecific differential gene expression induced in HT-29 cells. The current chapter describes the attempt at optimizing the differential display reverse transcription polymerase chain reaction (DDRT-PCR) for the scope of the present study.

5.1 Introduction to DDRT-PCR

The method described by Liang and Pardee (1992) is directed toward the identification of differentially expressed genes among the approximately 15 000 individual mRNA species in pair of mammalian cell populations, and then recovering their cDNA and genomic clones. The general strategy is to amplify partial cDNA sequences from subsets of mRNAs by reverse transcription and the polymerase chain reaction. The resultant products are then displayed on a sequencing gel. Pairs of primers are selected so that each will amplify DNA

from about 50 to 100 mRNAs because this number is optimal for display on the gel (Shima *et al,* 1995; Wang and Feuerstein 1994; Zhao *et al*, 1995).

Selection of 3' primers for RT-PCR takes advantage of the polyadenylate [poly(A)] tail present on most eukaryotic mRNAs to anchor the primer at the 3' end of the mRNA, plus two additional 3' bases. A primer such as 5' $T_{11}CA$ to mRNAs containing TG located just upstream of their poly(A) tails. By probability this primer will recognize one twelfth of the total mRNA population because there are twelve different combinations of the last two 3' bases, omitting T as the penultimate base. The primer permits initiation of reverse transcription of only this subpopulation (Liang and Pardee, 1992). Any reverse transcribed cDNA species would be amplified by PCR if the distance at which a second primer anneals is smaller than 2 to 3 kb from the beginning of the poly(A) tail (an average molecular size of 1.2 kb). Ideally this annealing position should be within 500 bp because cDNAs up to 500 bp can be resolved by size on a DNA sequencing gel. For a 5' primer of arbitrary base sequence, annaeling positions to cDNAs should be randomly distributed in distance from the ploy(A) tail. Therefore the amplified products from various mRNAs will differ in size. The PCR products may then be displayed by autoradiography as a ladder on a sequencing gel (Liang and Pardee, 1992).

Ross *et al.* (1997) have used the differential display technique to identify differentially expressed genes derived from three-day and freshly isolated Langerhans cells. Lee and Goetz (1998) have used differential display to characterize a differentially expressed mRNA in phorbol ester-stimulated ovarian tissue from the brook trout. Thomson *et al.* (1997) have used the DDRT-PCR technique to identify a product associated with apoptosis in chicken thermocytes.

5.2 Methods

The preparation of reagents and solutions is described in Appendix D.

For the scope of this project it did not appear necessary to do a full differential display using the prescribed kit as this would include reverse transcription of all the possible subsets of mRNAs and thus a display of virtually all the differences arising due to differentially expressed genes. It was preferable to use only one anchored primer which would permit the reverse transcription of only that subpopulation of mRNA for which it is specific. Thus we chose to use various commercial consumables to perform the differential display. The anchored primer used was an oligod$(T)_{15}$ obtained from Boehringer Mannheim(Germany). The methodology involved various trial experiments incorporating a number of combinations of variable parameters including annealing and extension temperatures, magnesium and primer concentrations, primer combinations, nucleotide concentration as well as template concentrations. For all experiments the PCR products were separated on mini denaturing polyacrylamide gels (7.5%) at 110 Volts and 50mA The gels were then silver stained to examine the reults.

The mRNA was isolated from undifferentiated HT-29 cells at confluence using an mRNA isolation kit obtained from Boehringer Mannheim. The kit isolation procedure was based on the principle of magnetic bead separation and was performed according to the manufacturers instructions. For the initial trial experiments the Promega (Madison, USA) access RT-PCR system was used to perform both the reverse transcription and PCR steps as a single step process in the one reaction tube. The reaction conditions are described in

Table 5.1. and in the text below. A positive control using control RNA and primers supplied as part of the kit was included for all experiments.

Table 5.1: Reaction components for trial experiments. AMV refers to Avian Myeloblastosis Virus as the source of the reverse transcriptase. The *Tfl* refers to the thermostable DNA polymerase from *Thermos flavus*. Upstream primer = Oligod$(T)_{15}$ and downstream primer = abitrary decamer.

Reaction component	**Volume**	**Final concentration**
Nuclease-free water	13.5 µl	
AMV/*Tfl* 5x reaction buffer	5 µl	1x
dNTP mix (10 mM each)	0.5 µl	0.2mM
Downstream primer	1 µl	1µmol
Upstream primer	1 µl	1µmol
25 mM $MgSO_4$	1 µl	1mM
AMV reverse transcriptase	0.5 µl	0.1u/µl
Tfl DNA polymerase (5u/µl)	0.5 µl	0.1u/µl
RNA template	2 µl	0.5µg
Final volume	25µl	

The outline for the procedure of RT-PCR is described below.

Phase 1 1 cycle **First strand cDNA synthesis**
48°C for 45 minutes reverse transcription

Phase 2 1cycle 94°C for 2 minutes AMV RT inactivation and RNA/cDNA/primer denaturation

Phase 3 40 cycles **Second strand cDNA synthesis and PCR amplification**
94°C for 30 seconds denaturation
37°C for 1 minute annealing
68°C for 2 minutes extension

Phase 4 1cycle 68°C for 7 minutes final extension

The initial trial experiments performed under the conditions described above did not yield any significant results in terms of amplification products, hence the images of the gels were not included in this document. The following section describes the steps taken to optimize the system further and the results which were obtained.

5.3 Further optimization and corresponding results

For all subsequent exepriments the outlined procedure for RT-PCR described above was divided into two separate stages. Firstly the reverse transcription was performed with reaction components as described in Table 5.1 with the exclusion of the downstream primer. The second stage (PCR) was performed using an aliquot (containing cDNA) from the first reaction and both downstream and upstream primers were included in the reaction mixture. *Tfl* DNA polymerase was replaced by Taq DNA polymerase as the Taq enzyme is known to be more processive for PCR reactions. Conseqently the appropriate PCR reaction buffer and $MgCl_2$ were used in the second reaction. In addition, the number of cycles for Phase 3 in the outlined procedure was changed to 5 cycles at 30°C and 35 cycles at 40°C to allow low-stringency primer annealing in order to obtain a more representative amplification. The first positive result was obtained with the optimization of magnesium concentration (Figure5.1). Reaction components for the second stage of this experiment are shown in Table 5.2.

Table 5.2: Reaction components for the second stage of RT-PCR. Five different magnesium concentrations were tested including 1.5mM, 1.75mM, 2mM, 2.5mM, and 5mM.

Reaction component	**Volume**	**Final concentration**
Nuclease-free water	Variable	
10x reaction buffer	7.5 µl	1x
dNTP mix (10 mM each)	0.5 µl	0.2mM
Downstream primer	1 µl	1µmol
Upstream primer	1 µl	1µmol
$MgCl_2$	Variable	Variable
Taq DNA polymerase (1u/µl)	1.25 µl	0.0625u/µl
RNA template	2 µl	0.5µg
Final volume	25µl	

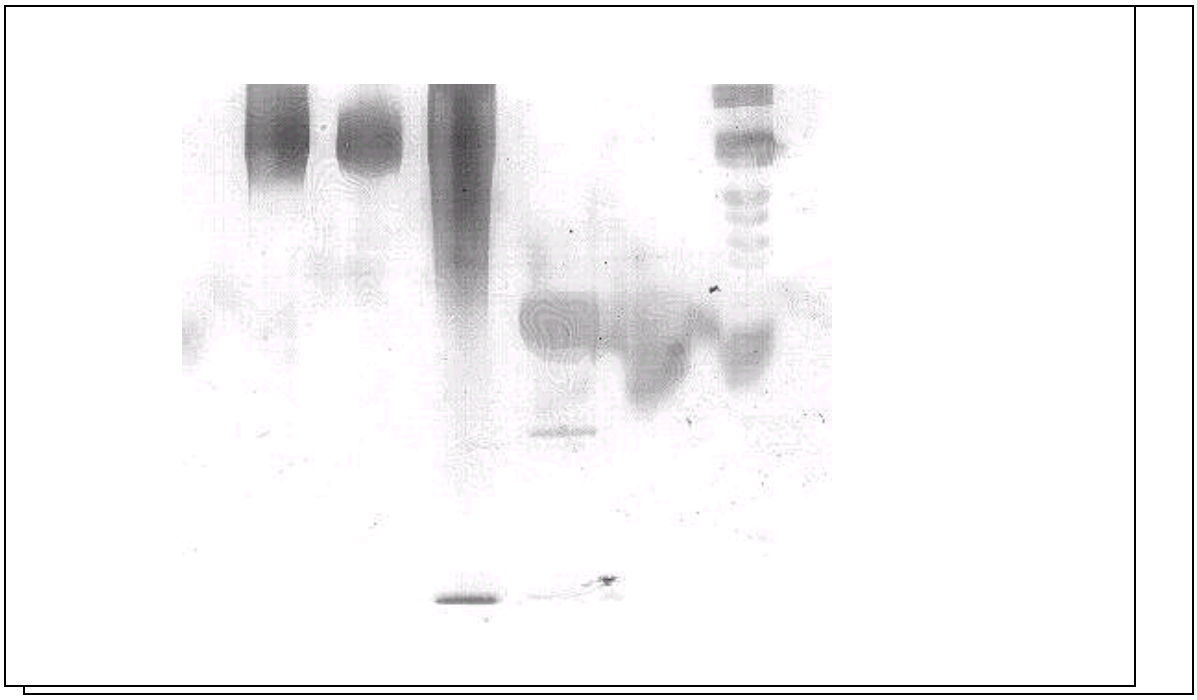


Figure 5.1: Silver-stained SDS-PAGE gel showing resultant amplifications of cDNA using varying concentrations of magnesium. The best result (lane 2) was obtained with 5mM magnesium. Lane 3 = 2.5mM, lane 4 = 2mM, lane 5 = 1.75mM, and lane 6 = 1.5mM. DNA molecular weight marker is in lane 7.

The magnesium optimization showed 5mM to be the optimum magnesium concentration for PCR amplification and was hence used in all subsequent experiments with reaction components as described in Table 5.2.

Brenz Verca et al. (1998) reported an improvement in the PCR amplification of cDNA using longer downstream primers of 25 base pairs (bp). Hence the following attempt was to use a longer primer (23bp in length) which was included as a control primer in the RT-PCR kit obtained from Promega. The result for the above exepriment (Figure 5.2) shows an improved amplification as compared to the reaction in which the 10bp primers were utilized as the downstream primers.

Figure 5.2: Silver-stained SDS-PAGE gel showing a positive result obtained using a 23bp downstream primer (lane 3) as opposed to 10bp primer combinations shown in lanes 1 and 2. The positive control is shown lane 4 and the DNA molecular weight marker is in lane 6.

The above experiment was repeated with the inclusion of a precipitation step following the reverse transcription reaction. The precipitation step (see Appendix D for details) was incorporated in order to remove any potential inhibitory reactants from the previous step. The precipitation proved to be effective as was indicated by an increase in the amplification of smaller fragments of cDNA (Figure 5.3).

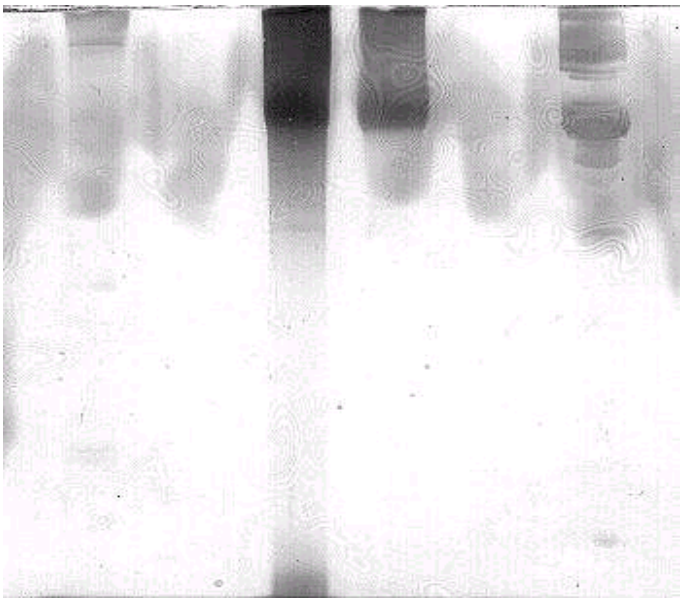
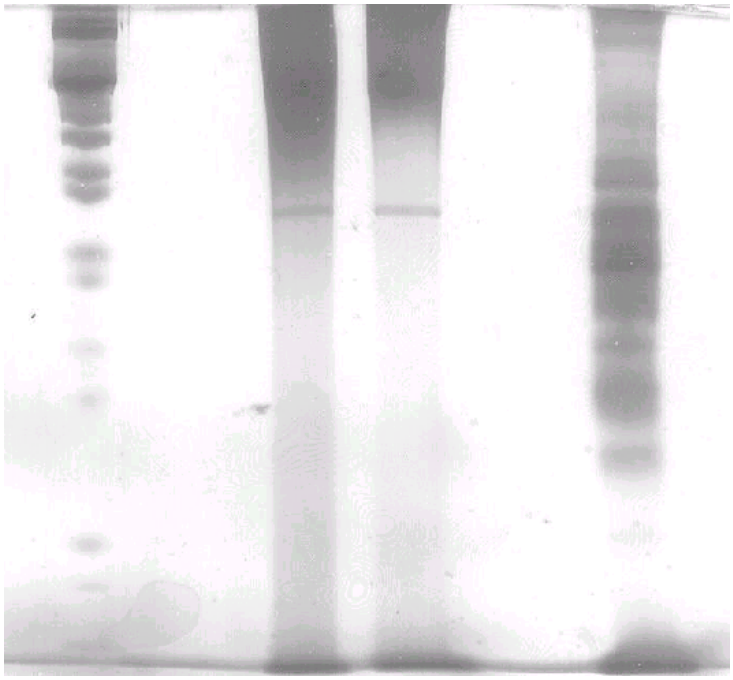


Figure 5.3: Silver-stained SDS-PAGE gel showing enhanced amplification as a result of ethanol precipitation of first strand cDNA synthesis products. The positive test result using the 23bp downstream primer is shown in lane 3. There are some faint bands visible below the dense smear at the top of lane 6. Lane 4 is that of the 10bp primer reaction showing comparitively less amplification product. The DNA molecular weight marker is in lane 1 and the positive control is in lane 6.

The next step in the process of optimization was to include a second longer primer (22bp) (Promega) to be used in combination with the 23 bp primer of the previous experiment. The PCR products of a reaction prepared as in the previous experiment using only the single 23bp primer was displayed on on the same gel. The result is shown in Figure 5.4. This was the best result obtained for RT-PCR showing faint amplification of numerous bands including very small fragments down to 100bp. A distinct band at approximately 600bp was amplified in both reactions indicating the potential for reproducibility within the system.

Figure 5.4: Silver-stained SDS-PAGE gel showing the PCR amplification products of a reaction obtained utilizing a combination of a 23bp and 22bp primer (lane 3). The reaction utilizing



only the 23bp primer is shown in lane 4. The result is very similar for the two reactions showing the amplification of a distinct band of approximately 600bp. The DNA molecular weight marker is in lane 1 and the positive control is in lane 6.

In a final attempt at DDRT-PCR the products of the reverse transcription step were subjected to a ribonuclease digestion to eliminate the possibilty of interference from template mRNA. The upstream and downstream primer concentrations were doubled for the same reason. An extra reaction was included using the 10bp primer used earlier. The results are shown in Figure 5.5.

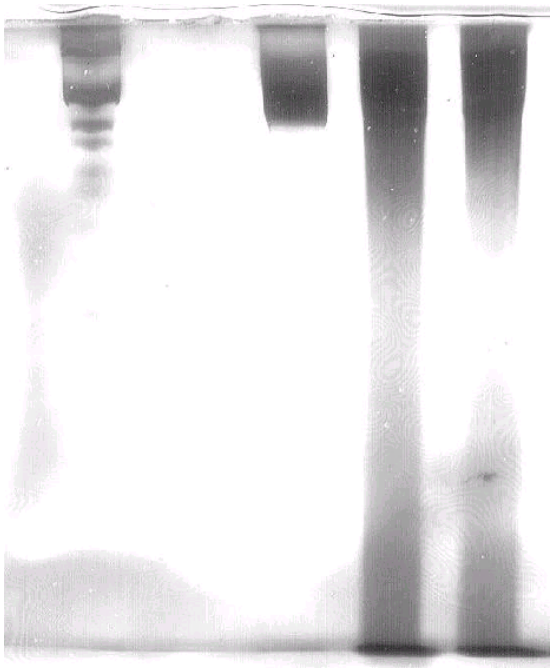


Figure 5.5: Silver-stained SDS-PAGE gel of the PCR amplification products of ribonuclease-digested first strand cDNA, synthesized by reverse transcription. Lane 1 = DNA molecular weight marker. Lane 3 shows the10bp primer reaction product. Lanes 4 and 5 shows the positive results of reactions using the 23bp and a combination of the 23bp and 22bp primers respectively.

5.4 Discussion

Subsequent to the experimental worked described above it was later learnt by personal communication with Miss M. Appel (Department of Biochemistry, University of Stellenbosch) that from months of personal experience with the DDRT-PCR techniques, the only system which provided the desired results was that obtained by using the original full differential display system as prescribed by Liang and Pardee (1992). This system allows for the full display of differentially expressed genes in a number of different species as

mentioned earlier and has a high success rate. Apparently all attempts at DDRT-PCR performed with kits other than the full system mentioned above have failed.

The results obtained in this study were positive and indicate the potential for validating the proposal of a non-specific (generalized) transcription of genes as induced in the HT-29 colonic carcinoma cell line. The methods used here did not yield the full differential display comparing induced to uninduced cells as would be achieved using the prescribed full DDRT-PCR system. However, obtaining a full differential display was beyond the scope of this research. Therefore under the circumstances the present study was succesful in achieving a positive result.

CHAPTER 6: FINAL CONCLUSION

The biochemical markers studied were shown to be effective as markers of the reported differentiation in HT-29 and Caco-2 colonic carcinoma cells allowing comparison of maturation induction between both HT-29 and Caco-2 cells. It was established that the closest correlation ($p < 0.05$) for the state of differentiation within the two cell lines was on days 4 and 12. Further the biochemical markers are applicable for both physiological inducers (i.e. the SCFAs) and non-physiological inducers (i.e. the cyclic dipeptides). The markers, however, are not specific for gastrointestinal gastrointestinal differentiation with the exception of carbonic anhydrase 1. Results from Chapter 4 (Figure 4.6) have shown that cAMP-dependent PKA causes a marked inhibition of carbonic anhydrase 1 in HT-29 cells treated with physiological and non-physiological inducers. Alkaline phosphatase expression was proven to be significantly inhibited indicating that alkaline phosphatase was nonspecific as a marker. Caoc-2 cells were unaffected by treatment with CDPs or by PKA inhibition indicating that an independent pathway of induction occurs in the spontaneously differentiating cell line.

Energy metabolism was found to be a significant role player in gastrointestinal differentiation as induced by both physiological and non-physiological inducers. The relevant energy metabolites can therefore be used as indicators of differentiation, but not as lineage-specific markers. Further, the energy-related metabolic markers are not conclusive as markers of natural differentiation as induced by contact inhibition in Caco-2 cells.

The results obtained (chapter 4) suggest that induction of differentiation in HT-29 cells could either be due to nonspecific transcription of genes by activation of the chromatin switch or specific by the activation of signal transduction pathways based on the flux of ATP through the cells. Differential display RT-PCR described in chapter 5 (Liang and Pardee, 1992) is probably the most sensitive method that could be used to validate the suggestion of either a nonspecific transcription of genes or specific differentiation as reported for HT-29 cells.

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APPENDIX A

All solutions were prepared with MQ water : MilliQ water (MilliQ Plus Millipore, Molsheim, France)

DMEM:

13.44g DMEM powder was dissolved in 1l H_2O. Once dissolved, 3.7g $NaHCO_3$ (Merck, Darmstadt, Germany) was added. The media was filtered through a 0.22μm filter (Micron Separations Inc., Elabtec, Port Elizabeth). 2ml pyruvate and 1ml Penicillin/Streptomycin were added to 500ml media. 5ml 50mg/ml glutamine (Sigma Chemicals, U.S.A) was also added to compensate for glutamine degradation within the standard medium.

INDUCER STOCK SOLUTIONS:

0.5M Sodium acetate (Sigma, St. Louis, U.S.A):

0.62g sodium acetate was dissolved in 15ml H_2O, and the pH was adjusted to pH 7.5 with 1M HCl.

0.5M Butyric Acid (BDH Chemicals, Poole, England):

0.688ml was dissolved in 15ml H_2O and the pH was adjusted to 7.4 with 1M NaOH

0.5M Acetoacetic Acid (Sigma, St. Louis, U.S.A.):

0.81g acetoacetic acid was dissolved in 15ml H_2O and the pH was adjusted to 7.4 with 1M NaOH.

0,5M Sodium Propionate (BDH Chemicals, Poole, England):

0.72g sodium propionate was dissolved in 15ml H_2O and the pH was adjusted to 7.4 with 1M HCl.

The stock solutions of the inducers were filter sterilised through a 0.22μm filter unit (Micron Separations Inc., Elabtec, Port Elizabeth, South Africa). For the 5mM solutions, 0.5ml of the stock solutions were added to 45ml DMEM and 5ml FCS.

Phosphate-buffered saline:

8g NaCl, 0.2g KH_2PO_4 (Protea Laboratory Services, Johannesburg, South Africa), 1. 44g $Na_2HPO_4.12H_2O$ (Unilab Saarchem, Chamdor, Krugersdorp), 0.2g KCl (Unilab, Saarchem, Krugersdorp), 0,2gEDTA were added to 900ml H_2O, the pH adjusted to 7.4 and the volume was adjusted to 1l.

Trypan Blue:

0.01g Trypan Blue was added to 100μl glycerol, which was then diluted in 900μl H_2O.

Solutions for Alkaline Phosphatase assays:

Alkaline phosphatase for standard :

0.00179mg alkaline phosphatase was dissolved in 1ml H_2O; this represented 700mU/ml. This stock solution was diluted 10X to obtain a 70mU/ml solution. This was serially diluted to obtained the concentrations needed for the standard curve.

1mM $MgCl_2$:

0.010165g $MgCl_2$ was dissolved in 50ml H_2O.

Glycine Buffer:

0.3754g Glycine was dissolved in 40ml H_2O, pH to 10.5 with 1M NaOH; 0.010165g $MgCl_2$ and 0.6814g $ZnCl_2$ were added and the volume was adjusted to 50ml.

0.03M *p*NPP:

0.05565g *p*NPP was dissolved in 5ml H_2O

Solutions for Carbonic anhydrase I assays:

Carbonic Anhydrase I for standard curve:

0.1mg enzyme was dissolved in 2ml H_2O, to give a 100U/ml stock, which

was subsequently serially diluted to obtain the concentrations needed for the standard curve.

1% Bromothymol Blue:

1g bromothymol blue was dissolved in 100ml H_2O

0.022M Veronal:

0.2818g Barbituric acid was dissolved in 100ml H_2O

0.022M Veronal Salt:

0.4536g Sodium Barbiturate was dissolved in 100ml H_2O.

Solutions for nuclei extractions:

CMFSW- Ca^{2+}, Mg^{2+}-free saline water:

28g NaCl, 0,8g KCl (Unilab, Saarchem, Krugersdorp) and 0.2g NaHCO in 1/ H_2O

0.5M sucrose, 10mM Tris pH 7.5:

1.21g Tris and 171.15g sucrose were dissolved in 900ml H_2O and the pH was aadjusted to 7.5 with 1M HCl. The volume was then adjusted to 1l.

20mM NaCl, 10mM $MgCl_2$, 20mM Tris pH 7.5; 1% Triton X 100 (BDH Chemicals, Poole, England):

1.17g NaCl, 2.42g Tris, 10ml Triton X 100 and 0.95g $MgCl_2$ were mixed in 900ml H_2O. The pH was adjusted to pH 7.5 with 1M HCl and the volume was adjusted to 1l.

0.25M sucrose, 10mM NaCl, 5mM $MgCl_2$, pH 7.5:

85.575g sucrose, 0.584g NaCl and 0.48g $MgCl_2$ were dissolved in 900ml H_2O after which the pH was adjusted to 7.5 with 1M HCl. The volume was brought up to 1l.

0.1mM Tris, 2mM $MgCl_2$, 0.25M sucrose, pH 7.4:

0.0121g Tris, 0.19g $MgCl_2$ and 85.575g sucrose were dissolved in 900ml water, and the pH was adjusted to 7.4 with 1M HCl. The volume was then adjusted to 1l.

Solutions for Histone extractions:

Nuclear disruption buffer:

10µl 1M Tris-HCl (pH 7.5), 26µl 0.5M EDTA, 5.2mg sodium bisulfite and 20µl 1M NaBut were mixed and H_2O was added to a final volume of 1ml.

Chromatin Wash Buffer:

10µl 1M Tris-HCl (pH 7.5), 28µl 5M NaCl, 26µl 0.5M EDTA, 5.2mg sodium bisulfite and 20µl 1M NaBut were mixed and H_2O was added to a final volume of 1ml.

1M Sodium Butyrate:

0.011009g of sodium butyrate was dissolved in 100µl 10mM Tris-HCl (pH 7.5) and stored at -20°C.

1M Tris-HCl pH 7.5:

1.2114g Tris was dissolved in 9ml H_2O, the pH was adjusted to 7.5 with 1M HCl and the volume adjusted to 10ml.

5M NaCl:

2.922g NaCl was dissolved in 10ml H_2O

Solutions for CREB-Ser 133 detection:

0.1mM Forskolin:

0.001g forskolin was dissolved in 24.36µl H_2O, which was then diluted in 24.11ml DMEM.

3%paraformaldehyde:

45µl of *para*formaldehyde was added to 1.5ml PBS, heated to 65°C

for a few minutes, after which it was cooled on ice. This solution was prepared prior to usage.

10X Tris-buffered saline (TBS):

24.2g Tris base, 80g NaCl were dissolved in 900ml. The pH was adjusted to 7.6 with HCl and the volume was adjusted to 1l.

TBST:

To 1xTBS, 0.1% Tween-20 was added.

Blocking buffer:

10ml 10X TBS was added to 90ml H_2O, 100µl Tween-20 (Serva, Feinbiochemica, Heidelberg, New York), and 5g non-fat dry milk (Protea) was added.

Primary antibody dilution buffer:

2ml 10X TBS was added to 18ml H_2O, 1g BSA and 20µl Tween-20 were added.

Phospho-specific CREB antibody was used at a 1:1000 dilution

Secondary antibody solution:

1µl of Anti-Rabbit IgG-AP conjugate was added to 1ml blocking buffer.

BCIP/NBT:

80µl BCIP/NBT stock solution was dissolved in 10ml 100mM Tris-HCl, 100mM NaCl, 50mM $MgCl_2$, pH9,5.

APPENDIX B

Parameters for detection of ^{14}C-acetate

Radioctivity was detected as counts per minute in duplicate

% 2 Sigma = 2.00

Time = 5 minutes

QIP = SIS

Parameters for detection of ^{32}P

Radioctivity was detected as counts per minute in duplicate

% 2 Sigma = 2.00

Time = 5 minutes

QIP = SIS

APPENDIX C

Reagents and gel preparations for SDS-PAGE slab gels used in PCR amplification analysis (Laemmli buffer system)

Stock solutions

A. Acrylamide/bis (30% T, 2.67%)
87.6 g acrylamide (29.2 g/100ml)
2.4 g N'N'-bis-methylene-acrylamide (0.8 g/100ml)
Constitute to 300ml with MilliQ water. Filter and store at 4°C in the dark (30 days maximum)

B. 1.5 M Tris-HCl, pH 8.8
18.15 g Tris base / 100ml
80 ml ddH_2O

Adjust to pH 8.8 with 1N HCl. Constitute to 100 ml and store at 4°C

C. 0.5 M Tris-HCl, pH 6.8
6 g Tris base
60 ml ddH_2O

Adjust to pH 6.8 with 1N HCl. Constitute to 100 ml and store at 4°C

D. 10 % SDS
Dissolve 10 g SDS in water with gentle stirring constitute to 100 ml with MilliQ water

E. 5X Electrode (Running) Buffer, pH 8.3 (enough for 10 runs)

Tris base	9 g	(15 g/l)
Glycine	43.2 g	(72 g/l)
SDS	3 g	(5 g/l)

constitute to 600 ml with MilliQ water.
Store at 4°C.

Dilute 80 ml E. with 320 ml of MilliQ water for one electrophoresis run.

Preparation of 7.5% Resolving Gel

MilliQ water	4.85 ml
Soln B	2.5 ml
Soln D	100 µl
Soln A	2.5 ml
TEMED	5 µl
10 % NH_4 Persulfate	50 µl

Preparation of 4% Stacking Gel

MilliQ water	6.1 ml
Soln B	2.5 ml
Soln D	100 µl
Soln A	1.3 ml
TEMED	10 µl
10 % NH_4 Persulfate	50 µl

Procedure for gel silver staining

1) **Fix solution:** ***(30 minutes)***

   10% (w/v) Ethanol

   5% (w/v) Acetic acid

2) **Oxidiser solution:** ***(10 minutes)***

   0.25g K_2CrO_7 / 250ml

   57 µl Nitric acid (55%)

3) **Water:** ***(15 minutes)***

   Until yellow colour has diappeared

4) **Silver solution:** ***(10 minutes)***

   0.2g Silver nitrate / 100ml

5) **Water:** ***(1minute)***

   wash away the remaining silver to prevent overstain

6) **Developer solution:** ***(until bands are visible)***

20g Na_2CO_3 . $10H_2O$ / 250ml

375µl Formaldehyde

1 small grain Sodium thiosulfate

7) **Water:** ***(a very quick wash ~ 30 seconds)***

To remove residual developer solution

8) **Stop solution:** ***(30 minutes)***

5% (w/v) Acetic acid