BIOCHEMICAL INVESTIGATIONS ON RAT SMALL INTESTINAL EPITHELIUM

Mitochondrial Protein Synthesis and Antibiotics
Cyclic Nucleotide Regulation and Function

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT TE ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. P.W. KLEIN
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN
DE OPENBARE VERDEDIGING ZAL PLAATS VINDEN OP
WOENSDAG 10 SEPTEMBER 1975
DES NAMIDDAGS TE 3.00 UUR PRECIES

DOOR

HUGO RÜDOLPHE DE JONGE

GEBOREN TE EINDHOVEN

1975
BRONDER OFFSET B.V. – ROTTERDAM

PROMOTOR:	PROF. DR. W.C. HÜLSMANN
CO-REFERENTEN:	PROF. DR. A.M. KROON
	PROF. DR. M. FRENKEL

CONTENTS

		page
CHAPTER I	GENERAL INTRODUCTION	9
CHAPTER II	MITOCHONDRIAL PROTEIN SYNTHESIS AND ANTIBIOTICS	11
	2.1 Introduction	11
	2.2 Some structural and functional properties of mitochondria from rat small intestinal epithelium	14
	2.3 Structure and function of intestinal mitochondria after antibiotic treatment	15
	2.4 Biochemical targets of the antibiotics in mammalian cells, not related to mitochondrial protein synthesis	19
	2.5 Concluding remarks	20
CHAPTER III	CYCLIC AMP AND FLUID TRANSPORT	22
	3.1 Introduction	22
	3.2 The mechanism of action of choleratoxin	23
	3.3 Cyclic AMP and the mechanism of fluid secretion	26
	3.4 Intestinal secretion evoked by other secretagogues	29
	3.5 Concluding remarks	32
CHAPTER IV	FORMATION AND FUNCTION OF CYCLIC GMP IN SMALL INTESTINAL EPITHELIUM	34

page

4.1 Introduction 34
4.2 Formation of cyclic GMP in a number of tissues other than small intestinal epithelium 35
4.3 Formation of cyclic GMP in small intestinal epithelium 38
4.4 Levels of cyclic GMP in epithelial cells, isolated from rat small intestine 41

CHAPTER V CYCLIC NUCLEOTIDES IN RELATION TO PROLIFERATION AND DIFFERENTIATION 43

5.1 Introduction 43
5.2 Growth and differentiation in small intestinal epithelium 45
5.3 Methods 48
5.4 Results 50
5.5 Conclusions and discussion 61

REFERENCES 67

SUMMARY 76

SAMENVATTING 81

CURRICULUM VITAE 91

APPENDIX PAPERS 93

1. Inhibition of Mitochondrial-Protein Synthesis in Rat Small-Intestinal Epithelium.
Hugo R. de Jonge and W.C. Hülsmann (1973) Eur. J. Biochem. 32, 356-364

2. Toxicity of Tetracyclines in Rat-Small-Intestinal Epithelium and Liver.
Hugo R. de Jonge (1973) Biochem. Pharmac. 22, 2659-2677

3. Adenylate Cyclase Activities and Adenosine 3',5'-Cyclic Monophosphate Concentration in Villus and Crypt Cells of Rat Small-Intestinal Epithelium. Response to Cholera Toxin, Prostaglandin E_1 and Hormones.
H.R. de Jonge and W.C. Hülsmann (1974) Biochem. Soc. Transact. 2, 416-419

4. The Response of Small Intestinal Villous and Crypt Epithelium to Choleratoxin in Rat and Guinea Pig. Evidence against a specific role of the crypt cells in choleragen-induced secretion.
H.R. de Jonge (1975) Biochim. Biophys. Acta 381, 128-143

5. The Localization of Guanylate Cyclase in Rat Small Intestinal Epithelium.
H.R. de Jonge (1975) FEBS Lett. 53, 237-242

6. Properties of Guanylate Cyclase and Levels of Cyclic GMP in Rat Small Intestinal Villous and Crypt Cells.
H.R. de Jonge (1975) FEBS Lett. 55, 143-152

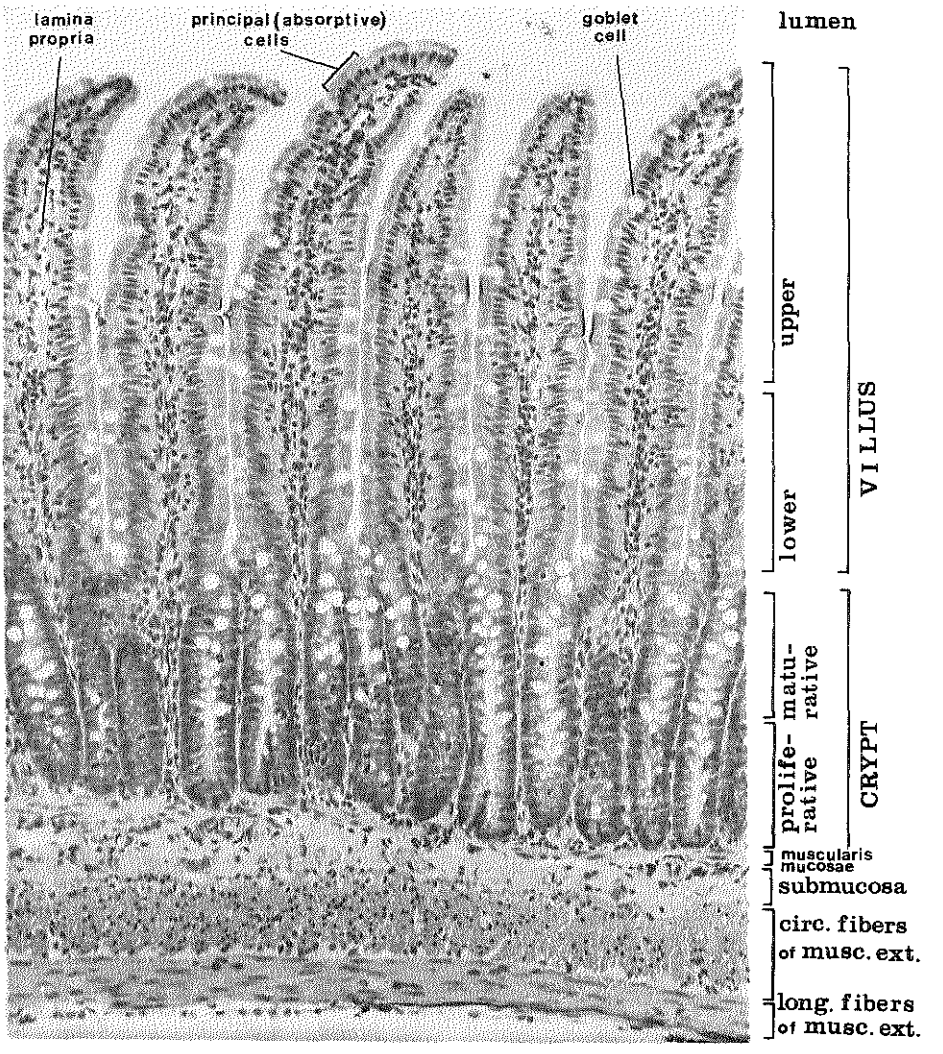

Fig 1.1 Small intestinal morphology

CHAPTER I GENERAL INTRODUCTION

Gastrointestinal disorders form an important category of diseases with an often obscure etiology or biochemical basis. Extension of the fundamental knowledge about structure and function of the enterocyte is essential for a better understanding of the intestinal pathophysiology and may probably permit a more rational approach to prevention or therapeutic treatment of these diseases.

In the small intestinal epithelium, absorptive villous cells continuously arise by rapid proliferation and differentiation of stem cells in the crypts of Lieberkühn (see Fig. 1.1). A drastic change in cell kinetics is frequently associated with gastrointestinal disfunction. Inhibition of biosynthetic processes in the crypt cell by way of irradiation or chemical inhibitors (colchicine, nitrogen mustard, cycloheximide) does not only influence the rate of cell renewal but may also affect cell maturation and the number and functional state of mature villous cells. One class of inhibitors (but seldom recognized in this quality) consists of two important groups of antibiotics, the tetracyclines and chloramphenicol with its derivatives. It is now commonly accepted that mitochondria contain an autonomous protein synthetizing system, possessing a high sensitivity for these inhibitors of bacterial protein synthesis. The intestinal crypt cell may be considered as an excellent target for this type of antibiotics. The first part of this thesis (Chapter II) is devoted to a biochemical study of the consequences of antibiotic treatment for small intestinal structure and function. The clinical experience that gastrointestinal complications are extremely rare during therapeutic application of these antibiotics has been made more understandable by the results of this study.

Pathological changes of gastrointestinal functions may also arise from a direct influence of toxic agents on the

non-proliferative epithelial cells. This is most clearly demonstrated in the case of diarrhoeal diseases in which the absorptive function of the intestine is disturbed by hormonal serum factors, bacterial toxins or bacterial invasion (dysentery). Investigation of the biochemical alterations in the cell in this situation would not only give a deeper insight in the regulation of absorptive and secretory processes in the epithelium but might also reveal a common basis for apparently different clinical syndromes. The recognition of cyclic AMP as the intracellular mediator of choleratoxin action was the first indication that also in this tissue cyclic nucleotides may function as important regulators of biological functions. The second part of this thesis (Chapters III-V) deals with some aspects of cyclic nucleotide formation and regulation in the small intestinal epithelium in relation to its absorptive and proliferative function. This study clearly demonstrates the advantages of using relatively homogeneous cell preparations from crypt and villous regions. Some basic properties of these isolated cells are discussed in ref. 1. An important feature of the intestinal epithelial cell is the clearcut distinction of luminal and basal-lateral plasma membranes. Both membrane fractions from enterocytes can be easily separated and the study of the biochemical differences between the two (e.g. in enzyme composition: see Papers 3-6) may contribute to the knowledge of membrane polarity in general.

CHAPTER II MITOCHONDRIAL PROTEIN SYNTHESIS AND ANTIBIOTICS

2.1 Introduction

The use of antibiotics as therapeutic agents is based upon some fundamental structural and functional differences between prokaryotic (bacterial) and eukaryotic (mammalian) cells. A number of widely used antibiotics (chloramphenicol, streptomycin and neomycins, lincomycin, macrolides, tetracyclines) exert their antimicrobial activity by inhibiting bacterial protein synthesis on the 70S-ribosomes. Cytoplasmic 80S-ribosomes in eukaryotic cells are almost completely (chloramphenicol) or relatively (tetracyclines) insensitive to their action. The mitochondrial protein synthesis in the animal cell however, carried out on 55S-ribosomes[2] is as much sensitive to the antibiotics as the bacterial system. The sensitivity for erythromycin and most of the other macrolides is latent because the inner membrane of intact mammalian mitochondria or the plasma membrane of the cell serves as a permeability barrier[3]. Unfortunately, a similar protection does not exist against chloramphenicol and tetracyclines. Although these compounds may be of great value in studies concerning the development and function of mitochondrial protein synthesis, their clinical use may harbour some potential hazards, because most of the mitochondrial translation products are essential components of the mitochondrial respiratory chain (see Section 2.3). A depletion of these components during antibiotic treatment is to be expected first in those cell types in which the biogenesis of mitochondria or mitochondrial constituents proceeds at a high rate: proliferating cells (neoplastic cells, regenerating liver, erythroid precursor cells, basal layers of epidermis, intestinal crypt cells) or non-dividing cells with a relatively high turnover or differentiation of mitochon-

drial proteins (e.g. villous epithelium, see Paper 1). Pioneering work of Firkin and Linnane (1968)[4] has shown that a drastic decrease of the level of mitochondrial translation products in cultured Hela cells grown for two generations in the presence of CAP (20 µg/ml) was accompanied by a large inhibition of cellular respiration and cell growth. In subsequent studies by the same authors[5] and, independently, by de Vries and Kroon[6], using the regenerating liver of rats as an in vivo model, it appeared impossible to evoke a similar drastic change in mitochondrial enzyme composition and function as in Hela cells because of the restricted number of cell divisions needed for regeneration (<2). As pointed out in the Introduction of the Papers 1 and 2, the small intestinal epithelium of adult rats seemed a superior model due to the presence of continuous and rapid cell divisions in the crypt and the differential development and turnover of mitochondrial components in the next stage of cell life. If it would be possible to inhibit the mitochondrial protein synthesis in the proliferating crypt cells by antibiotic treatment in vivo as efficaciously as in Hela cell cultures, an energy crisis in this cell type might be expected to occur as early as 1-2 days (corresponding with 2-3 cell divisions) following first contact to the antibiotics or even earlier if the mitochondrial energetic function in the crypt cells would be more sensitive to a partial loss of mitochondrial translation products than in Hela cells.

The first aim of our studies was to characterize the products of mitochondrial protein synthesis in rat small intestinal epithelium and to investigate the consequences of a continuous inhibition of their synthesis for the mitochondrial energetic function in this tissue. Secondly, we hoped to get more insight into the relative importance of mitochondrial and extra-mitochondrial energy production for the energy-demanding functions of the epithelium in vivo. In previous studies from this laboratory it had been

found that epithelial cells in vitro, isolated by means of the high-frequency vibration technique of Harrison and Webster, as well as everted pieces of intestine, displayed a high rate of aerobic glycolysis and missed a Pasteur effect[7]. Similar phenomena were also observed with mucosal sheets[8], everted sacs or rings[9] and small intestinal pieces[10] from adult rats in other laboratories. An explanation of this behaviour by assuming an uncoupled or "loosely" coupled state of oxidative phosphorylation, as suggested by earlier experiments with isolated mitochondria[7], could be excluded by our subsequent finding that tightly coupled mitochondria could be liberated from the isolated cells by using a mild homogenization procedure (see Paper 2). A strong resemblance seemed to exist between the metabolism of intestinal epithelium and that of cultured neoplastic cells, also characterized by a high rate of aerobic glycolysis in the presence of normal functioning mitochondria[11]. For this reason too, it seemed interesting to know whether this pattern of energy metabolism could be considered as an artifact of isolation or as a typical property of the epithelial cell in vivo, possibly related to its absorptive function[12]. One way to determine the importance of extra-mitochondrial energy production in vivo seemed to be the induction of a mitochondrial deficiency in the epithelium by a pretreatment with the antibiotics followed by a study of the various functions of the cell (proliferation, differentiation, absorption) under these conditions.

The results described in Paper 2 explain why this aim has never been reached: even prolonged antibiotic treatment was not able to impair the mitochondrial energetic function. However, the development of a new perfusion technique for rat intestine in situ has solved the question[13]. It is now clear that the epithelium in situ displays a normal Pasteur effect and low rates of lactate production[13]. The high level of glycolysis in vitro probably arises from two factors: tissue hypoxia, as has been shown for everted sacs[14] and other preparations in which the submucosal musculature and connective tissue is still present[15], and the stimulation of energy-consuming repair processes or ion pumps due to a slight tissue damage during the isolation (isolated cells). Both factors lower the energy charge of the cell to such a level that the glycolytic pathway functions at optimal rates (compare ref. 13).

2.2 Some structural and functional properties of mitochondria from rat small intestinal epithelium

The difference spectrum shown in Paper 1 indicates that mitochondria from villous cells contain quantities of the cytochromes aa_3, b and c and of flavoproteins comparable to those of other epithelial tissues[16]. There exists a differential development of some of these proteins (cytochromes aa_3 and c[18]) during the transition from crypt to villus. In contrast, matrix enzymes (e.g. glutamate dehydrogenase) and outer membrane markers (e.g. monoamine oxydase) are kept at constant levels during cell maturation. Isolated villous cell mitochondria oxidize pyruvate, β-hydroxybutyrate and glutamate with

appreciable rates (Paper 2). Malate could be oxidized in the presence of citrate and vice versa (unpublished results). This gives a strong indication for the existence of a tricarboxylate carrier in the enterocyte, previously found in liver[19]. Palmitate (in the presence of malate, ATP, CoASH and carnitine) could not further stimulate the endogenous respiration of the mitochondria. Addition of palmitoylcarnitine gave no better results (unpublished). Isolated cells may oxidize added fatty acids but with appreciably low rates[1] . Most probably, fatty acids may be used in the intact cell as endogenous substrates in the absence of sufficient amounts of aminoacids. However, glutamine and glutamate (but no other aminoacid) are the preferential substrates for the intestinal mucosa in vivo [15,20]; the intestine is a major site for metabolism of glutamine released by other tissues[20].

2.3 Structure and function of intestinal mitochondria after antibiotic treatment*

Inhibition of mitochondrial protein synthesis by chloramphenicol (CAP) and oxytetracycline (OTC) led to a rapid fall of the levels of the cytochromes aa_3, b and, most probably also of c_1, to about 30 per cent of their normal values. A large number of other mitochondrial and extra-mitochondrial enzyme activities were not affected in the antibiotic-treated rats (Papers 1 and 2). In a similar study of Gijzel et al.[17] the synthesis of F_1-ATPase appeared also unchanged but its sensitivity to oligomycin was strongly decreased. All these data together perfectly agree with the recent concept about the function of mitochondrial protein synthesis[22]. The following proteins are now considered as real mitochondrial translation products:

---

* Parallel morphological and histochemical studies (de Pijper and Hülsmann, 1972) are reported in ref. 21.

(1) The two or three largest polypeptides of the cytochrome c oxydase complex. The residual four polypeptides are synthetized on cytoplasmic ribosomes.
(2) The cytochrome b apoprotein.
(3) The four hydrophobic proteins of the ATPase complex. F_1-ATPase itself and the oligomycin sensitivity-conferring protein are cytoplasmic products. One of the four mitochondrial products is additionally needed for oligomycin-sensitivity.

The apoprotein of cytochrome c_1 is made on cytoplasmic ribosomes. Incorporation of its heme group is dependent on mitochondrial protein synthesis.

The normal content of most of the mitochondrial enzymes in the treated rats indicated that the cytoplasmic synthesis of these proteins was not controlled by or dependent on the mitochondrial protein-synthetizing system. This independency of both systems is in fact the basis for the preservation of the mitochondrial function during long-term treatment with the antibiotics, documented in Paper 2 (Table 2). Most of the experiments have been done with total homogenates to exclude a possible selection of intact mitochondria in the mitochondrial pellet after differential centrifugation. As a general conclusion from this study and that of others[5,23] it may be stated that in many tissues of the rat (liver, heart, brain, intestine) a 50-70 per cent reduction of cytochrome b and aa_3-content and a loss of oligomycin-sensitivity is not sufficient to impair the mitochondrial energetic function *in vitro*. After a further reduction of cytochrome levels, the electron transport chain would become rate-limiting for the oxidation of some substrates (e.g. succinate; see Table 2, Paper 2).

The maintenance of a normal energy charge and creatinephosphate level in freeze-clamped intestine from rats, treated for 48-72 h with CAP or for 48 h with OTC (Paper 2, Table 3) confirmed the intact function of the cytochrome-poor mitochondria. The preservation of a normal proliferative

activity in the crypt cells from rats, treated for 100 h with OTC, was an even stronger indication because thymidine incorporation into crypt cell DNA (at least *in vitro*) is strongly dependent on the intactness of oxidative energy metabolism (unpublished observations) and because a cytochrome depletion will become manifest first in the proliferative cells.

The incomplete inhibition of the mitochondrial protein synthesis in the enterocytes by CAP and OTC at continuous serum levels between 10 and 40 µg/ml may be explained by a relatively low affinity of the intestinal mitoribosomes (or a low permeability of the mitochondrial membrane) for the antibiotics, accumulation of these compounds in other regions of the cell (e.g. OTC in microsomes[24]), intracellular inactivation (glucuronic acid conjugation of CAP) or a low permeability of the plasma membrane for the antibiotics. In the case of tetracyclines this tissue permeability is mainly a function of lipid solubility and divalent cation binding, but there is also some indication that "cytoplasmic anion binding" proteins ("Y" and "Z"[25]) might participate in the overall transport of tetracyclines through the plasma membrane (Arias, personal communication). The accumulation of tetracyclines in liver and kidney would agree with the high concentration of Y-protein in these tissues. The luminal absorption is possibly regulated by a competition between fatty acids and tetracyclines for binding sites on the Z-protein. This also provides a possible explanation for the decreased absorption of fat after tetracycline-administration[26] (see Paper 2).

An apparently normal cell proliferation and/or development in the presence of low concentrations of CAP (<30 µg/ml) has also been shown for regenerating liver[5], mouse embryo cultures[27] and rat embryos *in vivo*[28], despite a 50 per cent reduction of cytochrome aa_3 content in these tissues. In these studies mitochondrial morphogenesis

Table 2.1 ANTIBIOTIC-SENSITIVE PROCESSES IN MAMMALIAN CELLS, NOT RELATED TO MITOCHONDRIAL PROTEIN SYNTHESIS*

Antibiotic	Concentration	Tissue	Function studied	Degree of inhibition (%)	Ref.
CAP	20 µg/ml (4h)	Bone marrow (human)	Haem and globin synthesis	40	31
	100 µg/ml	Hela cells	Mitochondrial respiration	50	4
	100 µg/ml	Bone marrow	"	30	29
	50 µg/ml	Lymphocytes	RNA synthesis**	50	32
	100 µg/ml	Bone marrow	"	50	32
	100 µg/ml	Heart muscle	Hexose transport	40	33
			Impaired mechanical performance	-	
OTC	150-300 µg/ml	Liver, brain, intestine	Respiration, oxidative phosphorylation	-	34
TC	250 µg/ml	Liver	(Cytoplasmic) protein synthesis	78	35
OTC			in vitro	27	35
TC	30-60 µg/ml	Yeast	Polyphenylalanyl synthesis on 80S ribosomes	50	36
TC	2x 125 mg/kg IM (34 h)	Liver	Aminoacid incorporation	25	37
		Intestine	"	35	37
TC	400 mg/kg IP (4 h)	Intestine	"	30	38
TC	250 mg/kg IM (4 h)	Pancreas	"	47	39
			Amylase secretion	-	
Anhydro-TC	5 µg/ml	Hela cells	Aminoacid incorporation	60	40
TC	35 mg/kg IM (4 weeks)	Liver	Fatty acid and cholesterol synthesis	80	41
OTC, DOC	10-100 µg/ml	Intestinal smooth muscle	Contraction amplitude	~(25-46)	42
	0.6-1 mg/ml	"	"	60-95	42
TC	50 µg/ml	Fat cell	Adenylate cyclase	20	43
DMC	Therapeutical	Nephron and	ADH-stimulated**	71	44
DOC	dosages	toad bladder	water flow	63	44
MC	"	"	"	56	44
TC	"	"	"	53	44
OTC	"	"	"	1	44
DC, TC, OTC	2-3 µg/ml	Serum	Bacteriocidal activity	-	45

* Our own results (Paper 2) are not included in this survey
** (Possibly) related to inhibition of adenylate cyclase activity
Abbreviations: TC, tetracycline; DOC, doxycycline; CTC, chlortetracycline; DMC, demeclocycline; MC, minocycline. IM = intramuscularly injected; ADH = anti-diuretic hormone (vasopressin); IP = intraperitoneally injected.

was also nearly normal except for the appearance of dilated and vesicular cristae. In studies concerning reversible bone marrow depression resulting from large doses of CAP[29,30], these ultrastructural changes are often taken as evidence for a causal relationship between the myelotoxic effect of CAP and its role as an inhibitor of mitochondrial protein synthesis. To our opinion direct measurements of an impaired mitochondrial function in the affected tissue are needed to make such a link.

2.4 Biochemical targets of the antibiotics in mammalian cells, not related to mitochondrial protein synthesis

A brief survey of literature data concerning inhibitory actions of CAP and tetracyclines on cellular processes other than mitochondrial protein synthesis is given in Table 2.1. At serum levels of CAP above 35 µg/ml (virtually uniformly accompanied by bone marrow suppression in man) there is an additional risk for direct inhibitory effects on haem synthesis[31] and RNA synthesis[32]. These effects could have played a role in the morphological damage of the intestinal mucosa in two rats after 120 h of CAP pretreatment[21]. In these animals plasma levels, however, were not registrated at this time. Extremely high concentrations of the drug are needed for a direct inhibition of mitochondrial respiration[4,29] or sugar transport[33]. An interference of these effects in our studies is highly unlikely.

Tetracyclines may exert a broad spectrum of action at high concentrations (Table 2.1). Possible inhibitory effects on cytoplasmic protein synthesis, respiration and oxidative phosphorylation in small intestinal epithelium and liver are extensively discussed in Paper 2. The suggestion made in this paper that the decrease of ATP level in the liver would be the result of tissue damage following the accumulation of lipid droplets ("fatty liver") is

probably erroneous, because a recent report[46] has made clear that most of the lipid accumulates outside the cell, in the space of Disse, after discharge from the sinusoidal surface of the cell. If the extremely high levels of tetracycline in the liver reported in this study (800 µg/g at 300 mg TC/kg/day) had also been reached in our studies where a similar OTC-regime was applied, the tissue damage and ATP depression may be simply explained by large inhibitory effects on overall protein synthesis or mitochondrial functions. Moreover, small amounts of OTC-degradation products may be formed in the tissue which might have a much stronger inhibitory effect on cytoplasmic protein synthesis than OTC itself[40] (Table 2.1).

A depression of adenylate cyclase activity by tetracyclines[43,44] could not interfere in our studies because OTC is inactive in this respect (Table 2.1 and unpublished experiments).

2.5 Concluding remarks

Extrapolating the results of our study to man, inhibition of mitochondrial protein synthesis is likely to occur at normal serum levels of the antibiotics (≤10 µg/ml) and will result in a rapid decrease of cytochrome levels in proliferating tissues. However, if the excess of these essential components in the respiratory chain in human tissues would be comparable to that found in most tissues of the rat, an impairment of mitochondrial function cannot be expected before at least 60-70 per cent of the normal cytochrome content has been lost. A drop of the cytochrome levels beneath this critical value will be an extremely rare phenomenon in clinical situations because the inhibition of mitochondrial protein synthesis is reversible and incomplete, even at high dosages. In human small intestinal epithelium, the chance that the antibiotics will induce such a critical depletion of cytochromes is even less than

in the rat, because the crypt cell division time in man (appr. 2 days) exceeds that in rats about four times[47]. This may explain why an impairment of gastrointestinal functions during clinical use of CAP and OTC in normal therapeutical dosages has hardly ever been reported, except in case of superinfections of the alimentary tract.

The antibiotics sometimes evoke severe complications in case of overdosage, an impaired conjugation system for CAP in the liver or renal insufficiency. These complications are not necessarily the consequence of a mitochondrial cytochrome depletion, but may result from the many side effects of CAP and tetracyclines mentioned in the preceeding Section.

CHAPTER III CYCLIC AMP AND FLUID TRANSPORT

3.1 Introduction

In the course of their investigations of the hyperglycemic effects of glucagon and epinephrine on liver, Rall and Sutherland discovered a small, heat stable compound capable of stimulating glycogenolysis in broken cell preparations[48]. In 1958 this compound was identified as adenosine 3',5'-monophosphate (cyclic AMP)[49]. Since then cyclic AMP has been detected in almost all prokaryotic and enkaryotic cell types where it fulfills an important and often essential role as an intracellular regulator ("second messenger") of biological functions. Its implication in entirely different processes like glycogenolysis and gluconeogenesis, lipolysis, muscle cell contraction and relaxation, sensory and neural excitation, cellular permeability and secretion, lymphocyte activation, cell adhesiveness and motility, growth inhibition (Chapter V) and hormone synthesis, illustrates the almost universal character of its function.

One of the earliest recognized actions of cyclic AMP was on membrane permeability of epithelial tissues. The discovery of its key-role as a mediator of the action of neurohypophyseal hormones on toad bladder[50] (1962) and mammalian kidney[51] (1966) was followed by a burst of investigations of cyclic AMP control in other transport processes, including gastric secretion, pancreatic secretion and fluid secretion by insect salivary glands. In 1968 Field and coworkers published their first report about an active secretion of both chloride and bicarbonate by rabbit ileum in response to cyclic AMP and theophylline[52]. These effects were clearly different from those in other epithelial tissues where cyclic AMP increased the permeability to water and urea (hydro-osmotic response of toad bladder[50] and kidney[51]) or the active transport of

sodium from mucosal to serosal side (toad bladder[50]). The first step in the recognition of cholera as a disorder of cyclic nucleotide metabolism was set by Schafer and co-workers (1970) who measured significantly elevated levels of cyclic AMP in canine small intestine after luminal contact to choleratoxin[53]. This enterotoxin, produced by Vibrio cholerae, is responsible for the massive secretion of water and electrolytes in the cholera syndrome. This discovery led to gigantic efforts to clarify further the mechanism of action of choleratoxin in the small intestinal epithelium. It was hoped that the knowledge gained from these studies would lead to new pharmacological tools in the prevention and treatment of cholera and other diarrhoeal diseases.

Thus far, the results of this research have contributed much to our present understanding of the mechanism of hormone action and ion transport. However, because choleratoxin acts by activating an enzyme system of fundamental importance for the functioning of all living cells, the chances for a chemotherapy, based on the administration of inhibitors of these systems must not be assessed too high.

The bacteriological, immunological and toxicological aspects of enterotoxin-induced diarrhoea and the characteristics of the cyclic AMP-mediated intestinal secretion have been extensively reviewed in articles of Banwell and Sherr[54], Field[55] and Kimberg[56]. Moreover, a lot of recent literature data is summarized in the Papers 3 and 4. Therefore, this chapter will be restricted to a brief review of the latest literature in this field and a discussion of some unpublished results.

3.2 The mechanism of action of choleratoxin

An indispensable role of choleratoxin (choleragen) in producing the cholera diarrhoea has been genetically proven

by the recent isolation of non-reverting tox⁻mutants that are totally avirulent[57]. In clinical cholera, other bacterial factors may contribute to the virulence, e.g. the production of mucinase[58], phospholipase and neuraminidase [59]. The latter enzyme may drastically increase the number of choleragen receptors at the mucosal surface of the epithelial cells[60].

Choleratoxin is an antigenic, neutral protein with a molecular weight of 84.000 constituting a multichain aggregate of at least two type of subunits[61]. The light (L) subunit (M ∿ 8.000) is responsible for the binding of the toxin to G_{M_1} ganglioside receptors in the plasma membrane and also for most of its antigenicity[61-63]. The heavy (H) subunit (M ∿ 28.000-36.000) is important for the biological action of the toxin[61-64], which appears to be associated with an activation of adenylate cyclase in the plasma membrane. This stimulation is preceeded by a characteristic lag phase of at least 30 min[63-65] (see Paper 4). Adenylate cyclase in broken cell preparations has lost its sensitivity to the choleragen (Paper 3 and unpublished results) although it still responds to prostaglandins and hormones (compare Paper 3). Both aspects of choleratoxin action can be adequately explained with the mobile receptor theory proposed by Cuatrecasas and coworkers[63]. In this scheme the binding of toxin to G_{M_1} receptors is followed by a lateral movement of the toxin-receptor complex in the plane of the membrane, which ultimately leads to a direct and persistent interaction of the strongly hydrophobic H-subunit of the complex with adenylate cyclase. Disruption of the plasma membrane should disturb this process and make the target-enzyme insensitive to the toxin[65]. This mode of action of choleragen is essentially different from that of a number of other toxic proteins (e.g. the bacteriocins colicin E_3[66] or cloacin DF_{13}[67] and diphteria toxin[68]) where a hydrophylic part of the molecule moves into the cytoplasma prior to its interaction with the

target.

The mobile receptor theory is based on the new concept of a relatively fluid state of biological membranes[69]. A similar mechanism is proposed for hormone-receptor-adenylate cyclase interactions[63]. Several recently detected properties of adenylate cyclase may be easily fitted in this frame work: first the increase in its affinity for hormones in the choleragen-affected state[63,70,71]. This may reflect stabilization by choleragen of the complex between hormone-receptors and the cyclase. The regulation of adenylate cyclase by guanyl and inosylnucleotides[72] may be explained on a similar basis. In this scheme it seemed also quite possible that choleratoxin, prostaglandins or hormones should simultaneously affect several membrane functions or enzymes (cyclases, (Na^+-K^+)-ATPase, phosphodiesterases) by interaction with a single receptor. This has actually been shown for prostaglandin E_1 and E_2 in lysates of erythrocytes and platelets[73].

The possible interaction of choleratoxin with membrane-bound enzymes in intestinal epithelial cells was tested during our study by measuring (Na^+-K^+)-ATPase (according to Ferard[74], Ca^{2+}-ATPase (Na^+ and K^+ replaced by 0.5 mM Ca^{2+}) and alkaline phosphatase (as described by Iemhoff et al.[7]) in brushborders and villous cell homogenates from control and choleragen-treated rats isolated 15 min, 1 h and 4 h after the luminal application of purified choleragen. Although at this time the adenylate cyclase response has nearly reached its maximum (Paper 4 and Chapter V), no significant changes were found in any of the other enzyme activities measured (results not shown). Thus, at least in the intestinal cell membrane, the action of choleratoxin seemed to be entirely confined to the adenylate cyclase system and the changes in ion transport could not be explained by a direct or indirect effect of the toxin on other membrane-bound enzymes. The inhibition of small intestinal (Na^+-K^+)-ATPase, reported by other authors[58,75,76]

is probably due to the presence of mucinase in their crude preparations of choleragen[58,75] or during clinical cholera[76].

3.3 Cyclic AMP and the mechanism of fluid secretion

Our knowledge about the steps lying between cyclic AMP accumulation and fluid secretion is still very poor. In analogy to studies in other epithelial tissues (toad bladder[77], kidney[78]) it may be assumed that cyclic AMP should act by changing the level of phosphorylation of some specific proteins in the plasma membrane, presumably at the luminal side of the enterocyte. This should ultimately lead to a change in the properties of the ion-transport system. Indeed it has been shown that the phosphorylated state of intestinal proteins particularly in the core of the microvilli, is increased during cholera[79]. A characterization of cyclic AMP-dependent protein kinases and their substrates in the epithelium is needed to substantiate this model. Cycloheximide, an inhibitor of protein synthesis, blocks the secretory response of the cell without inhibiting the cyclic AMP accumulation[80]. This means that protein synthesis is essential for a certain step in the secretory mechanism or that cycloheximide itself exerts a direct effect on the secretion process[80].

Much work has been done on the localization and characterization of the ion-transport system controlled by cyclic AMP. The stripped mucosa of rabbit ileum is the commonly used model system. According to the most recent scheme for cyclic AMP action, proposed by Powell and coworkers[81,82], cyclic AMP mainly stimulates the transcellular, active secretion of NaCl and/or $NaHCO_3$ from serosa to mucosa and reduces the electrical conductance, determined by a passive ionflux through a transepithelial shunt pathway. The latter phenomenon may be ascribed to a swelling of the cell in response to choleratoxin, probably reducing the width of the intracellular space[82]. The

existence of an active neutral NaCl absorptive mechanism in the brushborder of rabbit intestinal epithelial cells has been shown earlier by Frizzel[83] and Nellans[84]. According to these authors, cyclic AMP should inhibit this neutral influx rather than stimulate a reversal of this process. The model of Powell is more in agreement with our in vivo measurements of unidirectional flux rates of Na^+ and Cl^-, indicating a stimulation of the neutral efflux of NaCl from serosa to mucosa in the absence of a significant change of the opposite flux (Paper 4). The flux studies performed in vitro with stripped mucosa give no information about the individual role of the different cell types of the epithelium in active secretion. Our main contribution to this field is the finding that both villous and crypt regions are involved in fluid secretion after prolonged exposure to choleragen. Independently, Schwartz et al.[85] measured adenylate cyclase activation by choleratoxin in villous as well as in crypt cells from rat and rabbit intestine but did not solve the question of a possible cell type specificity of the secretory response.

The involvement of the goblet cells in the electrolyte secretion is not entirely clear so far. Some indication that this process is confined to the absorptive cells may be derived from a combination of the following data:

(1) Goblet cells react after exposure to choleratoxin with a rapid outpouring of mucus, probably stimulated by cyclic AMP[86]. In rat small intestine, the mucus secretion is completely compensated by an increased rate of mucus synthesis (Chapter V). Therefore, mucus synthesis is probably also controlled by cyclic AMP.

(2) High concentrations of isoproterenol and epinephrine (0.05-0.1 mM) cause a stimulation of mucus synthesis in intestinal slices of the rat[87] and a moderate elevation of cyclic AMP levels in isolated villous and crypt cell populations (β-adrenergic response, Paper 3).

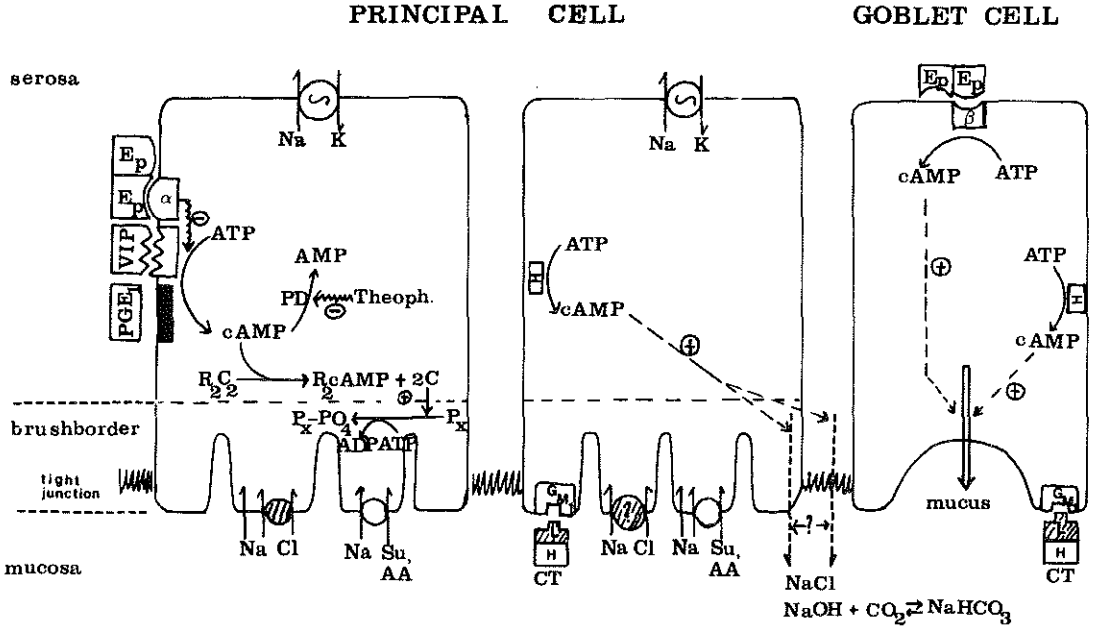

Fig. 3.1 Tentative scheme of cyclic AMP action on principle and goblet cells from rat small intestine

AC = adenylate cyclase
R_2C_2 = cyclic AMP-dependent protein kinase (R = regulatory subunit; C = catalytic subunit)
P_x = substrate protein for C
CT = choleratoxin (H = heavy subunit; L = light subunit)
G_{M_1} = ganglioside receptor
α = α-receptor
β = β-receptor
Ep = epinephrine
VIP = vasoactive intestinal peptide
PGE_1 = prostaglandin E_1
Su = sugar
AA = aminoacid
⏀ = Na^+, K^+-ATPase

(3) (Nor)epinephrine but not isoproterenol lowers cholera-gen- or PGE_1-stimulated cyclic AMP levels in rabbit mucosa (α-adrenergic response)[55] concomitant with an enhancement of active transport of NaCl and fluid from mucosa to serosa[88].

These facts may be easily fitted into the following hypothesis: epinephrine and isoproterenol stimulate a cyclic AMP-mediated mucus production in the goblet cell by interaction with a β-receptor. In absorptive cells, however, the α-adrenergic response predominates resulting in an increased rate of fluid and electrolyte absorption. Choleratoxin elevates cyclic AMP in both cell types resulting in a combined secretion of mucus and electrolytes. This situation is schematically represented in Fig. 3.1. It remains to be established whether epinephrine (or isoproterenol) indeed possesses the ability to stimulate mucus secretion from the goblet cells of an intact tissue. The observation of fluid secretion in the absence of mucus secretion in studies with shigella enterotoxin[89] provides a second argument against the involvement of the goblet cell in fluid secretion, if the shigella toxin should indeed share a common secretory mechanism with choleratoxin, as has been recently suggested[89].

3.4 Intestinal secretion evoked by other secretagogues

A number of hormones, prostaglandins, enterotoxins and other, unrelated agents have been reported to inhibit absorption or to cause net secretion of water and electrolytes in the small intestine. A list of these compounds is given in Table 3.1. The involvement of cyclic AMP as a mediator of the secretion is clearly established in the case of the prostaglandins (Paper 6), E-coli enterotoxin, VIP (vasoactive intestinal peptide, related in structure to secretin, glucagon and gastric inhibitory peptide), bile salts, and the phosphodiesterase inhibitors theo-

Table 3.1 CONTROL OF FLUID AND ELECTROLYTE SECRETION IN THE SMALL INTESTINE

Agent	Secretin (+) or absorption (-)	Cyclic AMP increase (+) or decrease (-)	Clinical syndrome
Choleratoxin[53,56]	++	++	Cholera
E.coli enterotoxin[54,56]	+	+	Neonatal diarrhea, traveller's "
C.perfringens enterotoxin[90]	+	?	
Shigella enterotoxin[89,91]	+	no effect	Bacillary dysentery
Prostaglandins E_1, E_2, $F_{2\alpha}$[56]	+	+[a]	?
VIP[92,93]	++	++	Pancreatic cholera
GIP, secretin[56,92,93] (penta)gastrin, glucagon, gastrin + glucagon, cholecystokinin, calcitonin	+	no effect[a]	
Acetylcholine[94]	+	no effect	
Angiotensin[95]	+ (high doses) - (low doses)	?	
Vasopressin[51,96,97]	+ (man) - (dog, rat)	no effect	
Epinephrine[88]	-	+	
Isoproterenol	no effect	+[b]	
Epinephrine/isoproterenol[88] + propranalol	-/no effect	no effect[b]	
(Hydroxy) fatty acids[98-100] (oleate, rincinoleate)	+	no effect[b]	Diarrhea in fat malabsorption
Bile acids[101,102] (desoxycholate, taurocheno-desoxycholate)	+ (colon)	+	
Theophylline[52,54-56], caffeine[103]	+	+	
Aspirin[104]	-	no effect	
Enterovirus[105]	+	?	Transmissible gastroenteritis

a) See also Paper 6

b) H.R. de Jonge, unpublished results.

phylline and caffeine. As discussed in the preceeding section, the stimulation of cyclic AMP levels by epinephrine and isoproterenol (Paper 3, Table 3.1) is probably a specific response of the goblet cells. DL-propranalol (1 mM) inhibited this response (unpublished results), confirming its β-adrenergic character.

The other hormones tested were not able to change cyclic AMP levels in isolated villous and crypt cells from the rat (Paper 6, Table 2) nor in stripped mucosa from the rabbit[92]. They were likewise unable to stimulate adenylate cyclase activity in human jejunal mucosa[93]. Guanylnucleotides increased the basal activity of the intestinal enzyme but did not change its hormone-responsiveness[93] (compare Section 3.2). As pointed out by Birnbaumer et al.[106] guanyl and inosyl nucleotides may increase or decrease the apparent affinity of the adenylate cyclase system for hormones, dependent on the type of tissue or hormone studied. The effect of hormones on intestinal adenylate cyclase, preactivated with choleragen, has not been investigated thus far. Such a study could be interesting because choleratoxin strongly increased the affinity of the enzyme for hormones in a number of other tissues[63,70,71] (compare Section 3.2). If a similar phenomenon could be reproduced in intestinal epithelium, the increased hormone sensitivity during cholera might be a complicating factor in the tissue response to the choleragen.

The inhibition of ileal and jejunal fluid absorption by fatty acids (C_{10}-C_{18}) and hydroxy fatty acids (ricinoleate, the active component of castor oil)[98,99] cannot be explained on the basis of a change in cyclic AMP levels. Even very high concentrations of ricinoleate (up to 5.0 mM) were unable to affect the cyclic AMP content of isolated villous cells (unpublished results). It has been suggested that these fatty acids may accumulate as acyl-CoA esters in the mucosa which, by inhibiting adenine nucleotide translocase, could alter mucosal transport functions by

limiting the ATP-supply of the cells[100].

Recent studies comparing the action of choleratoxin and shigella toxin (an enterotoxin of Shigella dysenteriae) on rabbit ileum and jejunum indicated that both toxins probably stimulate the same ion transport system but in an entirely different way, for the cyclic AMP response was totally absent in the case of shigella toxin[89,91]. The inability of this toxin and many hormones to change cyclic AMP levels despite their reported interaction with fluid transport indicates the existence of alternative, hitherto unknown, mechanisms to affect intestinal secretion. Several of these hormones have been reported to change the levels of the nucleotide cyclic GMP in other tissues. A possible role of this compound in intestinal transport processes is discussed in Chapter IV.

3.5 Concluding remarks

It is a general conclusion from our own work as well as that of others that even a slight increase of cyclic AMP levels in the intestinal epithelium leads to a drastic reduction of its absorptive capacity for water and electrolytes and may ultimately convert it into a secretory organ. The apparent insensitivity of the adenylate cyclase system of the enterocyte to a number of hormones (glucagon, insulin, secretin, catecholamines in a physiological concentration range) may therefore protect the absorptive function of the cell against large fluctuations in the levels of these hormones. At present, the only naturally occurring extracellular regulator of intestinal adenylate cyclase is believed to be VIP, produced in the gastrointestinal tract and in the pancreas. This protein may possibly play a physiological role in a fine regulation of the absorptive mechanism.

Cyclic AMP control of metabolic pathways in the small intestine, comparable to that in other tissues (liver,

muscle, fat cells) might offer important risks for a continuous functioning of the absorptive system. It may be suggested that the lack of glycogen[107], the absence of gluconeogenesis[108] and a possible absence of a hormone-sensitive lipase[109], at least in rat small intestine, is related to the need to hold its cyclic nucleotides at constant intracellular levels.

CHAPTER IV. FORMATION AND FUNCTION OF CYCLIC GMP IN SMALL INTESTINAL EPITHELIUM

4.1 Introduction

Besides cyclic AMP, the only other 3',5'-cyclic nucleotide ubiquitous present in living cells is guanosine 3',5'-monophosphate (cyclic GMP). This compound was discovered in 1963 by Ashman and coworkers[110] in urine and subsequently detected in a variety of mammalian tissues and other phylogenetic forms including prokaryotes[111]. Tissue levels of cyclic GMP are usually ten- to hundred fold lower than those of cyclic AMP[111]. The physiological significance of the agent is not fully understood yet. However, a number of studies suggest a role of cyclic GMP in the cellular response to cholinergic and α-adrenergic agents (neurotransmitters)[111,112], insulin[113], prostaglandin $F_{2\alpha}$[114], estrogens[115], plant lectins[116] and growth factors[117] (Chapter V).

According to the "Yin-Yang" hypothesis proposed by Goldberg (1973)[111], cyclic GMP is involved in promoting cellular events that are antagonistic to those mediated by cyclic AMP. This "dualism" should only apply to bidirectional control systems (e.g. muscle contraction and relaxation). In a monodirectional controlled system (e.g. stimulation of steroidogenesis by ACTH) the two cyclic nucleotides might promote similar events rather than opposing ones. This concept of biological control has now proven its value in several model systems, e.g. lysosomal enzyme release by human neutrophils[118], chemotaxis or cell motility of human monocytes[119], the platelet release reaction[120], muscle contraction and relaxation[112] and growth control[117]. The molecular basis of these apparent antagonistic actions of both nucleotides is not yet elucidated. Applying this hypothesis to the ion-transport system in the small intestine (possibly bidirectionally controlled; see Chapter III), cyclic GMP would antagonize the ion secretion stimulated by cyclic AMP.

In that case, a better knowledge of the factors that determine cyclic GMP formation and degradation in the epithelium could have important therapeutic implications. In the opposite case, assuming a similar action of both nucleotides on ion transport, this study would possibly provide an alternative basis to explain the stimulatory effects of certain hormones or toxins on intestinal fluid secretion in the absence of any change in cyclic AMP levels (Chapter III).

4.2 Formation of cyclic GMP in a number of tissues other than small intestinal epithelium

Cyclic GMP is formed by the enzyme guanylate cyclase (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2) in the following reaction:

$$GTP \xrightarrow{Mn^{2+}} 3',5'\text{-cyclic GMP} + PP_i$$

dGTP and ITP can be used as alternative (non-physiological?) substrates . The enzyme exists in a soluble and a particulate form, showing different kinetic and physical properties[121]. Their relative activities are strongly tissue-dependent and may vary with the stage of development[122]. The particulate enzyme is most active in sea urchin sperm[123] and glomerular membranes[124]. Human platelets[125] and rat lung[126] are the richest sources for the soluble enzyme. The guanylate cyclase from sea urchin sperm is largely confined to the flagellar membranes[127]. Reliable subcellular distribution studies for the particulate enzyme in other non-epithelial tissues with the use of marker enzymes have not been reported thus far. In rat heart[126], calf uterus[128] and bovine rod outer segments[129], most particulate activity resides in the nuclear pellet. In Table 4.1, the main differences and similarities of soluble and particulate enzymes are summarized and compared to those of adenylate cyclase.

Table 4.1 COMPARISON OF SOME PROPERTIES OF THE SOLUBLE AND PARTICULATE FORMS OF GUANYLATE AND ADENYLATE CYCLASE

	Guanylate cyclase		Adenylate cyclase	
	Soluble (rat lung[121], heart[126])	Particulate (sea urchin sperm[123], rat lung[121])	Particulate (liver)	Soluble (bacteria)
Substrate	MnGTP (dGTP, ITP)	MnGTP	MgATP (dATP)	MgATP
Cofactor	Mn^{2+} (Mg^{2+}, Ca^{2+})	Mn^{2+}	Mg^{2+} (Mn^{2+})	Mg^{2+}
Substrate-kinetics	Classic (=Michaelis-Menten)	Positive cooperativity (classic in presence of CaGTP)	?	?
Cofactor-kinetics	Negative cooperativity (Mn^{2+}_{free} <0.2mM) Classic (Mn^{2+}_{free} >0.2mM)	Classic	?	?
Activator	Ca^{2+} (>0.1mM at low Mn^{2+})	Detergent (Triton X-100) MnATP, ADP (<50µM)	F^-, GTP (50nM-0.1mM), hormones	α-Ketoacids
Inhibitor	PEP (1-10mM), oxalacetate (0.1-0.5mM) ATP (nucleotides; 0.5-2mM)	MnATP, ADP (>50µM)	Ca^{2+} (0.1-1mM), Li^+, alloxan, detergent	Oxalacetate
Molecular weight	∿450.000	∿800.000	?	?

Despite their different kinetic properties and molecular weights, both forms of guanylate cyclase possibly represent different physical states of the same molecule(s). This is strongly suggested by the recently reported apparent redistribution of both forms during growth and development of regenerating liver, fetal liver and hepatoma cell[122] in the absence of any change in total enzyme content.

The current state of knowledge concerning the physiological regulation of guanylate cyclase activity is still very poor. If the "second messenger" concept for cyclic AMP is translated in terms of cyclic GMP, it might be anticipated that hormonal stimulation of cyclic GMP levels would have its basis in a direct interaction of hormone receptors with a particulate guanylate cyclase localized in the plasma membranes. Evidence for this derives from at least two model systems: the stimulation of guanylate cyclase in fibroblast membranes by a specific growth factor[117] (Chapter V) and in liver plasma membranes by insulin[130]. The parallel with the adenylate cyclase system has even led to the suggestion that the guanylate cyclase activity in plasma membranes reflects a change in substrate specificity of the catalytic subunit of adenylate cyclase from ATP to GTP[113]. Efforts to show a direct hormonal stimulation of particulate guanylate cyclase in heart muscle failed thus far[126,131]. However, such effects could have been masked by the apparent high contamination of these crude preparations with nuclear enzyme (possibly insensitive to hormone action). Several reports describing stimulatory effects of secretin[132], cholecystokinin[133] and acetylcholine[134] on soluble guanylate cyclase in vitro could not be confirmed in other laboratories (ref.126 and Hardman, personal communication). Because stimulation of cyclic GMP levels by cholinergic or depolarizing agents generally requires the presence of Ca^{2+} in the medium it has been suggested that these agents may rise cyclic GMP by promo-

ting Ca^{2+}-uptake through the plasma membrane[118,135]. Indeed, it has been demonstrated repeatedly that the Ca^{2+}-ionophore A-23187 can mimic their action and raise cyclic GMP levels in several tissues[118,136,137]. As shown in Table 4.1, a stimulatory effect of Ca^{2+} on soluble guanylate cyclase *in vitro* requires high Ca^{2+}- and low Mn^{2+} levels. Whether these high levels of free Ca^{2+} will actually be reached during cholinergic stimulation is uncertain. As has been shown earlier for adenylate cyclase[138], also guanylate cyclase activity can be regulated by a number of cellular metabolites. Especially the strong inhibition by nucleotides (ATP, ADP, etc.) may be of physiological importance.

4.3 Formation of cyclic GMP in small intestinal epithelium

The presence of a distinct guanylate cyclase in mucosal scrapings of the rat has first been described by Ishikawa *et al*[139] (1969). They noted an exceptionally high activity of the particulate enzyme and a relatively low amount of soluble enzyme in this tissue. Despite its potential usefulness as a model for studying function and regulatory properties of the particulate guanylate cyclase, no further research on the enzyme has been reported since then, probably because the small intestinal epithelium is generally considered to be a hormone-insensitive tissue.

New interest in the role of cyclic GMP in the small intestine arose by the recent observation that acetylcholine may stimulate Cl^--secretion also in stripped mucosa[140] (in accordance with the histochemical findings of cholinergic nerve endings in the lamina propria) and the inability of a number of hormones to change cyclic AMP levels in the mucosa[56,92], though markedly influencing fluid and ion transport (e.g. vasopressin, pentagastrin; see Paper 6).

The main results of our studies dealing with a further characterization of the cyclic GMP-generating system in the

epithelial cells are presented in Papers 5 and 6.

The composition of the assay system described in Paper 5 needs some additional comment. The assay of guanylate cyclase in relatively crude preparations requires the use of a GTP-regenerating system to ensure constant levels of GTP. The PEP-PK system applied in the original publication of Ishikawa et al.[139] was replaced by CP-CK in view of the marked inhibition of guanylate cyclase by high concentrations of PEP (Paper 6, Table 1). Moreover, CP-consumption and product degradation could be kept low (<15 per cent) by the addition of theophylline (a strong inhibitor of alkaline phosphatase[141] and phosphodiesterases) and by using low amounts of protein and a short incubation time. Limitation of CP-degradation is essential to obtain reliable kinetic data, because otherwise deviations from linearity arise due to a change in $[Mn^{2+}_{free}]$ resulting from decreased amounts of Mn^{2+} complexed to CP. $MnCl_2$ must be added just prior to assay, to avoid the formation of Mn^{2+}-Tris precipitates and Mn^{2+} oxidation.

The results of our studies (Papers 5 and 6) can be summarized as follows:

(1) The soluble form of guanylate cyclase is nearly absent as well in villous- as in crypt cells.

(2) Most of the guanylate cyclase is incorporated into microvillous membranes of the brushborder during an early stage of development. The distribution study, according to Douglas, did not eliminate a possible nuclear localization but the large increase of specific enzyme activity after brushborder purification according to Harrison and Webster, in which adherent nuclear material is adsorbed to glass wool, indicated that most (but possibly not all) of the guanylate cyclase in these preparations was confined to the microvilli.

(3) The residual part of the guanylate cyclase is most probably localized in the basal-lateral plasma membranes. An accurate determination of its contribution

to the total enzyme activity in the cell is impossible in view of the different kinetic properties of the luminal and anti-luminal enzyme forms.

(4) The kinetic characteristics of the brushborder enzyme appeared to be very similar to those of particulate enzymes in other tissues. Inhibition by oxalacetate, PEP and nucleotides has been found earlier for the soluble enzyme from rat liver and lung[142]. The strong inhibition by ATP sharply contrasts with its stimulatory action on the nuclear enzyme in ovarian tissue[128]. The small stimulation by amino acids (also found in the absence of a regenerating system) have no parallel in other tissues; one can only speculate about its physiological significance.

(5) The microsomal preparation, weakly contaminated with brushborder enzymes, showed kinetic characteristics that were intermediate to those of particulate and soluble enzymes in other tissues.

(6) The apparent hormone-insensitivity of the brushborder enzyme seemed not very surprising, in view of a possible lack of hormone receptors at this side of the cell. However, also the microsomal enzyme was insensitive to the hormones. Possible reasons for this phenomenon are discussed in Paper 6.

A relationship between cyclic GMP and intestinal transport functions was considered on the basis of the following: (1) The presence of a highly active guanylate cyclase in the brushborder. (2) The essential role of the microvilli in intestinal transport processes. (3) The observation of 50-70 Å actin-like core filaments in the microvilli[143], and of pulsating movements of microvilli, under the phase contrast microscope[144]. Both data strongly suggest a contractile function of the microvilli, possibly related to its transport function. (4) The high levels of guanylate cyclase in other cell types/organelles with a contractile function (flagel-

lar membranes[127], platelets[125]).(5) A possible general role of cyclic nucleotides in the regulation of cytokinesis[119,145].

A search for the presence of cyclic GMP-dependent protein kinases in the microvilli and localization of their substrate-proteins (core proteins?) would possibly open the way to test this hypothesis experimentally.

Alternatively, a role of cyclic GMP in the development of microvilli may be suggested, based on the recent finding that cyclic AMP caused regression of microvilli in normal mouse fibroblasts and that a reduction of cyclic AMP levels by transformation led to formation of microvilli[146].

4.4 Levels of cyclic GMP in epithelial cells, isolated from rat small intestine

Direct measurements of cyclic GMP levels in isolated cells to test the hormone-responsiveness of the cyclic GMP-system, eliminates homogenization artifacts (e.g. receptor damage) and may provide additional information about hormonal effects on cyclic GMP degradation. However, this approach has two major disadvantages: (1) The metabolic changes in the cell following isolation (e.g. a lowered energy state) could profoundly influence the results and (2) small effects of hormones on the guanylate cyclase in the anti-luminal cell borders may be masked by a possibly large contribution of the (hormone-insensitive?) microvillous enzyme to the total cyclic GMP pool.

The results of these measurements are reported in Paper 6. The observed lack of correlation between cyclic GMP content and guanylate cyclase activities in villous- and crypt cells is not unique for the intestinal epithelium: also during the development of fetal rat heart and lung, cyclic GMP contents decreased and guanylate cyclase activities strongly increased[147]. Both studies suggest an important role of cyclic GMP-dependent phosphodiesterases in

the regulation of cyclic GMP levels.

As discussed in Paper 6, the inability to alter cyclic GMP levels in villous- and crypt cells by addition of hormones or hormone-like agents does not definitively exclude a role of cyclic GMP as a mediator of hormone action. The negative results would be of more value if the cyclic GMP measurements could be directly coupled to measurements of ion transport rates. The silicon oil filtration technique, particularly suited for rapid transport measurements in isolated epithelial cells [148](Chapter V) offers good perspectives to accomplish such studies.

CHAPTER V CYCLIC NUCLEOTIDES IN RELATION TO PROLIFERATION AND DIFFERENTIATION

5.1 Introduction

3',5'-Cyclic nucleotides have been implicated as important modulators of growth and differentiation of cells in culture. Cyclic GMP seems to act as an initiator of cell growth, probably by promoting the movement of cells out of the G_o or G_1 phase of the cell cycle[149]. In cultured fibroblasts and lymphocytes[116,150], specific growth factors (fibroblast growth factor (FGF), bacterial lipopolysaccharides (LPS), lectins) may elevate intracellular levels of cyclic GMP, possibly by stimulating the guanylate cyclase activity in the plasma membrane[117,149]. By contrast, increased cyclic AMP levels may evoke the opposite pleiotypic response[151]. Intracellular cyclic AMP concentrations are negatively correlated with growth rates among a variety of fibroblast cell lines[152]. Transformed cells have a lower cyclic AMP content than untransformed cells[153]. A rise of intracellular cyclic AMP, induced by exogenous cyclic AMP analogs, activators of adenylate cyclase (choleratoxin, β-adrenergic agents, prostaglandins, peptide hormones) or inhibitors of cyclic phosphodiesterases (methylxanthines, papaverine, cytokinins[154]) slows or stops growth in a great number of cell cultures[155]. In most cell lines studied, cyclic AMP arrests the cell cycle specifically in G_1, a common control point in growth regulation[156-159]. In exceptional cases the cyclic AMP-sensitive phase seemed to be G_2[156,160] or S[161]. Cyclic AMP most probably acts through activation of a protein kinase, for kinase-deficient mutants of mouse lymphoma cells do not respond to dibutyryl-cyclic AMP and maintain a normal cell cycle[157]. The latter result also proves that cylic AMP fulfills a non-essential function in cell cycle regulation. At which level the protein kinase regulates

growth is unknown thus far.

Cyclic AMP may play a more essential role as an inductor of enzymes or structural proteins[162]. In prokaryotes enzyme synthesis subjected to catabolite repression is under cyclic AMP control at the transcriptional level[162,163]. In this system, cyclic AMP, bound to a receptor protein (no protein kinase) facilitates the attachment of RNA polymerase to the promotor locus of the operon. Cyclic GMP inhibits enzyme induction by competition with cyclic AMP for its receptor protein. Catabolites (glucose, tryptophan) act by depressing cyclic AMP levels. Many eukaryotic cell types respond to cyclic AMP with a rapid induction of specific enzymes (e.g. catabolic enzymes in liver) or a repression of others (e.g. anabolic liver enzymes; see ref. 162 for a review). Both transcriptional and translational control may exist, probably based on phosphorylation, by cyclic AMP-dependent protein kinases, of chromatin proteins and ribosomal proteins respectively. There is no information about a possible role of cyclic GMP in enzyme induction.

In vivo studies of the role of cyclic nucleotides in cell growth and differentiation in the whole animal are complicated by the lack of synchronicity in most physiological cell renewal systems, the lack of tissue specificity of a number of agents used to alter cyclic nucleotide levels, the possible interference of degradation products of these agents (e.g. adenosine in case of cyclic AMP) and the difficulty to measure the nucleotide levels in situ in a well-defined cell type. Results obtained with epidermis[156,164], human lymphocytes[61,150,160,165-169], embryonal palatal shelves of the rat[170] and rat mammary tumors[171] tend to confirm the anti-mitotic action of cyclic AMP or the mitogenic properties of cyclic GMP. These studies led to the view that proliferative skin diseases, in particular psoriasis, have their origin in an unbalanced ratio of cyclic AMP and cyclic GMP in the epidermis. They also postulate an universal role of cyclic nucleotides as intracellular media-

tors of antigen-induced activation and proliferation of immunocytes. However, inertia or even positive effects of cyclic AMP on growth rates in vivo have been suggested in studies of regenerating liver[172], thymocytes[173], salivary gland acinar cells[174], Morris hepatoma cells[175], human breast tumors[176] or melanoma tumors in mice [159]. In the latter model system, where cyclic AMP levels were elevated by daily injections of melanocyte stimulating hormone (MSH), it has been proposed that the cells could escape the G_1-restriction point by a discontinuous reception of hormone signals, based on the observation that MSH-receptors were only available during the G_2-phase[159]. A recent reinvestigation of the salivary gland model in vivo[177] showed that the growth stimulation by isoproterenol and its analogs is based on an unusual elevation of cyclic GMP levels in the acinar cells by these β-adrenergic agents. In order to resolve further some of these discrepancies between in vivo and in vitro studies and to prove the universal character of the involvement of cyclic nucleotides in regulation of growth and differentiation, extension of these studies to a new model system of physiological importance seemed highly desirable.

5.2 Growth and differentiation in small intestinal epithelium

Despite its potential usefulness as a model for studies of growth regulation and cytodifferentiation in mammalian systems, the basic regulatory mechanisms for homeostasis in intestinal epithelium are poorly understood. The existence of a feedback control by differentiated villous cells on the proliferative activity in the intestinal crypts, possibly mediated by local growth inhibiting factors (chalones) is suggested but not convincingly proven[178]. Also a role for humoral factors has been advocated[179]. In rat small intestine, a number of agents (e.g.

bile acids[180], serotonin[181], epinephrine or noradrenalin[182], pentagastrin[183]) and conditions (e.g. lactation[184], irradiation[178], starvation[185], diabetes[186], germ-free state[187], flattened mucosa[188]) are known to affect crypt cell proliferation or differentiation. In none of these studies a possible involvement of cyclic nucleotides has been explored. However, it may be speculated that at least the growth inhibition (appr. 50-70 per cent) found during β-adrenergic interaction of the crypt cells with isoproterenol or epinephrine or the shortening of cell cycle time (appr. 50 per cent) after α-adrenergic stimulation by norepinphrine[182] is mediated by cyclic nucleotides.

The choice of the small intestinal epithelium as an experimental system for studying such relation may be motivated by the following considerations:

(1) Proliferative, maturative and functional absorptive cells are regularly distributed along the length of crypts and villi. Epithelial cells in successive stages of development can be isolated separately due to this distinct localization. Also other separation methods are available, based on different binding characteristics to plant lectins[189,190]. By measurements of biochemical parameters in these cell populations, changes in the developmental pattern in relation to cell age may be sensitively monitored. Moreover, suitable techniques are known to detect altered growth rates or a possible extension of the proliferative cell pool (autoradiography or biochemical analysis after pulse labelling with [^{3}H]thymidine, mitotic counting, cell cloning).

(2) Our previous work (Chapters III and IV) showed the way to alter cyclic nucleotide levels in both villous- and crypt cells. Whereas cyclic GMP levels could only be raised by a non-specific agent, alloxan, cyclic AMP was markedly elevated by prostaglandins, epinephrine and phosphodiesterase inhibitors <u>in vitro</u> and choleratoxin

in vivo. These agents were expected to be useful tools for studying a hypothetical relationship between cyclic nucleotides and cell development.

The study was carried out at two levels. In a first approach, macromolecular synthesis was measured in isolated crypt cells, exposed to some of the fore-mentioned agents. Side-effects on other tissues do not interfere in this system. However, in view of the short survival time of isolated crypt cells[1], this method could only be used to detect rapid effects of cyclic nucleotides on precursor transport, metabolism or macromolecular synthesis. The consequences of cell cycle blockade or changes in enzyme development cannot be adequately studied in this model. In a subsequent study choleratoxin was administered in vivo and its influence on some cell-kinetic parameters was examined during the next two days. Purified choleratoxin seemed highly preferable over the other compounds for the following reasons:

First, a chalone-like action of this agent has been confirmed in a number of fibroblast cell lines[191,197], mouse leukaemia cells[168], melanoma cells[193] and T-lympnocytes[61,167,168]. Also enzyme induction by the choleragen in vivo has been reported (e.g. alkaline phosphatase in the liver[194]). Secondly, if applicated intraluminally in adult rats, side effects on other tissues are completely avoided since the toxin does not pass the intestinal wall. Moreover, in contrast to the other agents, choleratoxin activates the adenylate cyclase in an irreversible manner. High intracellular levels of cyclic AMP may therefore persist for a long time period after short contact to choleragen[191-193]. Finally, its action on adenylate cyclase is very specific in all cell types studied so far and is not toxic to the animal if dehydration is prevented.

The technique described in the following section allowed a study of cell migration and enzyme development during cholera for any time period desired without need for

infusion of salt solutions. The results could also be of clinical interest, because the duration of choleragen-induced diarrhea probably depends on the transit time of the affected epithelial cells[195].

5.3 Methods

Male Wistar rats, weighing 220-250 g, were maintained on a normal laboratory diet. Prior to the choleratoxin-experiments, rats were fasted for two days.

Isolation and incubation of villous- and crypt cells and measurements of incorporation of radioactive label into TCA-insoluble material were carried out in principle as described in Paper 2. Concanavalin A-selected cells were prepared according to Paper 5.

The method for measuring cyclic AMP levels in isolated crypt cells is mentioned in Paper 6. Adenylate cyclase activities were measured in principle as described in Paper 4. In the purification procedure, Dowex columns were replaced by Alumina columns (Paper 5).

Uptake of [^{3}H]thymidine by isolated epithelial cells was followed with the silicon oil filtration technique, recently published by Lamers and Hülsmann[148]. Plastic (micro-) centrifuge tubes (Eppendorf No. 3810) were filled with a bottom layer of 0.1 ml 0.5 M $HClO_4$, an intermediate layer of 0.5 ml silicon oil (Tegiloxan® A.V. 100, Goldschmidt Ltd., Essen, W.Germany) and a top layer of 0.5 ml Krebs phosphate buffer (pH 7.4), containing in addition 10 mM glucose, 1.4 mM $CaCl_2$, 0.01-100 µM [^{3}H]thymidine (0.5 µCi) and 1 mM [U-^{14}C] sorbitol (0.25 µCi). For measurements of intracellular water space [^{3}H]thymidine was replaced by ^3H_2O (0.25 µCi). The tubes were equilibrated at 0° or 37° in a 95% O_2/5% CO_2 atmosphere. Samples of 50 µl from a concentrated cell suspension (25-30 mg protein/ml), preincubated for 1 min at 0° or 37°, were rapidly transferred with an Eppendorf pipette into the top layer.

After 1-5 min of incubation cells were separated from the medium within 15 sec by sedimentation at 10.000 g for 4 min. Samples, taken from the cell-free top layer and from the bottom layer were analysed for radioactivity. The perchloric acid pellets were washed and [^{3}H]thymidine incorporation measured as described in Paper 2. The method for calculating intracellular water space and uptake rates of [^{3}H]thymidine is given in ref.148.

The experiments using choleratoxin were carried out as follows: in anesthetized fasted rats, appr. 10 cm from the pylorus,two adjacent segments of small intestine, each 5 cm in length and cleared of intestinal contents, were closed at both sides with ligatures. The ropes were tightened around the stalk of a match, in order to facilitate the loosening afterwards and to reduce tissue damage as much as possible. The distal (control) segment was filled with 0.5 ml saline with a needle. The proximal segment received 10 µg purified choleragen in saline. Then the abdomen was closed with woundclips. Three hours later, the lumen of both segments was thoroughly perfused with 80 ml saline using two needles introduced at both ends of each segment. Residual amounts of unbound choleragen were removed by this procedure. Then the ligatures were cut and the abdomen again carefully closed with woundclips. One, six or twelve hours afterwards the animals were injected intraperitoneally with 10 µCi [^{3}H]thymidine (20 Ci/mmole) and sarcrificed at different time intervals after injection. Parts of the segments were fixed in neutral formalin and processed for autoradiography as indicated in Paper 2. Sections were poststained with hematoxylin-eosin or PAS. In at least two sections of each segment the position of the highest labelled cells along 20-30 longitudinally cut crypts and villous columns was determined. Residual part of the segments were used for measurements of [^{3}H]thymidine incorporation into TCA-insoluble material, as described in Paper 2.

Radioactive materials were obtained from The Radiochemical Centre, Amersham. In all studies [^{3}H-methyl]thymidine was used. Biochemicals usually came from Boehringer, Mannheim. Theophylline (1,3-dimethylxanthine) and caffeine (1,3,7-trimethylxanthine) came from Merck, Darmstadt and MIX (3-isobutyl-1-methylxanthine, SC-2964) from Aldrich (Milwaukee, Wis. USA). RO 20-1723 (1-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidone) was a gift from Hoffmann-La Roche, New York. Purified choleratoxin was kindly supplied by Dr. R.S. Northrup, SEATO Cholera Research Program.
The autoradiography could be done due to the cooperation of Dr. N.J. de Both, Department of Experimental Pathology, Erasmus University, for which he is gratefully acknowledged.

5.4 Results

Table 5.1 summarizes the effect of cyclic nucleotide analogs, phosphodiesterase inhibitors, PGE_1 and epinephrine on the initial incorporation of thymidine, uridine and aminoacids into macromolecules of isolated crypt cells under conditions that cylic AMP levels were significantly elevated. The RO 20-1723 inhibitor was included because of its specificity for cyclic AMP-dependent phosphodiesterases[196] and its inactivity as an inhibitor of alkaline phosphatase (in contrast to theophylline and MIX; ref.141 and H.R. de Jonge, unpublished results). The results are strongly suggestive for a rapid inhibition of thymidine transport or utilization by cyclic AMP. Uridine transport or incorporation was affected to a minor extent or appeared unchanged whereas aminoacid incorporation was not affected at all. The inhibition by dibutyryl-cyclic AMP was probably not caused by the butyryl group for dibutyryl-cyclic GMP was inactive. Epinephrine was likewise ineffective, despite its ability to raise cyclic AMP levels. However, this phenomenon fits well in a previous hypothesis,

Table 5.1 INCORPORATION OF THYMIDINE, URIDINE AND AMINOACIDS INTO PCA-INSOLUBLE MATERIAL AND LEVELS OF CYCLIC AMP IN ISOLATED CRYPT CELLS

After preincubation for 2 min at 37° with or without the additions made, crypt cell suspensions were supplemented with 1.0 μM [^{3}H]thymidine (1 μCi) or 1.0 μM [^{3}H]uridine (1 μCi) and [^{14}C]aminoacids (10 nAtC/ml, 0.25 μCi). Incubations were stopped after 5 min by addition of perchloric acid. Net incorporation rates were measured as described in Paper 2. Mean values are given, obtained with n cell preparations. Values for cyclic AMP levels are followed by the standard error.

Additions	Final concentration (mM)	% Inhibition (-) or stimulation (+) thymidine (n=6)	uridine (n=4)	aminoacids (n=6)	pmoles cyclic AMP / mg protein (n=4)
-	-	0	0	0	11.7 ± 0.8
Dibutyryl cyclic AMP	0.5	-25	- 2	0	-
	1.0	-40	+ 3	- 3	-
Cyclic AMP	1.0	- 5	+ 4	+ 1	-
Dibutyryl cyclic GMP	1.0	- 1	0	+ 3	-
Theophylline	2.0	-60	-21	- 8	23.2 ± 2.5*
MIX (SC-2964)	0.5	-58	-20	- 4	32.5 ± 3.6*
RO 20-1723	0.1	-40	+ 2	- 5	20.0 ± 1.9*
Caffeine	1.0	-55	-35	- 5	26.3 ± 3.1*
Papaverine	0.5	-49	-62	- 7	30.7 ± 2.9*
Prostaglandin E_1	0.05	-28	-15	+ 3	20.8 ± 1.7*
Epinephrine	0.05	- 4	+ 1	+ 4	16.2 ± 1.5*
Adenosine	0.5	-84	-37	- 2	10.2 ± 0.9
AMP	0.5	-62	-	0	11.0
ADP	0.5	-27	-	+ 1	10.9 ± 1.2
Adenine	1.0	- 2	+ 4	0	-
Hypoxanthine	1.0	0	-	- 2	-

* P-value <0.05 (Student t-test).

assuming a specific β-adrenergic action on the non-proliferative goblet cells (Chapter III). Adenosine and, to a minor extent, AMP and ADP, also inhibited thymidine incoporation. In astrocytoma[197] and neuroblastoma cells[198] adenosine could elevate cyclic AMP levels via interaction with adenosine-receptors at the plasma membrane. A similar action was not observed in the isolated crypt cells (Table 5.1). The inhibition seemed specific for nucleosides because adenine itself was completely inactive.

The rapid onset of the changes in thymidine incorporation rates indicated that the inhibition could take place at the transport level. Therefore, thymidine transport in the enterocytes was characterized and the influence of the inhibitors on this process was examined.

The uptake of thymidine by isolated crypt and upper villous cells increased linearly with time for about 2 min but gradually diminished in the next period (Fig. 5.1). Upper villous cells contain thymidine kinase in a latent form[199]. Thymidine transported into these cells is not converted to TMP but instead accumulates until the intracellular concentration equals the concentration in the medium (Fig. 5.1). No evidence was found for the existence of an active transport system for this nucleoside in the enterocyte, in accordance with conclusions for other cell types studied[200,201]. Due to the accumulation of phosphorylated intermediates, thymidine uptake by the crypt cells proceeded for longer periods. Here the deflection of linearity is most probably caused by the presence of non-proliferative cells in the preparation and a gradual detoriation of the crypt cells during incubation, demonstrated by an increased loss of soluble enzymes[1] and a diminished oxygen uptake (unpublished observations). For similar reasons, incorporation of thymidine into DNA also slightly decreased after 5 min of incubation (Fig. 5.1).

In subsequent studies initial transport rates were measured after 1 min to ensure linearity of uptake. Shorter times were not applied because the sorbitol marker needed appr. 45 sec to equilibrate fully with the extracellular space.

Thymidine uptake by villous and crypt cells showed a hyperbolic relationship with the extracellular thymidine concentration at levels up to appr. 20 μM, suggesting a carrier-facilitated diffusion (Fig. 5.2). At higher concentrations transport rates were perfectly linear and unsaturable, indicating a simple diffusion process. Incorporation of label into DNA likewise increased hyperbolically at thymidine levels below 5 μM as might be expected if thymidine transport would be rate-limiting for the incorporation. At 10 μM thymidine the salvage pathway is saturated and a plateau value is reached (Fig. 5.2)*.

Fig. 5.3 shows the Lineweaver-Burk plots of initial uptake rates, corrected for the contribution of the unsaturable component, calculated from the slope of the straight lines in Fig. 5.2. Thymidine uptake in the crypt cells followed normal Michaelis-Menten kinetics. A K_m-

---

* Under conditions that the endogenous synthesis of TMP from desoxyuridine monophosphate is inhibited by preincubating the crypt cells for 10 min in the presence of 30 μM hypoxanthine, 100 μM glycine and 10 μM amethopterin (a folic acid antagonist) as recommended by Siegers et al.[202], the maximal incorporation rate of thymidine amounted to 3.2 pmoles.min^{-1}.μl^{-1} intracellular space, equivalent to 27 pmoles.min^{-1}.mg^{-1} protein or appr. 270 pmoles.min^{-1}.mg^{-1} DNA (compare Paper 2). This accounts for the synthesis of appr. 16 μg DNA. hr^{-1}.mg^{-1} DNA, assuming 50 per cent thymidine-adenine base pairs in the DNA. This leads to a mean doubling time of the 30-50 per cent proliferative cells in the crypt cell preparation of appr. 24-40 h (in vivo: 10-14 h). Although part of the thymidine incorporation may be involved in DNA repair, this calculation justifies the conclusion that the DNA synthesizing system in the crypt cells is reasonably preserved during the isolation procedure.

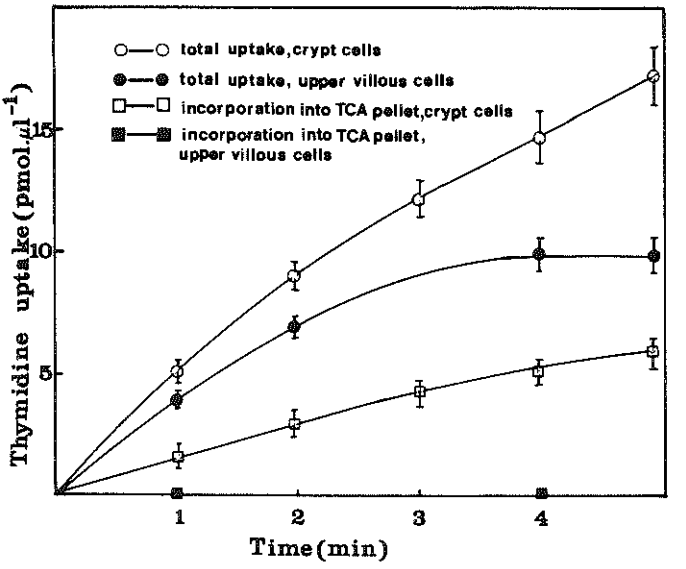

Fig. 5.1 Total uptake of thymidine (0-0, ●-●) and incorporation into the TCA-insoluble fraction (□-□, ■-■) by isolated crypt and upper villous cells as a function of time. Cells were incubated under a 95% O_2/5% CO_2 atmosphere at 37° in the presence of 10 µM [^{3}H]thymidine. Each point represent the mean of four experiments (± S.E.). Rate of transport/incorporation were expressed as pmoles thymidine per µl intracellular water space. Intracellular water volume of crypt and upper villous cells were 6.03±0.14 (n=6) and 8.43±0.22 (n=6) µl/mg protein respectively.

value of 5 µM and a V equal to 3.5 pmoles.min^{-1}.µl^{-1} intracellular water could be calculated from the Figure. The apparent V decreased to 0.4 pmoles.min^{-1}.µl^{-1} at 0° (average Q_{10}: 1.7). The uncorrected uptake rate at 100 µM decreased by 2.5 fold, confirming the simple diffusion character of this process. An equal value for the K_m and a slightly lower value for the V (3.0 pmoles.min^{-1}.µl^{-1}) was measured in upper villous cells (results not shown).

All compounds shown to inhibit thymidine incorporation into macromolecules (Table 5.1) caused a similar degree of transport inhibition (Figs. 5.2 and 5.3). The inhibition was of competitive nature for all agents tested (Fig. 5.3). Further experiments indicated that the trans-

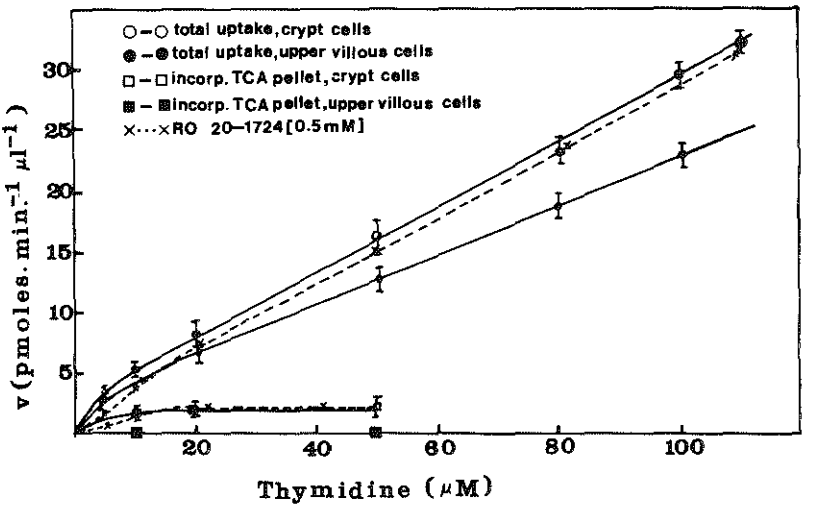

Fig. 5.2 Initial rates of thymidine uptake (0-0, ●-●) and incorporation (□-□, ■-■) by isolated upper villous cells and crypt cells as a function of the thymidine concentration in the medium. Incubations were carried out for 1 min at 37° under 95% O_2/5% CO_2. Each point represents the mean of three experiments (± S.E.).

port inhibition was the cause, and not the consequence, of the decreased rate of thymidine incorporation into DNA: similar inhibitory effects on transport were measured at 0°, where the DNA synthesis is negligible. Moreover, inhibition could be reproduced in upper villous cells, where thymidine-phosphorylation is lacking (results not shown). The inhibition at 0°, found for all inhibitors tested, may also be taken as a strong argument against the involvement of intracellular cyclic AMP that did not increase significantly under these conditions (not shown). Therefore, a competitive inhibition of carrier-mediated transport of thymidine in the plasma membrane seemed the best explanation for the effects of PGE_1, phosphodiesterase inhibitors and nucleosides on thymidine incorporation into crypt cell DNA *in vitro*. The inhibition of transport, and concomitant-

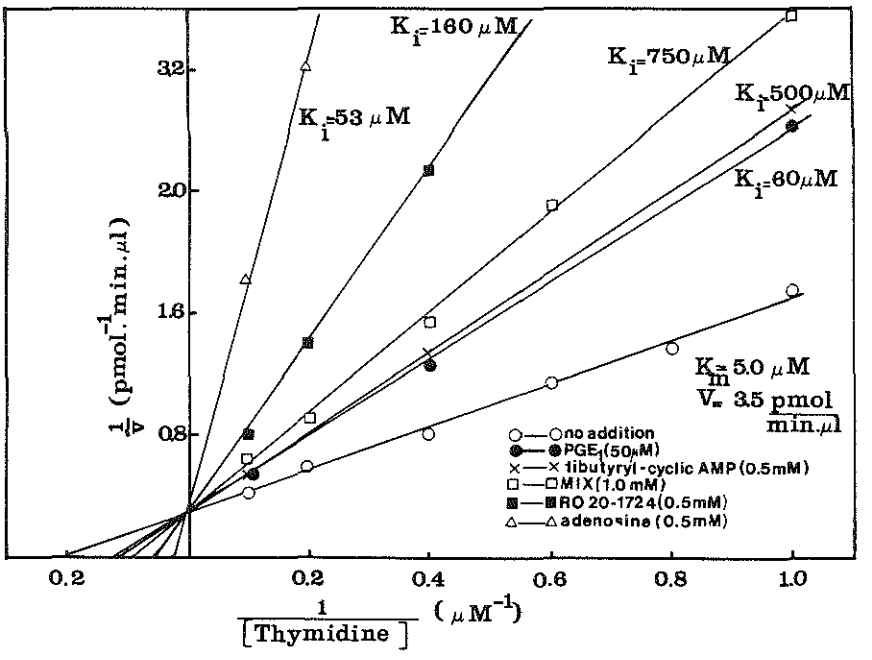

Fig. 5.3 Lineweaver-Burk plots of the initial rate of carrier-mediated thymidine transport into isolated crypt cells as a function of the extracellular thymidine concentration. Transport rates, calculated from the total amount of [^{3}H]thymidine taken up by crypt cells during incubation for 1 min at 37^o in a 95% O_2/5% CO_2 atmosphere in the presence or absence of inhibitors were corrected for the contribution of simple diffusion.

ly of incorporation, could be overcome at high thymidine levels (compare Fig. 5.2) indicating that simple diffusion was unaffected by the compounds.

In an alternative approach,cyclic AMP levels in the crypt region were elevated appr. two times by in vivo treatment of an intestinal segment with choleragen, and [^{3}H]thymidine incorporation and cell migration in the segment were compared to an adjacent control segment. It follows from Figs. 5.4-5.6 that both parameters of cell proliferation did not appreciably differ in control and

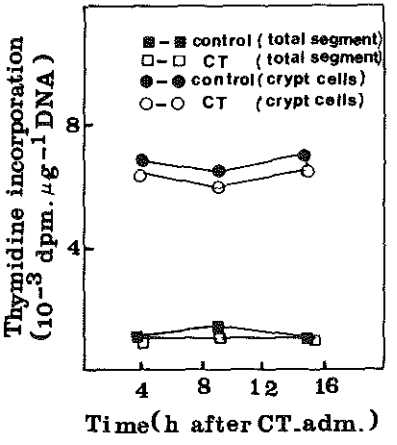

Fig. 5.4 Incorporation of [^{3}H]thymidine into PCA-insoluble material of control and CT-treated whole segments and crypt cells isolated from the segments. Mean values of three animals are given.

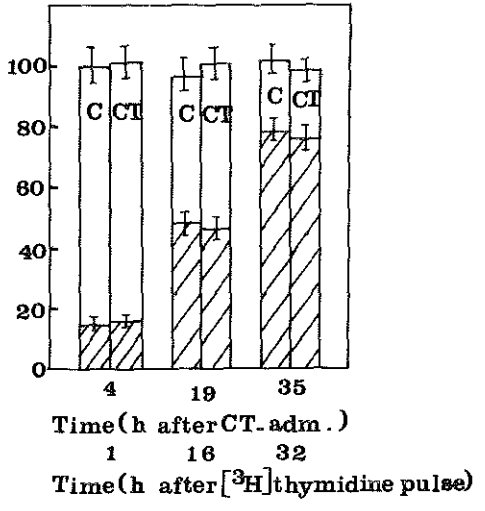

Fig. 5.5 Cell migration in duodenal rat epithelium at different time points after CT exposure. C=control, CT=choleragen segment. Ordinate: average distance of migration (arbitrary units ± S.E.) of labelled cells (shaded).
0 = crypt bottom
100 = main villus length in control segments 1 h after [^{3}H]thymidine pulse.

Table 5.2 ADENYLATE CYCLASE ACTIVITIES IN TOTAL CRYPT CELLS AND ConA-SELECTED CRYPT CELLS FROM CONTROL AND CHOLERAGEN-TREATED SEGMENTS OF RAT DUODENUM

Enzyme activities were measure 4 h after luminal administration of CT. n = number of experiments.

	Adenylate cyclase activity (pmoles cyclic AMP X 10 min^{-1} X mg^{-1} protein)			
	Control segments		Choleragen segments	
	Basal	Fluoride	Basal	Fluoride
Whole crypt population (n=4)	42	310	91	334
ConA-selected crypt cells (n=2)	48	330	64	339

choleragen segments during a period of 35 h following exposure to choleragen. In agreement with a supposed non-toxic action of the choleragen in the epithelium, intestinal morphology in experimental sections appeared to be perfectly normal. In sharp contrast to the findings in the rabbit[89] the mucus content of the goblet cells of the rat did not change during the whole period, including the acute phase of water-secretion (3-17 h) when the luminal fluid contained large amounts of mucus (Fig. 5.7).Therefore glycoprotein synthesis must be stimulated to a similar extent as mucus release during experimental cholera in the rat.

Finally, Fig. 5.8 illustrates the parallel changes of adenylate cyclase activities and net fluid movement found in the cholera segments. Both parameters returned to normal within 32 h. Because the total transit-time of the epithelial cells in control and cholera segments was appr.

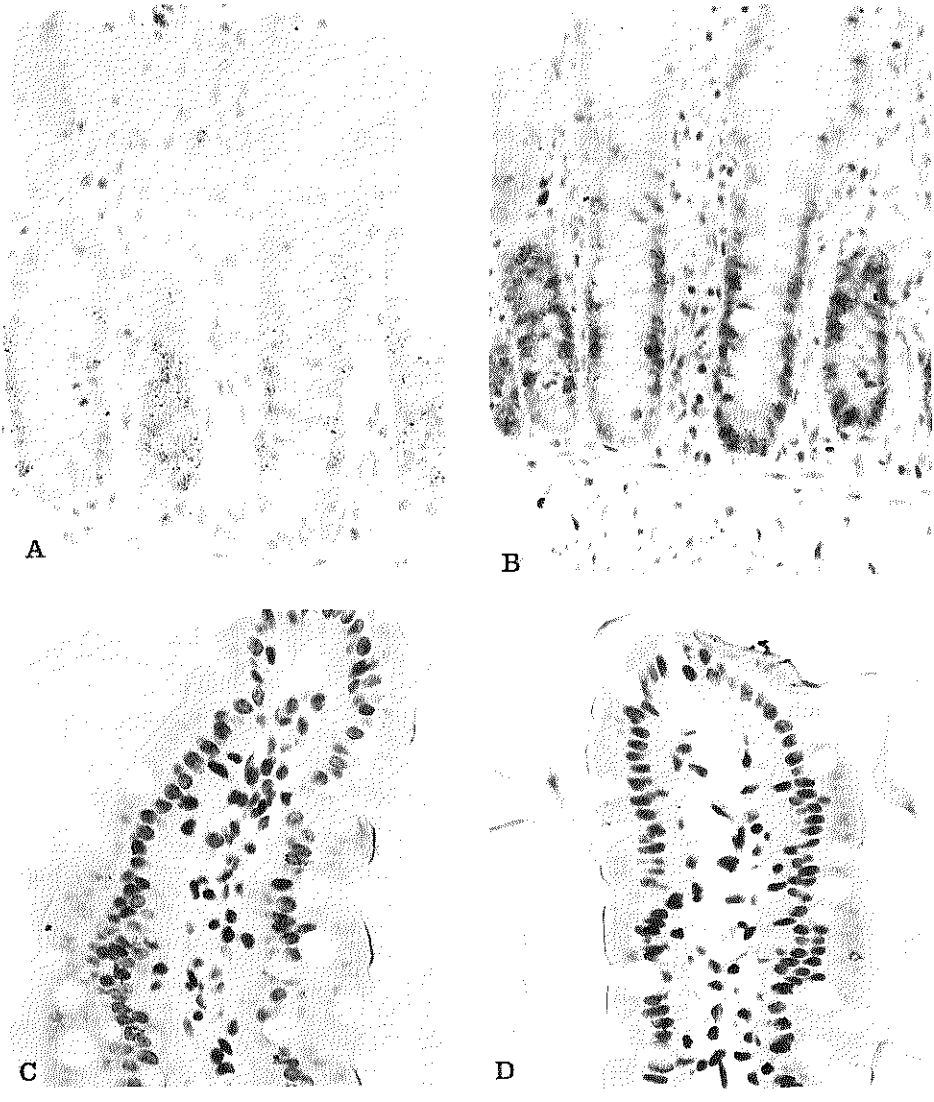

Fig. 5.6 Autoradiography in rat duodenal epithelium.
A and B: 1 h after [^{3}H]thymidine injection (4 h after CT-administration)
C and D:32 h after [^{3}H]thymidine injection (35 h after CT-administration)

A,C: Control segment
B,D: choleragen segment

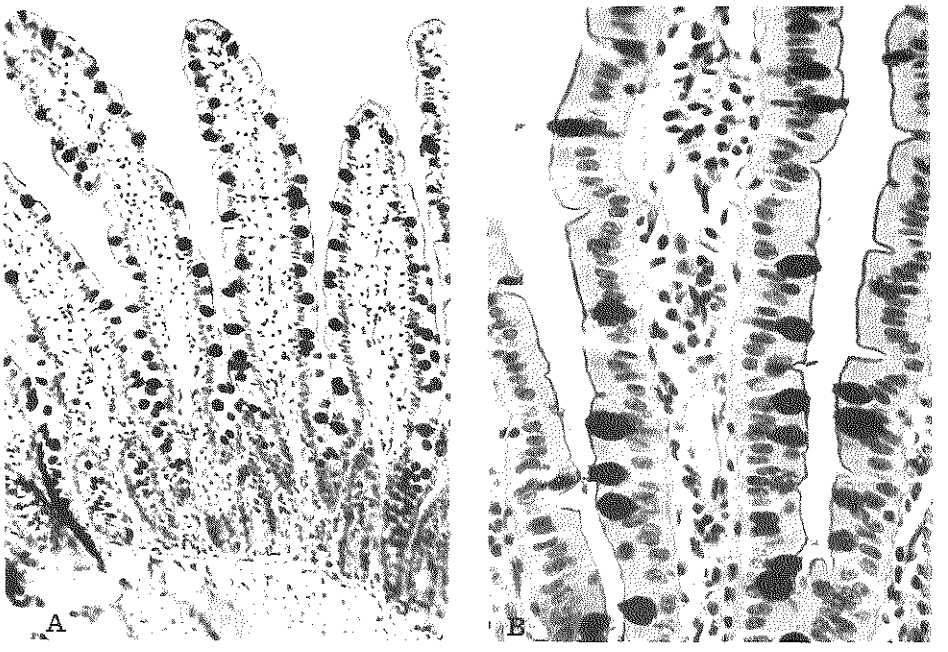

Fig. 5.7 Histological appearance of duodenal epithelium after PAS staining.
A = control segment
B = choleragen segment, 4 h after CT-administration.

48 h, the short duration of the choleratoxin action was rather surprising in view of its long term effects on other cell types. A gradual desensitization of the intestinal adenylate cyclase for the toxin, possible due to enzyme turnover, or a negligible stimulation of adenylate cyclase in the basal cell layers of the crypt during exposure to choleragen would explain the results. The latter possibility could be verified by measuring adenylate cyclase activities in conA-selected crypt cells, representing a subpopulation of the proliferative cell pool[189]. As shown in Table 5.2, the response of the enzyme in this cell type to the choleratoxin was indeed much lower than expected on the basis of its stimulatory effect on the total crypt population.

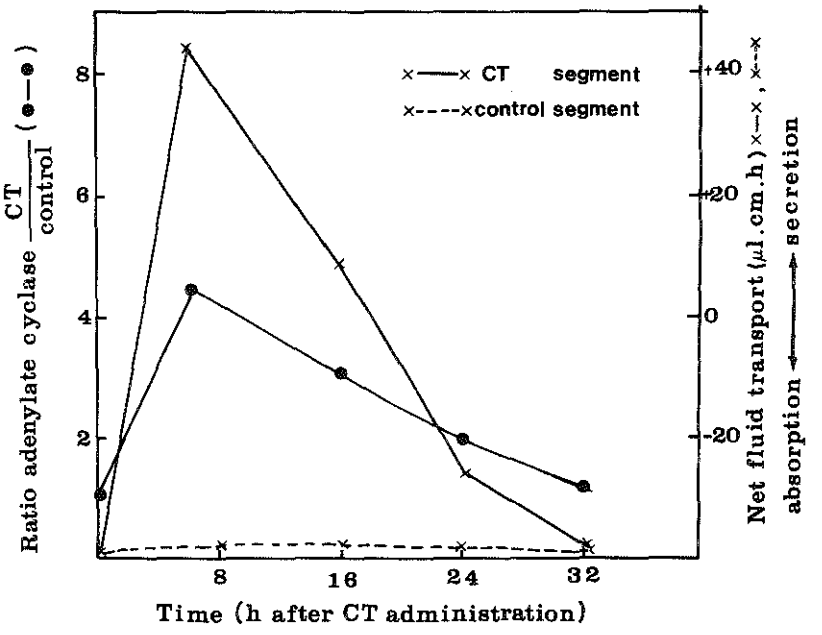

Fig. 5.8 Ratio of adenylate cyclase activities from upper villous cells in choleragen and control segments and net fluid transport in both segments.
Fluid transport was measured according to Paper 4. Mean values of three experiments are given.

5.5 Conclusions and discussion

The strong inhibitory effects of dibutyryl-cyclic AMP, several phosphodiesterase inhibitors and PGE_1 on the rates of thymidine incorporation into isolated crypt cells could be traced back to a competitive inhibition of thymidine transport at the plasma membrane level. Similar effects of theophylline, dibutyryl-cyclic AMP or prostaglandins on nucleoside transport have been reported earlier in serum-stimulated mouse embryo cells[205], virus-transformed mouse

fibroblasts[151] and rat hepatoma cells[204]. In these reports a role of cyclic AMP in this inhibition is suggested or at least not excluded. Because changes in the cellular uptake of nutritients may belong to the earliest symptoms of altered growth rates[155], transport inhibition might constitute an important basis to explain growth regulation by cyclic nucleotides[151]. However, an involvement of cyclic AMP was not very likely in the epithelial cells, for a similar degree of transport inhibition was found at 37° and 0°, despite the absence of changes in cyclic AMP levels at 0°. The phenomenon is better explained by assuming a competition by the heterologous compounds for thymidine-binding sites in the plasma membrane or a non-specific interaction with the membrane structure which would be overcome by increasing the thymidine concentration. The high $\frac{K_i}{K_m}$ ratios (10-150) indicate a much lower affinity of the transport system for the inhibitors than for thymidine. The inhibitory effects on uridine incorporation were much less pronounced (Table 5.1). If this inhibition would also be based on a competitive inhibition of uridine transport (compare refs.204 and 205), the K_i-values would largely exceed the K_i for thymidine transport. RO 20-1723, a specific inhibitor of cyclic AMP-dependent phosphodiesterase[196] and structurally unrelated to the methylxanthines, also showed a high specificity toward the thymidine transport system for incorporation of uridine was not affected by this compound (Table 5.1).

Another interesting feature of the thymidine transport system in the intestine is the conservation of its full capacity during the whole lifetime of the epithelial cell, despite the complete loss of thymidine-phosphorylating activity in an early stage of development. In other cell types studied the maximal velocity of thymidine transport appeared strongly dependent on growth rate[200].

The <u>in vitro</u> experiments learned that studies involving the use of labelled precursors to establish the

effects of phosphodiesterase inhibitors, prostaglandins or cyclic AMP analogs on metabolism or macromolecular synthesis must be interpreted very carefully. By using choleratoxin for activation of the intestinal adenylate cyclase, inhibitory effects at the transport level, not related to cyclic AMP, may be excluded. Initially the use of choleratoxin to assess a possible relationship between cyclic AMP and crypt cell proliferation seemed justified by the observations that 3 h after luminal application of the choleragen, toxin molecules were uniformly distributed along crypts and villi in rabbit intestine[206] while the adenylate cyclase activity in total crypt populations from rabbit[85] and rat (Paper 4) was increased appr. twofold over the basal level. Therefore it was expected that choleragen-affected cells at the bottom of the crypt would be exposed to increased intracellular levels of cyclic AMP for at least one generation time (10-14 h). This time, normally needed to pass the proliferation zone of the crypt, would be enough to detect an effect of cyclic AMP elevation on the cell cycle.

However, in the experimental cholera model, no indication was found for a change in cell kinetics resulting from choleratoxin-treatment. This led to a reinvestigation of the extent of adenylate cyclase activation in the proliferating cell layers, permitted by the availability of a new isolation technique (Table 5.2). Indeed, the response of this cell population in vivo appeared to be small as compared to the total crypt population isolated by the vibration technique. These preparations contain both proliferative and maturative cells and are contaminated to a variable degree with basal villous cells. Presumably the increase of adenylate cyclase activity is localized preferentially in the non-dividing cell types. The low sensitivity of the proliferative cells for the toxin may find its origin in a limited accessibility of the deeper crypt layers for luminal choleragen or a possible lack of ganglioside receptors

in the undeveloped luminal membranes; also in other tissues the responsiveness to growth inhibition and adenylate cyclase activation was governed by the availability of GM_1 receptors, and markedly reduced amounts of ganglioside G_{M1}, G_{M2} and $G_{DI\alpha}$ as well as UDP-GalNAc: G_{M3}-N-acetylgalactosaminyltransferase activity (involved in glycolipid synthesis) have been found in mouse and hamster cells after transformation[192]. The first explanation seemed rather unlikely in view of the uniform distribution pattern of the toxin examined in rabbit intestine. A direct proof of the second hypothesis by incubation of ConA-selected crypt cells with choleragen followed by measurements of cyclic AMP levels or adenylate cyclase activity could not be easily given in view of the short survival time of crypt cells at 37° and the characteristic lag time of appr. 30 min in cyclase activation. A direct determination of the number of ganglioside receptors in the proliferative cells using [^{125}I]-labelled choleragen[191] might elucidate the question. A proposed low sensitivity of the proliferating cells towards choleratoxin seems hard to reconcile with the threefold rise of adenylate cyclase activity in foetal rabbit intestine after <u>in vitro</u> exposure to choleragen[207]. This response was found in a period of active cell proliferation before the appearance of villi and microvilli. However, these enzyme determinations were done in stripped mucosa containing appr. one half the thickness of the muscularis, raising the possibility that mainly the effect of toxin on the non-proliferative tissue was measured. This is even more likely in the light of an enhanced permeability of foetal intestine for macromolecules as compared to the adult state. Therefore, a low response of foetal intestinal epithelium to choleragen due to a lack of receptors for this toxin is not definitely excluded by this study. The short duration of the choleragen effects on the intestine (Fig. 5.8) may be totally explained by the inability of choleragen to affect the proliferative cell pool.

If only the non-proliferative cells in crypt and villus should be affected by luminally administrated toxin, recovery from cholera was expected to be finished at appr. 34-38 h, the time needed for the maturative crypt cells to reach the villous tip (compare Fig. 5.5). This corresponds reasonably well with the period of adenylate cyclase activation in the upper villous region and a diminished rate of fluid absorption (Fig. 5.8). The data shown in Fig. 5.8 adds new evidence for an active role of the villous cells in cholera-induced secretion (Paper 3): a clearly diminished absorption rate could also be demonstrated in the period within 20-32 h, when the choleragen-affected population must be replaced by new unaffected cells (Fig. 5.5). The recovery of cholera patients is reported to take appr. 3 days following the first administration of antibiotics[208]. This period is also markedly less than the total cellular transit-time of human epithelium (appr. 5 days)[209]. Here the absence of net water secretion was used as a criterium for recovery. However, this situation appeared as soon as sufficient new cells have been formed to compensate fluid secretion by the choleragen-contacted cells. A considerably longer time may be required for complete recovery of the whole cell population (compare Fig. 5.8).

In summary, although the experimental cholera model failed to establish an effect of cyclic AMP on cell growth it ensured initially high levels of cyclic AMP in maturative crypt and absorptive villous cells. These levels are sustained for a sufficiently long period to enable a study of the possible role of cyclic AMP in the intestinal differentiation process in the intact animal. Interpretation of the results will be facilitated by the present demonstration of normal crypt cell kinetics during cholera. Preliminary results of a similar study in vitro with cultured rabbit intestine have revealed a marked depression of the glycolytic enzyme pryuvate kinase and a twofold stimulation of the gluconeogenic enzyme fructose-1,6-diphosphatase

after 6 h of exposure to choleragen[210]. It would be interesting to see whether the same phenomenon occurs *in vivo*, particularly because both enzymes, extensively characterized in previous studies from our laboratory[211,212], may play an important role in glycolytic regulation[211].

REFERENCES

1. Hülsmann, W.C., van den Berg, J.W.O. and de Jonge, H. R. (1974) Methods in Enzym. (ed. Fleischer, S. and Packer, L.) Academic Press, New York, Vol. XXXII, pp. 665-673
2. de Vries, H. and Kroon, A.M. (1974) in The Biogenesis of Mitochondria (ed. Kroon, A.M. and Saccone, C.) Academic Press, New York, pp. 357-365
3. Kroon, A.M., Arendzen, A.J. and de Vries, H. (1974) in The Biogenesis of Mitochondria (ed. Kroon, A.M. and Saccone, C.) Academic Press, New York, pp. 395-402
4. Firkin, F.C. and Linnane, A.W. (1968) Biochem. Biophys. Res. Commun. 32, 398-402
5. Firkin, F.C. and Linnane, A.W. (1969) Exp. Cell Res. 55, 68-76
6. de Vries, H. and Kroon, A.M. (1970) Biochim. Biophys. Acta 204, 531-541
7. Iemhoff, W.G., van den Berg, J.W.O., de Pijper, A.M. and Hülsmann, W.C. (1970) Biochim. Biophys. Acta 215, 229-241
8. Dickens, F. and Weil-Malherbe, H. (1941) Biochem. J. 35, 7-15
9. Wilson, T.H. (1956) J. Biol. Chem. 222, 751-763
10. Lohman, K., Graetz, H. and Langen, P. (1966) in Current Aspects of Biochemical Energetics (ed. Kaplan, N.O. and Kennedy, E.P.) Academic Press, New York, pp. 111-126
11. Wenner, C.E. (1967) Adv. Enzymol. 29, 321-390
12. Detheridge, J.F., Matthews, J. and Smyth, D.H. (1966) J. Physiol. 183, 369-377
13. Lamers, J.M.J. and Hülsmann, W.C. (1972) Biochim. Biophys. Acta 275, 491-495
14. Pietra, P. and Cappelli. V. (1970) Experientia 26, 514-515
15. Frizzel, R.A., Markscheid-Kaspi, L. and Schultz, S.G. (1974) Am. J. Physiol. 226, 1142-1148
16. Hersey, S.J. (1974) Biochim. Biophys. Acta 344, 157-203
17. Gijzel, W.P., Strating, M. and Kroon, A.M. (1972) Cell Differentiation 1, 191-198
18. Hülsmann, W.C., Iemhoff, W.G., van den Berg, J.W.O. and de Pijper, A.M. (1970) Biochim. Biophys. Acta 215, 553-555
19. Ferguson, S.M.F. and Williams, G.R. (1971) J. Biol. Chem. 241, 3696-3700
20. Windmueller, H.G. and Spaeth, A.E. (1974) J. Biol. Chem. 249, 5070-5079
21. de Pijper, A.M. and Hülsmann, W.C. (1972) Histochemie 33, 181-190
22. Schatz, G. and Mason, T.L. (1974) Ann. Rev. Bioch. 43, 51-88

23. Hallman, M. (1971) Biochem. Pharmac. 20, 1797-1809
24. Dale, E., Baxley, J.B. and Scatchfield, F.D. (1967) Experientia 24, 164-166
25. Levi, A.J., Gatmaian, Z. and Arias, I.M. (1969) J. Clin. Invest. 48, 2156-2167
26. Yeh, S.D.J. and Shils, M.E. (1966) Proc. Soc. Exp. Biol. Med. 123, 367-370
27. Pikő, L. and Chase, D.G. (1973) J. Cell. Biol. 58, 357-378
28. Oerter, D. and Bass, R. (1972) Naunyn-Schmiedeberg's Arch. Pharmacol. 272, 239-242
29. Martelo, O.J., Manyan, D.R., Smith, U.S. and Yunis, A.A. (1969) J. Lab. Clin. Med. 74, 927-940
30. Smith, U., Smith, D.S. and Yunis, A.A. (1970) J. Cell Sci. 7, 501-521
31. Becker, D., Metz, J. and Katz, J. (1974) Postgraduate Med. J. 50, 88-94
32. Ringrose, P.S. and Lambert, R.W. (1973) Biochim. Biophys. Acta 299, 374-384
33. Hamberger, A. and Isaksson, O. (1973) Exp. Cell Res. 76, 207-217
34. Brody, T.M., Hurwitz, R. and Bain, J.A. (1954) Antibiot. Chemother. 4, 864-870
35. Franklin, T.J. (1963) Biochem. J. 87, 449-453
36. Bottaner, E. and Vazques, D. (1971) Biochim. Biophys. Acta 254, 316-330
37. Greenberger, N.J. (1967) Nature, Lond. 214, 702-703
38. Ling, V. and Morin, C.L. (1971) Biochim. Biophys. Acta 249, 252-259
39. Tacker, P.C. and Webster, P.D. (1972) Am. J. Dig. Diseases 17, 675-681
40. Fuska, J., Podojil, M., Fusková, A. and Vaněk, Z. (1974) Experientia 30, 403-404
41. Mukherjee, D., Ghosh, H.N. and Mukherjee, S. (1971) J. Antibiotics 24, 263-265
42. Guiti, N. and Abtahi, F. (1973) Chemotherapy 19, 65-74
43. Counis, R., Koumanov, K., Raulin, J. and Infante, R. (1973) Eur. J. Biochem. 37, 244-247
44. Feldman, H.A. and Singel, I. (1974) J. Pharm. Exp. Ther. 190, 358-364
45. Forsgren, A. and Gnarpe, H. (1973) Antimicrob. Ag. Chemother. 3, 711-715
46. Gray, J.E., Weaver, R.N., Skinner, P., Mathews, J. Day, C.E. and Stern, K. (1974) Toxicol. and Appl. Pharmacol. 30, 317-332
47. Shorter, R.G., Moertel, C.G., Titus, J.L. and Reitemeiter (1964) Am. J. Dig. Diseases 9, 760-763
48. Rall, T.W., Sutherland, E.W. and Berthet, J. (1957) J. Biol. Chem. 224, 463-475
49. Rall, T.W. and Sutherland, E.W. (1958) J. Biol. Chem. 232, 1065-1076
50. Orloff, J. and Handler, J.S. (1962) J. Clin. Invest. 41, 702-709
51. Grantham, J.J. and Burg, M.B. (1966) Am. J. Physiol. 211, 255-259

52. Field, M., Plotkin, G.R. and Silen, W. (1968) Nature 217, 469-471
53. Schafer, D.E., Lust, W.D., Sircar, B. and Goldberg, N.D. (1970) Proc. Natl. Acad. Sci. USA 67, 851-856
54. Banwell, J.G. and Sherr, H. (1973) Gastroenterology 65, 467-497
55. Field, M. (1974) Gastroenterology 66, 1063-1084
56. Kimberg, D.V. (1974) Gastroenterology 67, 1023-1064
57. Holmes, R.K., Vasil, M.L. and Finkelstein, R.A. (1975) J. Clin. Invest. 55, 551-560
58. Richardson, S.H. (1968) Biochim. Biophys. Acta 150, 572-577
59. Leitch, G.J. (1972) Exp. and Mol. Path. 17, 187-197
60. van Heyningen, W.E. (1973) Naunyn-Schmiedeberg's Arch. Pharmacol. 276, 289-295
61. Holmgren, J., Lindholm, L. and Lönnroth, I. (1974) J. Exp. Med. 139, 801-819
62. Holmgren, J., Lönnroth, I. and Svennerholm, L. (1973) Infect. Immun. 8, 208-214
63. Bennet, V., O'Keefe, E. and Cuatrecasas, P. (1975) Proc. Natl. Acad. Sci. USA 72, 33-37
64. Lönnroth, I. and Holmgren, J. (1975) J. Gen. Microbiol., in press
65. Cuatrecasas, P. (1973) Biochemistry 12, 3547-3581
66. Bowman, C.M., Sidikaro, J. and Nomura, M. (1971) Nature 234, 133-137
67. de Graaf, F.K., Niekus, H.G.D. and Klootwijk, J. (1973) FEBS Lett. 35, 161-165
68. Gill, D.M., Pappenheimer, A.M. and Uchida, T. (1973) Fed. Proc. 32, 1508-1515
69. Edidin, M. (1974) Ann. Rev. Biophys. Bioeng. 3, 179-201
70. Bennet, V. and Cuatrecasas, P. (1975) J. Membrane Biol., in press
71. Field, M. (1974) Proc. Natl. Acad. Sci. USA 71, 3299-3303
72. Rodbell, M., Birnbaumer, L., Pohl, S.L. and Kraus, H.M.J. (1971) J. Biol. Chem. 246, 1877-1882
73. Johnson, M. and Ramwell, P.W. (1973) Prostaglandins 3, 703-719
74. Ferard, G., Sall, I. and Metais, P. (1975) Enzyme 19, 38-47
75. Parkinson, D.K., Ebel, H., Dibona, D.R. and Sharp, G.W.G. (1972) J. Clin. Invest. 51, 2292-2298
76. Hirschhorn, N. and Rosenberg, I. (1968) J. Lab. Clin. Med. 72, 28-39
77. Walton, K.G. LeLorenzo, R.J., Curran, P.F. and Greengard, P. (1975) J. Gen. Physiol. 65, 153-177
78. Schwarz, I.V., Shlatz, L.J., Kinne-Safran, E. and Kinne, R. (1974) Proc. Natl. Acad. Sci. USA 71, 2592-2599
79. Lucid, S.W. and Cox, A.C. (1972) Biochem. Biophys. Res. Commun. 49, 1183-1186

80. Kimberg, D.V., Field, M., Gershan, E., Schooley, R.T. and Henderson, A. (1973) J. Clin. Invest. 52, 1376-1383
81. Powell, D.W., Farris, R.K. and Carbonetto, S.T.(1974) Am. J. Physiol. 227, 1428-1435
82. Powell, D.W. (1974) Am. J. Physiol. 227, 1436-1443
83. Frizzel, R.A., Nellans, H.N., Rose, R.C., Markscheid-Kapsi, L. and Schultz, S.G. (1973) Am. J. Physiol. 224, 328-337
84. Nellans, H.N., Frizzel, R.A. and Schultz, S.G. (1973) Am. J. Physiol. 225, 467-475
85. Schwartz, C.J., Kimberg, D.V. and Ware, P. (1975) Gastroenterology 68, 94-104
86. Yardley, J.H., Bayless, T.M., Luebbers, E.H., Halsted, C.H. and Hendrix, T.R. (1972) Hopkins Med. J. 131, 1-10
87. Forstner, G., Shih, M. and Lukie, B. (1973) Can. J. Physiol. Pharmacol. 51, 122-129
88. Field, M. and McColl, I. (1973) Am. J. Physiol. 225, 852-857
89. Steinberg, S.E., Banwell, J.G., Yardley, J.H., Keusch, G.T. and Hendrix, T.R.(1975) Gastroenterology 68, 309-317
90. Mc.Donel, J.L. (1974) Infection and Immunity 10, 1156-1162
91. Flores, J., Grady, G.F., McIver, J., Witkum, P., Beckman, B. and Sharp, G.W.G. (1974) J. Infect. Dis. 130, 374-379
92. Schwartz, C.J., Kimberg, D.V., Sheerin, H.E., Field, M. and Said, S.I. (1974) J. Clin. Invest. 54, 536-544
93. Klaeveman, H.L., Conlon, T.P., Levy, A.G., Gardner, J.D. (1975) Gastroenterology 68, 667-675
94. Tidball, C.S. (1961) Am. J. Physiol. 200, 309-312
95. Bolton, J.E., Munday, K.A. and Parsons, B.J. (1974) J. Physiol. (Lond.) 27P
96. Soergel, K.H., Whalen, G.E., Harris, J.A. and Greenen, J.E. (1968) J. Clin. Invest. 47, 1071-1082
97. Kimberg, D.V., Field, M., Johnson, J., Henderson, A. and Gershon, E. (1971) J. Clin. Invest. 50, 1218-1230
98. Ammon, H.V. and Philips, S.F. (1974) J. Clin. Invest. 53, 205-210
99. Ammon, H.V., Thomas, P.J. and Philips, S.F. (1974) J. Clin. Invest. 53, 374-379
100. Gaginella, T., Bass, P., Olsen, W. and Shug, A. (1975) FEBS Lett. 53, 347-350
101. Binder, H.J., Filburn, C., Volpe, B.T. (1975) Gastroenterology 68, 503-508
102. Conley, D.R., Coyne, M.J., Chung, A., Bonorris, G.G. and Schoenfield, L.J. (1975) Abstr. Gastroenterology 68, 877
103. Wald, A. and Bayless, T.M. (1975) Abstr. Gastroenterology 68, 1008
104. Powell, D.W. and Farris, R.K. (1975) Abstr. Gastroenterology 68, 968

105. Kerzner, B., Mc.Clung, J., Kelly, M. and Butler, D. (1975) Abstr. Gastroenterology 68, 909
106. Birnbaumer, L. and Yang, P.C. (1974) J. Biol. Chem. 24, 7867-7873
107. Anderson, J.W. and Jones, A.L. (1974) Proc. Soc. Exp. Biol. Med. 145, 268-272
108. Lamers, J.M.J., Kurpershoek-Davidov, R. and Hülsmann, W.C. (1974) Biochim. Biophys. Acta 343, 427-430
109. Gangl, A. and Ockner, R.K. (1975) Gastroenterology 68, 167-186
110. Ashman, D.F., Lipton, R., Melicow, M.M. and Price, T.D. (1963) Biochem. Biophys. Res. Commun. 11, 330-334
111. Goldberg, N.D., O'Dea, R.F. and Haddox, M.K. (1973) in Advances in Cyclic Nucleotide Research (ed. Greengard, P. and Robison, G.A.) Raven Press, New York, Vol. 3, pp. 155-222
112. Tee-Ping Lee, Kuo, J.F. and Greengard, P. (1972) Proc. Natl. Acad. Sci. USA 69, 3287-3291
113. Illiano, G., Tell, G.P.E., Siegel, M.I. and Cuatrecasas, P. (1973) Proc. Natl. Acad. Sci. USA 70, 2443-2447
114. Dunham, E.W., Haddox, M.K. and Goldberg, N.D. (1974) Proc. Natl. Acad. Sci. USA 71, 815-819
115. Kuehl, F.A., Ham, E.A., Zanetti, M.E., Sanford, C.H. Nicol, S.E. and Goldberg, N.D. (1974) Proc. Natl. Acad. Sci. USA 71, 1866-1870
116. Hadden, J.W., Hadden, E.M., Haddox, M.K. and Goldberg, N.D. (1972) Proc. Natl. Acad. Sci. USA 69, 3024-3027
117. Rudland, P.S., Gospodarowicz, D. and Seifert, W. (1974) Nature 250, 741-742, 773
118. Smith, R.J. and Ignarro, L.J. (1975) Proc. Natl. Acad. Sci. USA 72, 108-112
119. Sandler, J.A., Clyman, R.I., Manganiello, V.C. and Vaughan, M. (1975) J. Clin. Invest. 55, 431-435
120. Chiang, T.M.H. and Kang, A.H. (1975) Abstr. Fed. Proc. 34, 695
121. Chrisman, T.D., Garbers, D.L., Parks, M.A. and Hardman, J.G. (1975) J. Biol. Chem. 250, 374-381
122. Murad, F. and Kimura, X. (1975) Abstr. Fed. Proc. 34, 232
123. Garbers, D.L., Hardman, J.G. and Rudolph, F.B. (1974) Biochemistry 13, 4166-4171
124. Helwig, J.J., Bollack, C., Mandel, P. and Goridis, C. (1975) Biochim. Biophys. Acta 377, 463-473
125. Böhme, E., Jang, R. and Meckler, I. (1974) in Methods in Enzym. (ed. Hardman, J.G. and O'Malley, B.W.) Academic Press, New York, Vol. XXXVIII, pp. 199-202
126. Kimura, H. and Murad, F. (1974) J. Biol. Chem. 249, 6910-6916
127. Gray, J.P. and Drummond, G.I. (1973) Proc. Can. Fed. Biol. Soc. 16, 79

128. Siegel, M.I., Puca, G.A. and Cuatrecasas, P. (1975) Abstr. Fed. Proc. 34, 232
129. Bensinger, R.E., Fletcher, R.T. and Chader, G.J. (1974) Science 183, 86-87
130. Cuatrecasas, P. (1974) Ann. Rev. Bioch. 43, 169-214
131. Limbird, L.E. and Lefkowitz, R.J. (1975) Biochim. Biophys. Acta 377, 186-196
132. Thompson, W.J., Williams, R.H. and Little, S.A. (1973) Biochim. Biophys. Acta 302, 329-337
133. Amer, M.S. and McKinney, G. (1973) The Pharmacologist 15, 157
134. White, L.E., Ignarro, L.H. and George, W.J. (1973) The Pharmacologist 15, 157
135. Schultz, G., Hardman, J.G., Schultz, K., Davis, J.W. and Sutherland, E.W. (1973) Proc. Natl. Acad. Sci. USA 70, 1721-1725
136. Van Sande, J., Decoster, C. and Dumont, J.E. (1975) Biochem. Biophys. Res. Commun. 62, 168-175
137. Ferrendelli, J.A., Rubin, E.H. and Kinscherf, D.A. (1975) Abstr. Fed. Proc. 34, 231
138. Birnbaumer, L., Pohl, S.L., Rodbell, M. (1972) J. Biol. Chem. 247, 2038-2043
139. Ishikawa, E., Ishikawa, S., Davis, J.W. and Sutherland, E.W. (1969) J. Biol. Chem. 244, 6371-6376
140. Corbett, C.L., Isaacs, P.E.T., Rilet, A. and Turnberg, L.A. (1975) Abstr. 9th Meet. Eur. Soc. Clin. Invest. 14
141. Fawaz, E.N. and Tejirian, A. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 1779-1783
142. Hardman, J.G. and Sutherland, E.W. (1969) J. Biol. Chem. 244, 6363-6370
143. Tilney, L.G. and Mooseker, M. (1971) Proc. Natl. Acad. Sci. USA 68, 2611-2615
144. Thuneberg, L. and Rostgaard, J. (1969) Abst. Commun. 6th Meet. Fed. Eur. Biochem. Soc. 864
145. Rasmussen, H. (1970) Science 170, 404-412
146. Willingham, M.C. and Pastan, I. (1975) Proc. Natl. Acad. Sci. USA 72, 1263-1267
147. Kim, G. and Silverstein, E. (1975) Abstr. Fed. Proc. 34, 231
148. Lamers, J.M.J. and Hülsmann, W.C. (1975) Biochim. Biophys. Acta 376, 561-572
149. Seifert, W. and Rudland, P.S.(1974) Proc. Natl. Acad. Sci. USA 71, 4920-4924
150. Watson, J. (1975) J. Exp. Med. 141, 97-111
151. Kram, R., Mamont, P. and Tomkins, G.M. (1973) Proc. Natl. Acad. Sci. USA 70, 1432-1436
152. Otten, J., Johnson, G.S. and Pastan, I. (1971) Biochem. Biophys. Res. Commun. 44, 1192-1198
153. Sheppard, J.R. (1972) Nature (New Biol.) 236, 14-16
154. Hecht, S.M., Faulkner, R.D. and Hawrelak, S.D. (1974) Proc. Natl. Acad. Sci. USA 71, 4670-4674

155. Ryan, W.L. and Heidrick, M.L. (1974) in Advances in Cyclic Nucleotide Research (ed. Greengard, P. and Robison, G.A.) Raven Press, New York, Vol. 4, pp. 81-116
156. Voorhees, J.J., Duell, E.A., Stawiski, M. and Harrell, E.R. (1974) ibidem, pp. 117-162
157. Coffino, P., Gray, J.W. and Tomkins, G.M. (1975) Proc. Natl. Acad. Sci. USA 72, 878-882
158. Froehlich, J.E. and Rachmeler, M. (1972) J. Cell Biol. 55, 19-31
159. Pawelek, J., Sansone, M., Koch, N., Christie, G., Halaban, R., Hendee, J., Lerner, A.B. and Varga, J.M. (1975) Proc. Natl. Acad. Sci. USA 72, 951-955
160. Luckasen, J.R., White, J.G. and Kersey, J.H. (1974) Proc. Natl. Acad. Sci. USA 71, 5088-5090
161. van Wijk, R., Wicks, W.D., Bevers, M.M. and van Rijn, J. (1973) Cancer Res. 33, 1331-1338
162. Wicks, W.D. (1974) in Advances in Cyclic Nucleotide Research (ed. Greengard, P. and Robison, G.A.) Raven Press, New York, Vol. 4, pp. 335-438
163. Pastan, I. and Perlman, R.L. (1970) Science 169, 339-344
164. Williams, J.P.G. (1972) Cell Differentiation 1, 317-323
165. Henney, C.S., Lichtenstein, L.M., Gillespie, I. and Rolley, R.I. (1973) J. Clin. Invest. 52, 2853-2857
166. Plescia, O.J., Yamamoto, I. and Shimamura, T. (1975) Proc. Natl. Acad. Sci. USA 72, 888-891
167. DeRubertis, F.R., Zenser, T.V., Adler, W.H. and Hudson, T. (1974) J. Immun. 113, 151-161
168. Sultzer, B.M. and Craig, J.P. (1973) Nature (New Biol.) 244, 178-180
169. Kateley, J.R. and Friedman, H. (1975) Ann. N.Y. Acad. Sci. 249, 413-423
170. Pratt, R.M. and Martin, G.R. (1975) Proc. Natl. Acad. Sci. USA 72, 874-877
171. Cho-Chung, Y.S. and Gullino, P.M. (1973) Science 183, 87-88
172. MacManus, J.P. and Whitfield, J.F. (1974) Prostaglandins 6, 475-487
173. MacManus, J.P. and Whitfield, J.F. (1969) Exp. Cell Res. 58, 188-191
174. Guidotti, A., Weiss, B. and Costa, E. (1972) Mol. Pharmacol. 8, 521-530
175. Thomas, E.W., Murad, F. and Looney, W.B. (1973) Biochim. Biophys. Acta 297, 564-567
176. Minton, J.P., Wisenbaugh, T. and Matthews, R.H. (1974) J. Nat. Canc. Inst. 53, 283-284
177. Durham, J.P., Baserga, R. and Butcher, F.R. (1974) Biochim. Biophys. Acta 372, 196-217
178. Galjaard, H. and Bootsma, D. (1969) Exp. Cell Res. 58, 79-92

179. Loran, M.R. and Carbone, J.V. (1966) in Gastrointestinal Radiation Injury (ed. Sullivan, M.F.) Excerpta Medica Foundation, pp. 127-139
180. Raicht, R.F., Deschner, E. and Salen, G. (1975) Abstr. Gastroenterology 68, 979
181. Tutton, P.J.M. (1974) Virchows Arch. B Cell Path. 16, 79-87
182. Tutton, P.J.M. and Helme, R.D. (1974) Cell Tissue Kinet. 7, 125-136
183. Johnson, L.R. (1975) Abstr. Gastroenterology 68, 920
184. Cairnie, A.B. and Bentley, R.E. (1967) Exp. Cell Res. 46, 428-440
185. Brown, H.O., Levine, M. and Lipkin, M. (1963) Am. J. Physiol. 205, 868-872
186. Jervis, E.L. and Levin, R.J. (1966) Nature 210, 391-393
187. Khovry, K.A., Floch, M.H. and Hersh, T. (1969) J. Exp. Med. 130, 659-665
188. Wright, N., Watson, A., Morley, A., Appleton, D., Marks, J. and Douglas, A. (1974) Virchows Arch. A Path. Anat. and Histol. 364, 311-323
189. Podolsky, D.K. and Weiser, M.M. (1973) J. Cell Biol. 58, 497-500
190. Etzler, M.E. and Branstrator, M.L. (1974) J. Cell. Biol. 62, 329-343
191. Hollenberg, M.D. and Cuatrecasas, P. (1973) Proc. Natl. Acad. Sci. USA 70, 2964-2968
192. Hollenberg, M.D., Fishman, P.H., Bennet, V. and Cuatrecasa, P. (1974) Proc. Natl. Acad. Sci. USA 71, 4224-4228
193. O'Keefe, E. and Cuatrecasas, P. (1974) Proc. Natl. Acad. Sci. USA 71, 2500-2504
194. Baker, A., Kaplan, M. and Kimberg, D.V. (1973) J. Clin. Invest. 52, 2928-2930
195. Guerrant, R.L., Chen, L.C. and Sharp, G.W.G. (1972) J. Infect. Dis. 125, 377-381
196. Posternak, T. (1974) Ann. Rev. Pharmacol. 14, 23-33
197. Clark, R.B., Gross, R., Su, Y.F. and Perkins, J.P. (1974) J. Biol. Chem. 249, 5296-5303
198. Blume, A.J., Dalton, C. and Sheppard, H. (1973) Proc. Natl. Acad. Sci. USA 70, 3099-3102
199. Salser, J.S. and Balis, M.E. (1973) Cancer Res. 33, 1889-1897
200. Cunningham, D.D. and Remo, R.A. (1973) J. Biol. Chem. 248, 6282-6288
201. Plagemann, P.G.W. and Erbe, J. (1972) J. Cell Biol. 55, 161-178
202. Siegers, M.P., Schaer, J.C. Hirsiger, H. and Schindler, R. (1974) J. Cell Biol. 62, 305-315
203. Rozengurt, E. and de Asua, L.J. (1973) Proc. Natl. Acad. Sci. USA 70, 3609-3612
204. Plagemann, P.G.W. and Sheppard, J.R. (1974) Biochem. Biophys. Res. Commun. 56, 869-875

205. Plagemann, P.G.W. (1971) Biochim. Biophys. Acta 233, 688-701
206. Peterson, J.W., Lo-Spalluto, J.J., Finkelstein, R.A. (1972) J. Infect. Dis. 126, 617-628
207. Grand, R.J., Torti, F.M. and Jaksina, S. (1973) J. Clin. Invest. 52, 2053-2059
208. Francis, T.I., Lewis, E.A., Oyediran, A.B.O.O. et al. (1971) J. Trop. Med. Hyg. 74, 172-176
209. MacDonald, W.C., Trier, J.S. and Everett, N.B. (1965) Gastroenterology 46, 405-417
210. Sherr, H.R., Stifel, F.B. and Herman, R.H. (1975) Abstr. Gastroenterology 68, 984
211. van Berkel, Th.J.C., de Jonge, H.R., Koster, J.F. and Hülsmann, W.C. (1974) Biochem. Biophys. Res. Commun. 60, 398-405
212. van Tol, A. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 1261-1262.

SUMMARY

The first part of this thesis (Chapter II, Papers 1 and 2) describes the consequences of a prolonged treatment of rats with inhibitors of mitochondrial protein synthesis (chloramphenicol and oxytetracycline) for mitochondrial biogenesis and function in the small intestinal epithelium. This tissue is extremely sensitive to the action of these antibiotics due to its unequalled high cellular turnover rate, implying a rapid formation of intestinal mitochondria. At serum levels of both antibiotics above 10 µg/ml the content of the cytochromes aa_3, b and c_1 in villous and crypt cells decreased to about 25-35 per cent of the control value within two days. The activity of numerous other mitochondrial enzymes did not change in this period. It is concluded that these proteins are made on cytoplasmic ribosomes prior to their integration into the mitochondrial structure. These processes are apparently not disturbed by a partial loss of mitochondrial translation products. Also the energy stores in the epithelium in vivo and the rate of oxidative phosphorylation in isolated mitochondria appeared to be unaffected, indicating an intact mitochondrial energetic function.

Prolonged treatment with oxytetracycline (but not with chloramphenicol) caused a severe structural damage of the villous epithelium and accumulation of lipid droplets in the liver ("fatty liver"). The crypt cell proliferation rate was not appreciably affected, suggesting again the preservation of a normal energy metabolism in this cell type. Because chloramphenicol, producing a similar degree of cytochrome depletion, was non-toxic under similar conditions, the toxicity of oxytetracycline in intestine and liver was ascribed to side effects on other cellular processes, arising after drug accumulation in blood and tissues. An investigation of several other potential targets for tetracyclines in the cell (not affected by chloram-

phenicol) suggested that a partial inhibition of cytoplasmic protein synthesis was responsible for most of the toxic effects. Although the degree of inhibition caused by oxytetracycline during short incubations of isolated epithelial cells appeared to be small, a gradual accumulation of the antibiotic in the microsomes or formation of highly active degradation products might have amplified its action in vivo.

During prolonged clinical application serum levels of the antibiotics seldom raise above 20 µg/ml, allowing little chance for toxic side-effects. On the basis of the present study a decrease of the level of some mitochondrial cytochromes in the intestinal mucosa of patients subjected to antibiotic therapy may be expected, although this change will probably occur much more slowly than in rat intestine in view of the considerably lower dividing activity of human crypt cells. However, the reversibility of the antibiotic action and the apparent excess of mitochondrial cytochromes in mammalian intestine will guarantee the preservation of a normal mitochondrial function under nearly all circumstances, even in this rapidly proliferating and differentiating tissue.

Chapter III (Papers 3 and 4) is devoted to a study of the adenylate cyclase system and cyclic AMP levels in isolated villous and crypt cells from rat small intestine. The major part of the adenylate cyclase activity appeared to be localized in the basal-lateral plasmamembranes of the enterocyte. The enzyme of both villous and crypt cells was stimulated by NaF, PGE_1 and high levels of epinephrine but did not respond to a number of other hormones, reported to affect the transport function of the epithelium in vivo. A similar hormone-insensitivity was observed in measurements of intracellular cyclic AMP levels. The stimulation by epinephrine was interpreted as a β-adrenergic effect on the mucus-secreting goblet cells. Prolonged

luminal contact of the epithelium in vivo to purified choleratoxin led to an irreversible activation of adenylate cyclase in villous and crypt compartments. Short exposure (1-2 min) to choleragen was followed by a maximal activation of this enzyme exclusively in the upper villus and not in the crypt. By comparing the effects of short and prolonged contact with choleratoxin on the unidirectional and net fluxes of ions and water in vivo, it could be established that both villous and crypt cells contribute to the choleragen-induced secretion. Choleragen stimulated the serosal to mucosal fluxes of Na^+, Cl^- and HCO_3^- without impairing the opposite flux. These results imply that absorptive and secretory processes may occur within the same epithelial compartment and conflict with the view that fluid secretion is a specific function of the crypt.

The cholera studies clearly showed that even a slight increase of cyclic AMP levels in the intestinal mucosa leads to a drastic reduction of its absorptive capacity for water and electrolytes. The apparent insensitivity of the adenylate cyclase system in the enterocyte to hormones (e.g. insulin, glucagon, catecholamines) is interpreted as a protection of the absorptive function against large fluctuations of hormones levels under physiological conditions.

Chapter IV (Papers 5 and 6) deals with some aspects of cyclic GMP formation and regulation in the enterocyte. Appr. 65-75 per cent of the total guanylate cyclase activity in the villous cell was found in the brushborder region, the other part was mainly localized in the basal-lateral plasma membranes. Crypt cell activities were much lower, probably due to the immature state of their luminal membranes. Whereas the kinetic properties of the brushborder enzyme were comparable to those of the particulate form of guanylate cyclase in rat heart and lung, the kinetics of the enzyme in the anti-luminal membranes showed more resemblance to the soluble form in other

tissues. Both types of guanylate cyclase were completely insensitive in vitro to a great number of hormones, neurotransmitters, prostaglandins and plant lectins but could be inhibited to an appreciable extent by physiological concentrations of ATP and ADP. L-aminoacids had a small stimulatory effect.

The levels of cyclic GMP in isolated villous and crypt cells were appr. 4-6 times lower than the cyclic AMP levels and decreased slightly from crypt to villus. Because the guanylate cyclase activity strongly increased in the same direction, it is suggested that cyclic GMP-degrading enzymes (phosphodiesterases) may play an important role in the physiological regulation of cyclic GMP levels. Also the cyclic GMP concentration could not be changed by incubation with hormones, hormone-like agents or the divalent cation-ionophore A-23187. Possible reasons for this insensitivity are discussed. It is concluded that the function of cyclic GMP in the intestinal epithelium is difficult to assess because of a lack of suitable and specific agents that could regulate its intracellular concentration. The preponderance of guanylate cyclase in the brushborder is suggestive for a relationship between cyclic GMP and transport processes. The existence of pulsating movements of the microvilli and the high activity of guanylate cyclase in some other organs with a contractile function (e.g. flagellae from sea urchin sperm) lend some support to the hypothesis that cyclic GMP plays a role in the assembly or action of intestinal microfilaments.

In Chapter V the relationship between cyclic AMP and crypt cell proliferation was investigated. Isolated crypt cells were used to measure direct effects of cyclic AMP on thymidine incorporation rates. All agents capable to elevate intracellular cyclic AMP levels (PGE_1, dibutyryl-cyclic AMP, phosphodiesterase inhibitors) caused a profound inhibition of this incorporation process. By measurements of thymidine transport rates in isolated villous and crypt

cells, using the silicon oil filtration technique, the effects on incorporation could be traced back to a direct competitive inhibition of thymidine transport at the plasma membrane level not related to a change in cyclic AMP-content. This result warns against thymidine incorporation measurements as a tool to investigate a relationship between cyclic AMP and rates of DNA synthesis.

In an alternative approach, cyclic AMP levels in crypt and villous cells were elevated by pretreatment of short segments of rat small intestine with choleragen to explore the influence of the cholera-state on cell proliferation and migration in vivo. It was found that cholera-toxin treatment did not lead to a retardation or acceleration of epithelial cell turnover and had no influence on cell morphology at the light-microscopal level. This result could not be used to deny a possible role of cyclic AMP in intestinal cell proliferation because application of a new selection technique for the proliferative cell types in the crypt showed that the cyclic AMP levels in these cells did not change during cholera. Although the experimental cholera model failed to establish an effect of cyclic AMP on cell growth, it ensured high levels of cyclic AMP in maturative crypt and absorptive villous cells for a sufficiently long period to enable a future study of the possible role of cyclic AMP in intestinal differentiation processes in the intact animal.

SAMENVATTING

In het eerste deel van dit proefschrift (Hoofdstuk II, Publicaties 1 en 2) worden de gevolgen beschreven van een langdurige behandeling van ratten met remmers van de mitochondriële eiwitsynthese (chlooramphenicol en oxytetracycline) voor de biogenese en functie van de mitochondriën in het dunnedarm epitheel. Dit weefsel is buitengewoon gevoelig voor deze antibiotica wegens de hoge proliferatiesnelheid van de stamcellen in de crypt, die gepaard gaat met een snelle vorming van darmcel mitochondriën. Bij plasma concentraties van beide antibiotica tussen 10 en 30 µg/ml nam het gehalte aan cytochroom aa_3, b en c_1 in de villus en crypt cellen binnen twee dagen af tot ongeveer 25-35 procent van de normale waarde. Activiteitsveranderingen van andere mitochondriële enzymen werden niet waargenomen. Hieruit volgt dat deze eiwitten op ribosomen in het cytoplasma worden vervaardigd vóór hun integratie in de mitochondriële structuur. Beide processen worden blijkbaar niet gestoord door een gedeeltelijk verlies van mitochondriële translatieprodukten. Ook het gehalte aan energierijke verbindingen in het epitheel in vivo was onveranderd, evenals het proces van de oxydatieve fosforylering in geïsoleerde mitochondriën, hetgeen duidt op een intact gebleven energielevering door deze organellen.

Een langdurige behandeling met oxytetracycline (maar niet met chlooramphenicol) veroorzaakte een ernstige structurele beschadiging van het villus epitheel en de ophoping van vet in de lever. De delingssnelheid van de cryptcellen was vrijwel onveranderd, hetgeen opnieuw wijst op een ongestoorde energiehuishouding in dit celtype. De chlooramphenicol behandeling leidde niet tot toxische effecten op het epitheel hoewel het cytochroom niveau in dezelfde mate was verlaagd. De toxiciteit van oxytetracycline in darm en lever werd daarom toegeschreven aan neveneffecten op andere cellulaire processen die

kunnen optreden na ophoping van dit antibioticum in bloed en weefsels. Een nader onderzoek van verscheidene andere potentiële aangrijpingspunten van de tetracyclines in de cel (niet gestoord door chlooramphenicol) leidde tot de suggestie dat een partiële remming van de cytoplasmatische eiwitsynthese verantwoordelijk was voor de meeste toxische effecten.
Hoewel de door oxytetracyline veroorzaakte remming tijdens kortstondige incubaties van geïsoleerde darmcellen slechts gering was, zou een geleidelijke ophoping van het antibioticum in de microsomen van de cel of de vorming van zeer toxische afbraakprodukten deze remming in vivo kunnen versterken.
In de klinische situatie zullen serumconcentraties van de antibiotica zelden boven de 20 µg/ml uitstijgen, zodat ontplooiing van de besproken neveneffecten weinig waarschijnlijk is. Op basis van deze studie kan verwacht worden dat het niveau van enkele mitochondriële cytochromen in de darmmucosa van patiënten die een antibioticumkuur ondergaan zal dalen hoewel dit proces veel langzamer zal verlopen dan in de rat wegens de aanzienlijk lagere delingscapaciteit van menselijke crypt cellen. De reversibiliteit van de remming door deze antibiotica en de klaarblijkelijke overmaat aan cytochromen in darmmitochondriën van zoogdieren zal echter onder vrijwel alle omstandigheden een normaal functioneren van de mitochondriën garanderen zelfs in dit sneldelende en differentiërende weefsel.

Hoofdstuk III(Publicaties 3 en 4) is gewijd aan een studie van het adenylcyclase systeem en de cyclisch AMP niveau's in geïsoleerde villus en crypt cellen van de dunnedarm van de rat. Een aanzienlijk deel van de adenyl cyclase activiteit kon worden gelocaliseerd in de basolaterale plasmamembranen van de enterocyt. Zowel het villus als het crypt enzym werd gestimuleerd door fluoride, prostaglandine E_1 en hoge concentraties epinephrine, maar reageerde niet op een aantal andere hormonen, waarvan bekend

is dat zij de transport functie van het epitheel in vivo beïnvloeden. Dezelfde hormoonongevoeligheid werd gevonden voor de intracellulaire cyclisch AMP niveau's. De stimulering door epinephrine werd geïnterpreteerd als een β-adrenergisch effect op de slijmbekercellen. Een langdurig luminaal contact van het epitheel met gezuiverd choleratoxine in vivo veroorzaakte een irreversibele activering van het adenylcyclase in villus en crypt. Door een kortstondige expositie (1-2 min) kon het adenylcyclase in de bovenste helft van het villus epitheel maximaal gestimuleerd worden, terwijl het crypt enzym zijn basale activiteit behield. Door de effecten van kort- en langdurig contact met het choleratoxine op de éénrichtings- en netto flux van ionen en water in vivo te vergelijken, kon worden vastgesteld dat zowel crypt als villus cellen bijdragen tot de door choleratoxine-geïnduceerde secretie. Choleratoxine stimuleerde de flux van Na^+, Cl^- en HCO_3^- van serosa naar mucosa zonder de flux in omgekeerde richting te beïnvloeden. Hieruit werd geconcludeerd dat absorptie- en secretieprocessen kunnen plaatsvinden binnen hetzelfde epitheliale compartiment. De resultaten zijn duidelijk in strijd met de opvatting dat vloeistofsecretie een specifieke functie van de crypt zou zijn.
De cholera studies toonden aan dat zelfs een geringe toename van de cyclische AMP niveau's in het darmslijmvlies een drastische verlaging teweegbrengt van de absorptiecapaciteit voor water en electrolyten. De ongevoeligheid van het adenylcyclase systeem in de enterocyt voor hormonen (bijv. insuline, glucagon, catecholamines) kan worden opgevat als een bescherming van de absorptieve darmfunctie tegen de aanzienlijke fluctuaties van hormoonspiegels die kunnen optreden onder physiologische condities.

Hoofdstuk IV (Publicaties 5 en 6) behandelt enkele aspecten van de cyclisch GMP productie en regulatie in de enterocyt. Ongeveer 65-75 procent van de totale guanyl-

cyclase activiteit in de villus cel werd aangetroffen in de borstelzoom, de overige activiteit was hoofdzakelijk gelocaliseerd in de baso-laterale plasmamembranen. De activiteit in de crypt cellen was veel lager, vermoedelijk als een gevolg van de onvolgroeide toestand van hun luminale membraan. De kinetische eigenschappen van het borstelzoom enzym bleken vergelijkbaar met die van de membraangebonden vorm van het guanylcyclase in rattehart en -long. De kinetica van het enzym in de baso-laterale membraan kwam daarentegen meer overeen met die van het oplosbare guanylcyclase in andere weefsels. Beide types guanylcyclase getest in vitro bleken volledig ongevoelig voor een groot aantal hormonen, neurotransmitters, prostaglandines en plant lectines maar konden aanzienlijk worden geremd door physiologische concentraties van ATP en ADP. L-aminozuren bezaten een geringe stimulerende werking. De cyclisch GMP niveau's in geïsoleerde villus en crypt cellen waren ongeveer 4-6 maal lager dan de cyclisch AMP niveau's en namen enigszins af van crypt naar villus. Omdat de guanylcyclase activiteit juist toenam in deze richting, wordt verondersteld dat cyclisch GMP-afbrekende enzymen (fosfodiesterases) een belangrijke rol zouden kunnen spelen in de physiologische regulatie van het intracellulaire cyclisch GMP gehalte. Ook de concentraties van cyclisch GMP konden niet worden gewijzigd door incubatie van de cellen met hormonen of daarmee vergelijkbare agentia en de ionofoor A-23187, specifiek voor divalente kationen (Ca^{2+}, Mg^{2+}). Mogelijke oorzaken voor deze ongevoeligheid worden aangegeven. De negatieve resultaten betekenden dat de functie van het cyclische GMP in het darmepitheel moeilijk was vast te stellen wegens een gebrek aan geschikte en specifieke middelen om de intracellulaire concentratie van cyclisch GMP te reguleren. De verrijking van guanylcyclase activiteit in de borstelzoom suggereert een mogelijke relatie tussen cyclisch GMP en transport processen. Het bestaan van pulserende bewegingen van de

microvilli en de hoge activiteit van guanylcyclase in enkele andere organen met een contractiele functie (bijv. de flagellen van zeeëgel sperma) werden gebruikt als argumenten voor de hypothese dat cyclisch GMP een rol speelt in de aanleg of activiteit van microfilamenten in de borstelzoom van de cel.

In Hoofdstuk V werd de relatie onderzocht tussen cyclisch AMP en de prolifererende activiteit van de crypt cellen. Geïsoleerde crypt cellen werden gebruikt om rechtstreekse effecten te meten van cyclisch AMP op de incorporatiesnelheid van thymidine in het DNA. Alle stoffen die in staat bleken intracellulaire cyclische AMP niveau's te verhogen (PGE_1, dibutyryl-cyclisch AMP, remmers van fosfodiesterases) veroorzaakten een aanzienlijke remming van dit inbouw proces. Door metingen van thymidinetransportsnelheden in geïsoleerde villus en crypt cellen met behulp van de siliconenoliefiltratie techniek kon worden vastgesteld dat de incorporatie remmingen waren terug te voeren op een rechtstreekse competitieve remming van het thymidine transport door het plasmamembraan die onafhankelijk was van de veranderingen in cyclisch AMP gehalte. Op grond van dit resultaat moet worden gewaarschuwd tegen de toepassing van thymidine incorporatie metingen als een middel om een relatie te onderzoeken tussen cyclisch AMP en DNA-synthese snelheden.
In een poging deze problemen te omzeilen werd het cyclisch AMP gehalte in crypt en villus cellen verhoogd door preincubatie van korte darm segmentjes met choleratoxine teneinde de invloed van de cholera-toestand op celdeling en migratie in vivo te onderzoeken. De choleratoxine-behandeling bleek niet van invloed op de "turnover"-snelheid van de epitheelcellen en ook de celmorfologie op lichtmicroscopisch niveau was niet verschillend van controle segmenten. Dit resultaat kan echter niet dienen om een mogelijke rol van cyclisch AMP in het darm cel proliferatie proces uit te sluiten: door toepassing van een nieuwe

techniek voor de selectie van prolifererende cellen uit de totale crypt cel populatie kan worden aangetoond dat het peil van cyclisch AMP in dit celtype niet verhoogd was in de cholera periode. Terwijl dit experimentele cholera-model dus ongeschikt bleek om een mogelijke invloed van cyclisch AMP op de celgroei aan te tonen, opent het goede perspectieven voor een toekomstig onderzoek naar een mogelijke rol van cyclisch AMP in het differentiatieproces van dunnedarm epitheelcellen in vivo omdat sterk verhoogde cyclisch AMP niveau's in absorptieve villus cellen en matu-rerende crypt cellen konden worden gegarandeerd gedurende een lange periode zonder enige schade aan de normale lichaamsfuncties van de rat.

NASCHRIFT

In dit naschrift wil ik graag iedereen dank betuigen die op de een of andere wijze het tot stand komen van dit proefschrift mogelijk hebben gemaakt.

In de eerste plaats ben ik veel dank verschuldigd aan mijn ouders voor de geestelijke en materiële steun die zij mij tijdens mijn scheikundestudie geboden hebben.

Willem Hülsmann, aan jou heb ik het te danken dat deze studie niet werd bekroond met een tweejarig verblijf in een militaire inrichting. Je brede kennis en groot associatief vermogen (zowel biochemisch als taalkundig) zijn de werkbesprekingen van de Darmgroep vaak ten goede gekomen en hebben het plezier in het onderzoek sterk gestimuleerd. De vrijheid die je me gelaten hebt in de bepaling van de richting van onderzoek heeft wellicht het werk (en dit proefschrift) een enigszins heterogeen karakter gegeven en het invullen van het FUNGO-projectformulier tot een jaarlijks bezoeking gemaakt. Deze flexibiliteit gaf echter ook de gelegenheid tot een verbreding van inzicht in de darmbiochemie binnen onze werkgroep en tot een tijdige aanpassing van het onderzoek aan de actuele ontwikkelingen in dit specialisme.

Prof. Dr. A.M. Kroon, beste Ab, ondanks je gastro-intestinale complicaties (enterovirus?) heb je de energie opgebracht het manuscript van dit proefschrift kritisch en nauwgezet door te nemen. Aangezien je één van de pioniers bent geweest in het onderzoek van de mitochondriële eiwitsynthese, heb ik in het bijzonder je oordeel over het eerste deel van het proefschrift op hoge prijs gesteld.

Ook Prof. Dr. M. Frenkel dank ik voor de bereidwilligheid waarmee hij als coreferent van dit proefschrift wilde fungeren. De belangstelling die hij als clinicus bezit voor biochemische vraagstellingen zoals ook bleek uit gezamenlijke werkbesprekingen is een stimulans voor een meer op de klinische problemen afgestemde instelling

van de onderzoeker.

Leo Scheek, het optimisme en de energie waarmee je in vrijwel alle fasen van het experimentele werk hebt geassisteerd is voor dit onderzoek van buitengewone waarde geweest. Het isoleren van darmcellen werd door jou tot een ongeëvenaarde graad van perfectionisme opgevoerd.

Cecile Hanson ben ik bijzonder dankbaar voor het uittypen van diverse artikelen en dit proefschrift in een adembenemend tempo en op een zeer accurate wijze. De foutloze ontcijfering van mijn handschrift heeft steeds mijn grootste respect afgedwongen. Ook Mieke d'Hamecourt dank ik voor de voortreffelijke uitvoering van het typewerk in de eerste periode van het onderzoek.

De vele discussies met Wim van den Berg, Jos Lamers en (in de beginfase) met Willem Iemhoff, zijn voor mij zeer waardevol geweest. Van de door hen ingevoerde technieken heb ik meerdere malen dankbaar gebruik gemaakt. Anna Maria Leeflang-de Pijper ben ik erkentelijk voor de informatie verkregen uit het histologisch onderzoek van het darmepitheel.

Frans Angenent dank ik voor de snelheid en bekwaamheid waarmee hij onverwachte technische storingen wist op te heffen en Rinus Machielse voor de efficiënte verwerking van spoedbestellingen en het op peil houden van een schier onuitputtelijke chemicaliënvoorraad in het magazijn.

Ook alle andere, niet met name genoemde medewerkers dank ik hartelijk voor hun bijdrage tot een aangenaam werkklimaat binnen de afdeling en voor hun belangstelling tijdens het onderzoek.

Bijzonder erkentelijk ben ik tenslotte voor de goede contacten met medewerkers van andere afdelingen, in het bijzonder met Dr. N.J. de Both (Pathologische Anatomie) en Drs. J.M. van Dongen (Celbiologie) die een vraagbaak vormden voor problemen van morfologische of enzymhistochemische aard; Nico wil ik speciaal danken voor zijn medewerking aan de autoradiografische experimenten beschreven in het laat-

ste hoofdstuk. Hun directe bijdrage aan dit proefschrift is zichtbaar op de omslag (een scanning-electronenmicroscopische opname van het dunnedarm oppervlak gemaakt door Han van Dongen) en in de eerste figuur (Fig. 1.1), een lichtmicroscopisch beeld van een dunnedarmcoupe vervaardigd door Nico. De autoradiografische bijdrage aan het antibioticumonderzoek was mogelijk dankzij de medewerking van Prof. Dr. H. Galjaard (Celbiologie) en werd met vakmanschap uitgevoerd door Pim Visser.

Dr. B.A. Cooke, beste Brian, je adviezen bij het opzetten van de eerste cyclische AMP bepalingen hebben het onderzoek zeer bespoedigd. Ik hoop dat onze gezamenlijke belangstelling voor cyclische nucleotiden, zij het in een qua functie totaal verschillend weefsel, ook in de toekomst een samenwerking op methodologisch gebied zal blijven verzekeren.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren in 1944 te Eindhoven en behaalde in 1962 het diploma Gymnasium-β aan het Protestants Lyceum ter stede. In hetzelfde jaar werd de chemiestudie begonnen aan de Vrije Universiteit te Amsterdam. Het kandidaatsexamen in de wiskunde en natuurwetenschappen (richting e) werd in 1967 afgelegd. Als hoofdrichting werd vervolgens biochemie gekozen, als bijvak microbiologie. Van de volgende periode werden vier maanden doorgebracht in Groningen voor het opdoen van electronenmicroscopische kennis (Rijksuniversiteit, Laboratorium voor Structuurchemie). In 1970 werd het doctoraalexamen scheikunde afgelegd . Sinds december 1970 is hij als wetenschappelijk medewerker verbonden aan de afdeling Biochemie I van de Erasmus Universiteit te Rotterdam waar het hier beschreven onderzoek werd verricht.

PAPERS

Eur. J. Biochem. *32*, 356–364 (1973)

Inhibition of Mitochondrial-Protein Synthesis in Rat Small-Intestinal Epithelium

Hugo R. DE JONGE and Willem C. HÜLSMANN

Afdeling Biochemie I, Medische Faculteit Rotterdam

(Received June 16/October 18, 1972)

Because of its very fast turnover, implying a rapid biogenesis of intestinal mitochondria, rat-small-intestinal epithelium was regarded as an excellent target for the action of chloramphenicol and oxytetracycline, specific inhibitors of mitochondrial protein synthesis. With constant blood levels of chloramphenicol and oxytetracycline above 10 μg/ml the levels of the mitochondrial cytochromes aa_3, b and c_1 in villous and crypt cells decreased gradually until a minimum level was reached after about 48 h of treatment. The rapid onset of the fall in cytochrome aa_3 content within the villous cells indicated a further synthesis of this enzyme outside the proliferative compartment of the crypt. No significant change was observed in the cellular contents of numerous other mitochondrial and extramitochondrial enzymes, confirming the restricted function of the mitochondrial protein-synthesizing machinery for the biosynthesis of mammalian mitochondria. Prolonged treatment with oxytetracycline, but not with chloramphenicol, caused a severe damage of the villous epithelium. This toxic effect was probably not related to the lowered cytochrome content of the mitochondria.

The small intestinal epithelium of the rat was regarded as an excellent target for inhibitors of mitochondrial protein synthesis, such as chloramphenicol or oxytetracycline, since the turnover of the cells is extremely rapid. In the intestinal crypts the cell cycle of the principal cells is about 10—14 h [1], implying rapid formation of mitochondrial enzymes. In the transitional zone between crypt and villus, differentiation may require extra synthesis of some mitochondrial proteins [2, 3] and in the villous cell mitochondrial protein synthesis may be continued to compensate for possible breakdown. Since the lifetime of the small intestinal cells is only about 2 days, antibiotic treatment of short duration might be expected to result in a selective influence on the constituents of these cells, without disturbing the vital functions of other organs in the rat.

This report, dealing with the changes in enzymic composition of the mitochondria in villous and crypt cells during antibiotic treatment, provides information concerning the participation of mitochondrial protein synthesis in the biogenesis of intestinal mitochondria and the site of synthesis of mitochondrial enzymes in the intestinal epithelium. Studies on the energy metabolism of the epithelial cells and mitochondria during long-term treatment with chloramphenicol or oxytetracycline and histochemical observations will be published elsewhere [4, 5].

MATERIALS AND METHODS

Animals

In all experiments male, specific-pathogen-free. Wistar rats, weighing 200—230 g were utilized in order to eliminate the contamination of villous and crypt cell preparations with protozoa (*cf.* [6]). Normal laboratory chow and drinking water were provided. Rats were sacrificed by cervical fracture and subsequent bleeding.

Chemicals

Enzymes and cofactors were purchased from Boehringer and Sons (Mannheim, Federal Republic of Germany), antimycin A, phenazine methosulphate and rotenone were from the Sigma Chemical Co.

Enzymes. Succinate: (acceptor) oxidoreductase or succinate dehydrogenase (EC 1.3.99.1); L-glycerol-3-phosphate: (acceptor) oxidoreductase or glycerol-3-phosphate dehydrogenase (EC 1.1.99.5); D-3-hydroxybutyrate: NAD oxidoreductase of D-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30); L-aspartate: 2-oxoglutarate aminotransferase or aspartate aminotransferase (EC 2.6.1.1); L-glutamate: NAD(P) oxidoreductase (deaminating) or glutamate dehydrogenase (EC 1.4.1.3); Ferrocytochrome *c*: oxygen oxidoreductase or cytochrome *c* oxidase or cytochrome aa_3 (EC 1.9.3.1); Monoamine: oxygen oxidoreductase (deaminating) or monoamine oxidase (EC 1.4.3.4); L-malate hydro-lyase or fumarate hydratase or fumarase (EC 4.2.1.2); L-lactate: NAD oxidoreductase or lactate dehydrogenase (EC 1.1.1.27).

(St. Louis, Missouri U.S.A.), fatty-acid-poor bovine serum albumin was from Pentex (Kankakee, Illinois, U.S.A.), chloramphenicol succinate was from Nogepha (Amsterdam, Holland) and oxytetracycline (terramycin) was from Chas. Pfizer & Co. (New York, U.S.A.). All other chemicals were A. R. Grade.

Preparation of Cell Suspensions and Homogenates

Removal of the small intestine and harvesting of villous and crypt cells were performed according to the method of Harrison and Webster [7], as described in detail in previous reports [6, 8]. For histochemical assays, small pieces of duodenum, jejunum and ileum were frozen immediately after flushing the intestinal lumen with cold saline (*cf.* [5] for histochemical data). The remaining parts of the intestine, with a total length of about 60 cm, were used as starting material for the isolation procedure. The isolation medium for the epithelial cells contained 0.01 M Tris-HCl buffer, 0.13 M NaCl and 5 mM EDTA (final pH 7.6). The cells, collected by centrifugation at $800 \times g_{max}$ for 15 s, were suspended in 10 ml of a medium containing 0.25 M sucrose, 20 mM Tris-HCl buffer, 1 mM EDTA, 5 mM $MgCl_2$ and 1 mg/ml bovine serum albumin (final pH 7.4) and homogenized for 60 s with a small Polytron homogenizer (type PT_{10}, Kinematica Luzern, Switzerland) set at position 4.5 (8000 rev./min).

Administration of the Antibiotics

Oxytetracycline was injected intramuscularly every 8 h in a total dose of 300 mg/kg body weight in 24 h. Chloramphenicol succinate was administered every 4 h intramuscularly to a total amount of 1680 mg/kg body weight in 24 h. Control animals received corresponding volumes of saline by a similar route.

Assays

Determination of concentrations of the antibiotics in serum samples was performed by the microbiological method of Hoette and Struyk [9] in collaboration with the Department of Clinical Microbiology of the Rotterdam Medical School.

Protein was determined according to Lowry *et al.* [10] in sonicated samples, using bovine serum albumin as standard.

Except when indicated all enzyme assays were carried out at 30 °C in sonicated preparations (for sonication method see [3]). In order to avoid autolytic damage as much as possible, the enzyme solutions were sonicated just before the start of the assays and labile enzymes (for instance cytochrome *c* oxidase) were tested first.

All data concerning the assays of the following enzymes are given in [3]: glycerol-3-phosphate dehydrogenase; succinate dehydrogenase; glutamate dehydrogenase; D-3-hydroxybutyrate dehydrogenase and aspartate aminotransferase.

Cytochrome *c* oxidase was estimated spectrophotometrically according to Smith *et al.* [11]. Purified cytochrome *c* was reduced with a few grains of dithionite. Excess of dithionite could be effectively removed by gassing the solution with oxygen. The cuvette contained 0.04 mM cytochrome *c* (reduced), 75 mM potassium phosphate buffer (pH 7.4) and 1 mM EDTA (total volume 2.07 ml). In the blank cuvette cytochrome *c* was omitted. During the first 10 min the oxidation of cytochrome *c* followed first order kinetics. The activity is expressed as the value of the first-order rate-constant (s^{-1}) per mg protein.

For the measurement of monoamine oxidase two different substrates were used. The oxidation of kynuramine was determined according to a slight modification of the method of Kraml [12] as described by Blokhuis [13]. The production of 4-hydroxyquinolin as measured fluorimetrically at 20 °C (excitation 318 nm and emission 386 nm) increased linearly with time and proportionally to enzyme concentrations during an incubation of the test solution for 30 min at 37 °C and was expressed quantitively with the aid of known product concentrations added to a blank without kynuramine. The monoamine oxidase activity with benzylamine as substrate was measured spectrophotometrically according to Tabor *et al.* [14], following the rate of benzaldehyde production at 250 nm.

Fumarase was determined in a cuvette containing 125 µmol sodium phosphate and 150 µmol potassium-L-malate. Total volume 3.0 ml, final pH 7.3. The formation of fumarate was followed at 240 nm. The molar absorption coefficient used was 1740 M^{-1} cm^{-1}.

Rotenone-insensitive NADH- and NADPH-cytochrome *c* reductase were tested according to Hogeboom [15] by following the reduction of cytochrome *c* at 550 nm. The assay mixture contained 75 mM potassium phosphate (pH 7.4), 1 mM EDTA, 0.1 mM NADH or NADPH, 0.05 mM cytochrome *c* (oxidized), 1 mM KCN and 1.5 µM rotenone. Total volume: 2.11 ml.

Lactate dehydrogenase was measured at 340 nm in a cuvette containing 150 mM potassium phosphate buffer (pH 7.4), 0.3 mM NADH and 1 mM KCN. Final volume: 2.07 ml. The reaction, except the blank, was started with 1 mM sodium pyruvate.

Cytochrome contents were calculated from the difference spectra of the reduced minus the oxidized pigments of a sonicated villous cell suspension in Ca^{2+}-free Krebs-Ringer-phosphate buffer, containing 4—10 mg protein/ml. Registration was performed with an Aminco-Chance wavelength scanning spectrophotometer in the split-beam mode at room temperature. After dividing the homogenate into two cuvettes and correcting for deviations of the baseline, the

sample cuvette was reduced with a few grains of dithionite while 1 µl H_2O_2 (5%) was added to the reference cuvette. The difference spectrum of cytochrome *b* in homogenates of villous cell suspensions, containing 250 mM sucrose, 20 M Tris-HCl (pH 7.4), 5 mM $MgCl_2$ and 5 µM rotenone, was recorded after reduction of cytochrome *b* in the sample cuvette by the addition of succinate (10 mM) and antimycin A (4 µg/ml). The contents of both cuvettes were saturated with oxygen. The data from these spectra were used for the calculation of a corrected value of the cytochrome *b* content.

The following absorption coefficients were used: Cytochrome aa_3: ε (605−630 nm) = 24.0 mM^{-1} cm^{-1} (*cf.* [16]); ε (445−455 nm) = 164 mM^{-1} cm^{-1} (*cf.* [16]); Cytochrome *b*: ε (562−575 nm) = 20.0 mM^{-1} cm^{-1} (*cf.* [17]); Cytochrome $c + c_1$: ε (551−540 nm) = 19.1 mM^{-1} cm^{-1} (*cf.* [17]); Cytochrome b_5: ε (424 to 405 nm) = 160 mM^{-1} cm^{-1} (*cf.* [18]).

RESULTS AND DISCUSSION

Administration and Blood-Levels of Chloramphenicol and Oxytetracycline

D(−)-*threo* Chloramphenicol and oxytetracycline are considered as effective and selective inhibitors of protein synthesis in mitochondria of a number of eucaryotic cell types [19].

To obtain optimal inhibition conditions, a level of 10−50 µg/ml chloramphenicol has been applied in the medium of cultivated mammalian cells [20, 21] as well as in blood during experiments with regenerating rat liver *in vivo* [22, 23]. After injection the blood concentration of biologically active chloramphenicol in adult rats decreases extremely rapidly because of partial inactivation, resulting from glucuronic acid conjugation, and a rapid secretion of free and conjugated forms in the urine [24]. In order to maintain sufficiently high levels during the course of our experiments, it appeared necessary to inject the rats intramuscularly every 4 h with a total dosage of 1680 mg chloramphenicol succinate ester per kg body weight in 24 h. Under these conditions the serum levels fluctuated between 10 and 30 µg/ml (see Fig. 1). Even after longer periods of treatment (7 days) these levels never exceeded 50 µg/ml or decreased below 8 µg/ml, indicating that the drug intake balanced the clearance.

Oxytetracycline has been reported to exhibit a similar action as chloramphenicol in yeast [25], cultured heart cells [26] and liver [22] at about the same concentrations. The biological half-life of this compound in rats appeared to be much higher than that of chloramphenicol (Fig. 1). Constant serum levels between 10 and 40 µg/ml were obtained by means of intramuscular injections of terramycin (Pfizer) every 8 h at a dosage of 300 mg per kg body

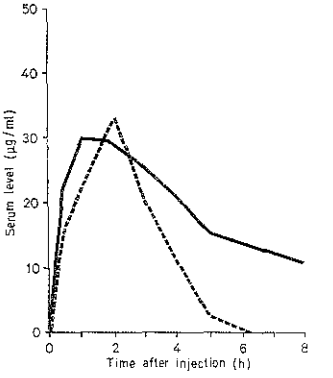

Fig. 1. *Concentrations of chloramphenicol and oxytetracycline in the serum of specific-pathogen-free rats.* Serum levels of oxytetracycline (——) and chloramphenicol (– – – –) in adult specific-pathogen-free rats were measured after a single intramuscular injection of 100 mg/kg body weight terramycin and 280 mg chloramphenicol succinate/kg body weight respectively

weight in 24 h. Long term administration (more than 2 days) led to temporary concentration rises of the drug up to 120 µg/ml. Tetracyclines are concentrated in the bile and excreted by the liver in the gut where they probably enter an enterohepatic circulation [27]. Because penetration of these antibiotics into small intestinal mucosa has been established [27, 28], oxytetracycline was expected to be an useful inhibitor for our purpose, although high tissue concentrations (above 50 µg/ml) might give rise to a disturbance of other cellular processes (see [29, 30, 4]).

Influence of Chloramphenicol and Oxytetracycline on Various Enzyme Activities in Homogenates of Isolated Villous Cells

The rather large range of values found for the specific activities in cell homogenates of the control rats may be caused by individual differences in the animals used, the variation in cross contamination of villous and crypt cells and a fluctuating degree of leakage of soluble enzymes from the intestinal cells during the isolation procedure (*cf.* [8]). It is noteworthy that treatment of the animals with either of the two antibiotics for 48 h did not result in a significant body weight or mucosal-protein loss, anorexia or diarrhea. Therefore possible influence of greatly diminished food uptake on the enzyme activities can be ruled out (*cf.* [31]). Furthermore, it is unlikely that reduction of the bacterial population during the treatment could explain some of the differences

Table 1. *Antibiotic treatment and the yield of villous- and crypt-cell protein*
Villous and crypt cell yield (expressed as mg protein ± standard error isolated from 60 cm of the small intestine) after various times of antibiotic treatment. The number of determinations is given in parenthesis. *P*-values were calculated from the Student *t*-test

Treatment	Cell fraction	Protein yield after treatment for			
		0 h	48 h	72 h	96 h
		mg			
Control	villus	128 ± 8 (19)			
	crypt	30 ± 3 (11)			
Chloramphenicol	villus		130 ± 15 (5)		120 ± 18 (4)
	crypt		25 ± 5 (5)		28 ± 6 (3)
Oxytetracycline	villus		121 ± 17 (6)	70 ± 12[a] (4)	45 ± 15[a] (4)
	crypt		26 ± 6 (6)	31 ± 5 (4)	25 ± 6 (3)

[a] $P < 0.01$.

Table 2. *Specific activities of enzymes in sonicated homogenates of villous cells isolated from the small intestine of control and antibiotic-treated rats*
Control animals were injected intramuscularly with 0.3 ml saline every 8 h for 2–3 days n = number of experiments, P probability value, calculated from the Student t-test

Enzyme	Control	Specific activity after						
		15-h oxytetracycline ($n = 4$)	24-h oxytetracycline ($n = 4$)	24-h chloramphenicol ($n = 3$)	48-h oxytetracycline ($n = 8$)	48-h chloramphenicol ($n = 5$)	130-h oxytetracycline ($n = 2$)	140-h chloramphenicol ($n = 2$)
	$s^{-1} \times mg^{-1}$							
Cytochrome *c* oxidase	0.112 ±0.010	0.075[b] ±0.011	0.058[a] ±0.009	0.046	0.033[a] ±0.008	0.035[a] ±0.007	0.029	0.030
	nmol × min^{-1} × mg^{-1}							
Succinate dehydrogenase	15.9 ±1.6	15.4 ±1.1	16.3 ±1.0	13.9	13.9 ±1.1	13.4 ±1.3	14.1	13.9
Glutamate dehydrogenase	78.5 ±7.9	81.2 ±6.6	71.1 ±5.0	67.0	73.7 ±5.6	82.4 ±4.8	95.6	84.1
Monoamine oxidase (kynuramine as substrate)	0.38 ±0.04		0.32 ±0.05	0.30	0.35 ±0.04	0.37 ±0.06	0.37	0.37
NADPH-cytochrome *c* reductase (rotenone-insensitive)	4.7 ±0.3		4.0 ±0.2	3.9	4.3 ±0.3	4.1 ±0.2	4.8	4.3
Lactate dehydrogenase	3200 ±68		2810 ±90	3250	2210 [a] ±135	3180 ±85	1050	2500

[a] $P < 0.01$.
[b] $P = 0.01$.

between treated and control rats because no significant difference in specific activity of the enzymes tested had been detected between specific-pathogen-free and germ-free rats (unpublished observations). After prolonged treatment only the oxytetracycline-injected rats showed symptoms of lethargy, weight loss and loosening of villous cells (see also [5]). The villous-cell protein yield after isolation appeared to be markedly reduced whereas the amount of crypt-cell protein remained relatively constant (Table 1).

Table 2 shows the effect of treatment of rats with oxytetracycline or chloramphenicol on the specific activities of a variety of enzymes tested in sonicated homogenates of villous cells.

The mitochondrial inner-membrane enzyme cytochrome *c* oxidase clearly has a lower specific activity after treatment with either of the antibiotics. The activity level decreases gradually with time and reaches a minimum, after about 48 h, equal to about 30% of the control value. Prolonged treatment did not result in a further lowering of this value. On the other hand, succinate dehydrogenase, also a mitochondrial inner-membrane marker, is not influenced by the antibiotic treatment to a significant

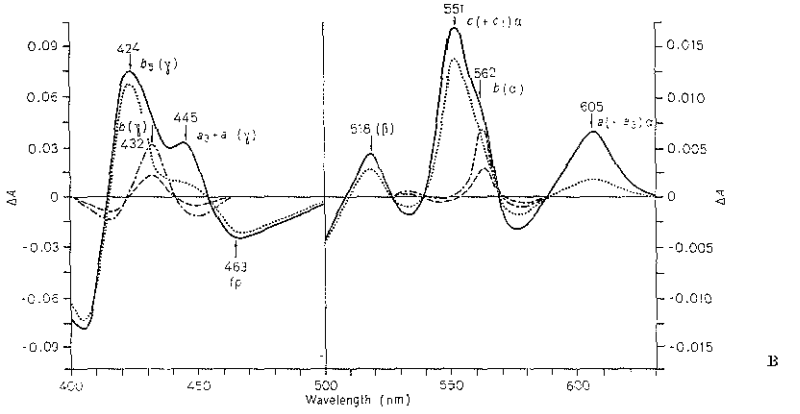

Fig. 2. *Reduced-minus-oxidized spectra of villous-cell homogenates from control (A) and oxytetracycline-treated (B) rats.* Experimental details are shown in the legend of Table 3. Protein concentration, 4.6 mg/ml. (——) Dithionite-reduced minus H_2O_2-oxidized difference spectra obtained with control rats. (·····) Analogous spectra obtained with rats, treated for 48 h with oxytetracycline. (–·–·–) Cytochrome *b* in sample cuvette reduced with succinate and antimycin A; control experiments; (– – – –) Comparable curves obtained with rats after 48 h of antibiotic treatment

extent. The same (not shown in Table 2) holds for mitochondrial glycerol-3-phosphate dehydrogenase (specific activity 11.6 ± 1.1 nmol × min^{-1} × mg protein^{-1}; $n = 12$) and D-3-hydroxybutyrate dehydrogenase (specific activity 11.4 ± 0.6 nmol × min^{-1} × mg protein^{-1}; $n = 12$). The specific activities of glutamate dehydrogenase, fumarase and aspartate aminotransferase, generally considered as mitochondrial matrix enzymes [32,33] (the latter two also occurring in the cytosol), appear unchanged in the treated rats. The control values for fumarase and aspartate aminotransferase in sonicated homogenates (not presented in Table 2) were 370 ± 21 and 189 ± 9.4 nmol substrate × min^{-1} × mg protein^{-1} respectively ($n = 12$). Neither the mitochondrial outer-membrane markers, monoamine oxidase, tested with kynuramine as substrate (Table 2), or benzylamine (specific activity 0.62 ± 0.09 nmol × min^{-1} × mg^{-1}), and rotenone-insensitive NADH-cytochrome *c* reductase (specific activity 23.1 ± 1.2 nmol × min^{-1} × mg^{-1}), nor the microsomal marker enzyme, rotenone-insensitive NADPH-cytochrome *c* reductase (Table 2) are affected by the antibiotics.

The activity of lactate dehydrogenase (a cytosol marker) decreases by about 30% after 48 h treatment with oxytetracycline whereas after 130 h only about 30% of the normal activity remained. On the contrary, chloramphenicol-treated animals did not show such a decrease even after a 140-h injection period. Because we observed no difference in specific activity of lactate dehydrogenase in whole pieces of intestine between control rats and rats, treated with oxytetracycline for 48 h, in accordance with the histochemical results [5], we must assume that the severe damage of intestinal villous cells caused by oxytetracycline after prolonged treatment is preceeded by an increased lability of cell membranes which results in an enlarged permeability for soluble protein molecules during the vibration procedure. The lability could not be induced with chloramphenicol and seems therefore not to be related to the cytochrome deficiency evoked by both of the antibiotics.

Cytochrome Content of Villous-Cell Homogenates of Control and Chloramphenicol- or Oxytetracycline-Treated Rats

In order to obtain further insight into the consequences of the inhibition of mitochondrial protein synthesis for the composition of intestinal mitochondria, difference spectra of villous-cell homogenates were measured.

Fig. 2 illustrates the results obtained with control rats and rats treated for 48 h with oxytetracycline. About the same picture was found after treatment of rats for two days with chloramphenicol (Table 3). Antibiotic treatment causes a significant decrease of the peak at 445 nm (γ-maximum of cytochrome aa_3), a disappearance of the shoulder at 562 nm (α-band of cytochrome *b*) and a strong reduction of the maximum at 605 nm (α-band of cytochrome aa_3). A slight decrease is observed at 551 nm (α-bands of

Table 3. *Cytochrome content of villous cell homogenates from control and chloramphenicol- or oxytetracycline-treated rats*
Details of the experimental procedure and calculation of cytochrome contents are given in Methods. n = number of experiments, P-values were calculated according to the Student t-test

Treatment	Cytochrome					
	$a + a_3$ (605—625 nm)	$a + a_3$ (445—455 nm)	b (562—575 nm)	b (corrected value)	$c + c_1$ (551—540 nm)	b_5 (424—405 nm)
	nmol/mg					
Control (n = 8)	0.061 ± 0.007	0.046 ± 0.004	0.135 ± 0.020	0.102[b] ± 0.012	0.205 ± 0.022	0.209 ± 0.022
24-h oxytetracycline (n = 3)	0.036	0.030	0.110	—	0.210	0.210
48-h oxytetracycline (n = 5)	0.015[a] ± 0.004	0.011[a] ± 0.005	0.079[a] ± 0.010	0.044[a] ± 0.007	0.167 ± 0.026	0.198 ± 0.023
48-h chloramphenicol (n = 5)	0.014[a] ± 0.003	0.010[a] ± 0.004	0.081[a] ± 0.007	0.046[a] ± 0.006	0.178 ± 0.021	0.220 ± 0.019
120-h oxytetracycline (n = 2)	0.014	0.011	0.107	—	0.180	0.230
120-h chloramphenicol (n = 2)	0.014	0.010	0.085	—	0.192	0.200

[a] $P \leq 0.01$.
[b] n = 4.

cytochromes c and c_1) and in the Soret region at 424 nm (γ-band of cytochrome b_5 with contributions of the γ-bands of cytochrome b and c; hemoglobin is absent in the preparations).

Table 3 shows that the amount (per mg protein) of cytochrome aa_3 decreases after a 48 h period of antibiotic treatment by about 75%, in good agreement with the data shown in Table 2 for cytochrome c oxidase. The levels of the cytochromes a and a_3 are equally affected since the absorption peak at 445 and 605 nm are lowered to the same degree. For the calculation of the cytochrome aa_3 contents, based on the height of the γ-maximum at 445 nm, no corrections were made for the absorption difference due to cytochrome b at this wavelength. This may explain the different absolute values for the cytochrome aa_3 content measured at the wavelength pairs 605—630 and 445—455 nm respectively. Again, a new steady-state level of cytochrome c oxidase is reached after about 48 h, indicating an incomplete inhibition of mitochondrial protein synthesis under the conditions *in vivo* or a strong reduction of the turnover rate of the intestinal cell population. The latter possibility could be excluded by further experiments [4].

The decrease of cytochrome b, determined from the dithionite-reduced minus H_2O_2-oxidized difference spectra (Table 3, uncorrected value), is small as compared to cytochrome aa_3. However, this determination is interfered with by the spectral contributions of the α-bands of cytochrome c, c_1 and b_5. Better values could be derived from the difference spectra obtained after reduction of cytochrome b in the sample cuvette with succinate in the presence of antimycin A (Fig. 2 and Table 3, corrected values). It can be seen that the maximal decrease of cytochrome b content (about 60%) is still slightly lower than the drop of cytochrome aa_3.

The decrease of the 424 nm maximum is not significant and can be easily explained by the diminished contribution of the γ-bands of the cytochromes b and $c + c_1$ at this wavelength (Fig. 2). Therefore we conclude that the antibiotics do not influence the level of cytochrome b_5, occurring in microsomes and the outer membrane of mitochondria, in agreement with the results of other microsomal and outer membrane markers already mentioned (Table 2). If it is assumed that the inhibitors of mitochondrial protein synthesis do not block the synthesis of cytochrome c, which, at least in other tissues, is synthesized in the microsomes [34], and that the contribution of cytochrome b_5 at 551 nm does not change during the treatment, it is likely that the slight decrease observed in the cytochrome $c + c_1$ peak at 551 nm is caused by a drop in cytochrome c_1 content (*cf.* [23]). In liver this cytochrome accounts for about one third of the absorption maximum at 551 nm at room temperature [35]. In HeLa cells [36] and regenerating liver [23], chloramphenicol-treatment led to a significant increase in the amount of cytochrome c. If a similar increase has occurred in the intestinal cells, it is obscured in the difference spectra by the fall of cytochrome c_1 content.

Finally, no significant change in flavoprotein content, measured at 463 nm, was observed (Fig. 2).

The results, obtained so far, confirm and replenish the work of other investigators who reported a similar specific action of chloramphenicol *in vivo* on the cytochromes aa_3, b and c, in regenerating liver [23] and during the rapid biogenesis of mitochondria in

Table 4. *Comparison of specific activities of several enzymes in villous- and crypt-cell homogenates from control and treated rats*
Separate villous- and crypt-cell fractions were obtained as described in Methods. The Table shows the specific activity of some enzymes in sonicated homogenates of villous cells (expressed as nmol substrate metabolized $\times$ min^{-1} $\times$ mg $protein^{-1}$), divided by their specific activity in crypt-cell homogenates, originating from the same animal, followed by the standard errors of the ratios. n = number of experiments

Enzyme	Ratio of activity villi/crypts				
	Control (n = 10)	15-h oxytetracycline (n = 4)	15-h chloramphenicol (n = 4)	48-h oxytetracycline (n = 3)	48-h chloramphenicol (n = 3)
Cytochrome *c* oxidase	0.97 $\pm$ 0.09	0.90 $\pm$ 0.13	0.90 $\pm$ 0.11	0.76	0.92
Succinate dehydrogenase	1.35 $\pm$ 0.12	1.33 $\pm$ 0.15	1.27 $\pm$ 0.09	1.46	1.34
Glycerol-3-phosphate dehydrogenase	1.61 $\pm$ 0.14	1.61 $\pm$ 0.14	1.95 $\pm$ 0.10	2.30	1.90
D-3-Hydroxybutyrate dehydrogenase	0.67 $\pm$ 0.04	0.72 $\pm$ 0.12	—	0.61	—
Glutamate dehydrogenase	0.87 $\pm$ 0.06	0.84 $\pm$ 0.11	0.81 $\pm$ 0.18	0.71	0.84
Lactate dehydrogenase	2.8 $\pm$ 0.5	3.0 $\pm$ 0.6	2.9 $\pm$ 0.7	2.40	—

liver, heart, kidney and brain of neonatal rats [37]. Recent studies of Cooke and Work [38] indicate that only about 7% of the total protein content of liver mitochondria, comprising at least 10 distinct proteins, is synthesized *in vivo* and *in vitro* on mitoribosomes. The major part of these products, with molecular weights in the range from 14000 to 50000, is considered to consist of "organizer" proteins which promote the insertion of the insoluble cytochromes of the mitochondrial inner-membrane into their active configuration in the cristae [38,19]. Whereas earlier investigations with the "petite" mutant of yeast [19] and mitochondria from rat liver [39] and *Neurospora crassa* [40] indicated that the protein moiety of the chloramphenicol-inhibited cytochrome aa_3 is entirely synthesized outside the mitochondria, very recent reports of Weiss *et al.* [41—43] suggest that in *Neurospora crassa* at least one of the precursor polypeptides of the functional cytochrome aa_3 complex is synthesized on mitochondrial ribosomes.

The Influence of Antibiotic Treatment on Enzyme Activities in Intestinal-Crypt Cells as Compared to Villous Cells

As shown in Table 4, we found different values for the villous/crypt ratios of homogenate activities (on a protein base) of several mitochondrial enzymes in the control rats, in agreement with the concept of a differential development of mitochondrial enzymes during the short life of the epithelial cells (*cf.* [3]). However, the homogenates showed appreciably lower ratios of glutamate dehydrogenase (Table 4), exclusively localized in the matrix mitochondrialis, and of aspartate aminotransferase (villous/crypt ratio: 0.83 $\pm$ 0.6, n = 10), as compared to the mitochondrial preparations used in earlier studies [2,3]. In those experiments, a more vigorous homogenization procedure (75 s with a type-20 Polytron homogenizer) was applied, which improved the yield but impaired the quality of the intestinal mitochondria. The latter could be inferred from a considerable leakage of mitochondrial matrix enzymes to the 14000 $\times$ g supernatant (see [2]) especially from crypt mitochrondria. The higher fragility of crypt mitochondria may reflect an inherent property of these particles or may be due to an artifact, caused by homogenization of a more dilute and less intact crypt-cell suspension [8] as compared to villous-cell preparations. By using the present homogenization procedure, no discrepancy of villus/crypt ratios of matrix enzymes was found between mitochondrial preparations and cell homogenates (unpublished results).

According to Harrison and Webster [7] and in agreement with our results (Table 4), the antibiotic-sensitive enzyme, cytochrome *c* oxidase, displays a constant activity (expressed per mg protein) from the crypt to the top of the villus. Since the amount of protein per crypt cell seems to be significantly lower than the amount per villous cell [7,44], the equal value found for the specific activity of cytochrome *c* oxidase in villous and crypt cells probably corresponds with an increase of the cellular content of this cytochrome in the maturating compartment of the crypt. An extra synthesis of cytochrome aa_3 in the crypt is also suggested by the lower values of the villus/crypt ratios for 3-hydroxybutyrate dehydrogenase and a number of matrix enzymes, if degradation of these enzymes in the villus is neglected. Further evidence was obtained by treatment of the rats with chloramphenicol or oxytetracycline during a very short period (15 h), corresponding with about one mitotic cycle. After this time the cells, present in the proliferation zone of the crypt as the treatment

started, have migrated not far beyond the junction of crypt and villus (*cf.* [45]). If the synthesis *de novo* of cytochrome *c* oxidase is limited to the proliferation zone of the crypt, the short treatment should only result in a decreased activity in the crypt and not in the villous cells. However, after 15 h treatment the level of cytochrome *c* oxidase in villous- and crypt-cell fractions are equally affected (Tables 2 and 4), indicating an extra synthesis of this cytochrome in the maturating zone of the crypt and/or in the villous cells. According to histochemical observations (see [5]) the strongest decrease of cytochrome *c* oxidase occurs in the crypt and the lower part of the villus, whereas a much smaller loss of activity has been shown at the upper half of the villus. The latter result suggests the existence of a slow rate of synthesis of this enzyme *de novo* in the villus. Since the specific activity of cytochrome *c* oxidase does not increase along the villus, the latter assumption seems to imply a remarkably rapid turnover of this mitochondrial inner-membrane protein in the villous epithelium if compared with other tissues [19]. The existence of rapid degradation processes in the villous region of small intestinal epithelium is suggested by James *et al.* [46] for brush-border proteins and has been demonstrated by Imondi *et al.* [47] for other extramitochondrial enzymes. However, no similar studies have been done on intestinal mitochondrial enzymes.

Also after a 48-h injection period, when a minimum level of cytochrome aa_3 in the villus was reached (Table 2 and 3, *cf.* [5]), we found the same extent of inhibition in villous and crypt cells. Apparently a new steady state is obtained in which an equal degree of inhibition of protein synthesis during the proliferation and the maturative phase of the cell life occurred.

As shown in Table 4, the unchanged values of the villous/crypt ratios for succinate dehydrogenase, glycerol-3-phosphate dehydrogenase and the matrix enzymes, confirm the data of Table 2 and prove that the drastically lowered level of cytochrome aa_3 has no effect on the differential development of other mitochondrial enzymes.

Since the influence of the antibiotics on the crypt cell activities never exceeded the influence on the villus, most of our work was done with villous-cell fractions which can be harvested with a greater yield and in a more intact state than the crypt cells (*cf.* [8]).

Long-Term Effects of the Antibiotics on the Small-Intestinal Epithelium

Chloramphenicol treatment for at least 3 days did not influence intestinal protein content (Table 1), absorptive function (judged from the absence of diarrhea and weight loss) and, most probably, did not alter the cellular turnover rate [4]. It was found that this treatment, although it led to strong reduction of the cytochrome content of small intestinal mitochondria, did not impair the energetic function of these organelles. The evidence for this was based on measurements of oxidative phosphorylation in cell homogenates and of creatine phosphate and adenine nucleotide levels in freeze-clamped samples of jejunum [4]. Lack of impairment of energetic function by chloramphenicol treatment has also been observed in regenerating liver [23] and organs of neonatal rats [37].

The damage of the villous epithelium after long-term treatment with oxytetracycline, mentioned before, occurred at the same cytochrome levels as measured in the chloramphenicol-treated animals [4] and was not attended by a significant reduction of the proliferation of crypt cells. The intestinal necrosis is therefore difficult to explain as the consequence of an impaired mitochondrial function in crypt or villous cells. Several other mechanisms of action of oxytetracycline, which may become manifest only after accumulation of the drug in blood and tissues, were investigated. Our present results [4] indicate that the inhibition of microsomal protein synthesis by high concentrations of tetracyclines (50—100 µg/ml), also described by other authors [30, 48—50], must be seriously considered as another possible base to account for the toxicity of oxytetracycline in long-term experiments.

We gratefully acknowledge the skilful technical assistance of Mr L. M. Scheek.

REFERENCES

1. Cairnie, A. B., Lamerton, L. F. & Steel, G. G. (1965) *Exp. Cell. Res. 39*, 528.
2. Hülsmann, W. C., Iemhoff, W. G. J., Van den Berg, J. W. O. & De Pijper, A. M. (1970) *Biochim. Biophys. Acta, 215*, 553.
3. Iemhoff, W. G. J. & Hülsmann, W. C. (1971) *Eur. J. Biochem. 23*, 429.
4. De Jonge, H. R. *Biochem. Pharmacol.* in the press.
5. De Pijper, A. M. & Hülsmann, W. C. (1972) *Biochimie*, in the press.
6. Iemhoff, W. G. J., Van den Berg, J. W. O., De Pijper, A. M. & Hülsmann, W. C. (1970) *Biochim. Biophys. Acta, 215*, 229.
7. Harrison, D. D. & Webster, H. L. (1969) *Exp. Cell. Res. 55*, 257.
8. Hülsmann, W. C., Van den Berg, J. W. O. & De Jonge, H. R. *Methods Enzymol.* in the press.
9. Hoette, I. & Struyk, A. P. (1958) *J. Lab. Clin. Med. 51*, 638.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
11. Smith, L. (1955) in *Methods of Biochemical Analysis* (Glick, D., ed.) vol. 2, p. 427, Wiley (Interscience), New York.
12. Kraml, M. (1965) *Biochem. Pharmacol. 14*, 1683.
13. Blokhuis, G. G. D. & Velstra, H. (1970) *FEBS Lett. 11*, 197.
14. Tabor, C. W., Tabor, H. & Rosenthal, S. M. (1954) *J. Biol. Chem. 208*, 645.

15. Hogeboom, G. H. (1949) *J. Biol. Chem. 177*, 847.
16. Van Gelder, B. F. (1966) *Biochim. Biochim. Biophys. Acta, 118*, 36.
17. Estabrook, R. W. & Holowinsky, A. J. (1961) *Biophys. Biochem. Cytol. 9*, 19.
18. Estabrook, R. W. (1958) *J. Biol. Chem. 230*, 735.
19. Ashwell, M. & Work, T. S. (1970) *Ann. Rev. Biochem. 39*, 251.
20. Kroon, A. M. & Jansen, R. J. (1968) *Biochim. Biophys. Acta, 155*, 629.
21. Firkin, F. C. & Linnane, A. W. (1968) *Biochem. Biophys. Res. Commun. 32*, 398.
22. De Vries, H. & Kroon, A. M. (1970) *Biochim. Biophys. Acta, 204*, 531.
23. Firkin, F. C. & Linnane, A. W. (1969) *Exp. Cell. Res. 55*, 68.
24. Della Bella, D., Ferrari, V., Marca, G. & Bonanomi, L. (1968) *Biochem. Pharmacol. 117*, 2381.
25. Clark-Walker, G. D. & Linnane, A. (1966) *Biophys. Res. Commun. 25*, 8.
26. Kroon, A. M. (1969) in *Inhibitors Tools in Cell Research* (Bücher, Th. & Sies, H., eds), pp. 159—166, Springer-Verlag, Berlin, Heidelberg, New York.
27. Kelly, R. G. & Kanegis, L. A. (1967) *Toxicol. Appl. Pharmacol. 11*, 128.
28. Shils, M. E. (1962) *Clin. Pharmacol. Ther. 3*, 321.
29. Brody, T. M., Hurwitz, R. & Bain, J. A. (1954) *Antibiot. Chemother. 4*, 864.
30. Ling, V. & Morin, C. L. (1971) *Biochim. Biophys. Acta, 249*, 252.
31. McManus, J. P. A. & Isselbacher, K. J. (1970) *Gastroenterology, 59*, 214.
32. Ernster, L. & Kuylenstierna, B. (1970) in *Mitochondria Structure and Function* (Ernster, L. & Drahota, Z., eds), pp. 5—31. Academic Press, London and New York.
33. De Duve, C., Pressman, B. C., Gianetto, R., Wattiau, R. & Appelmans, F. (1955) *Biochem. J. 60*, 604.
34. Sherman, F. & Stewart, J. W. (1971) *Ann. Rev. Genet. 5*, 257.
35. Klingenberg, M. (1958) *Arch. Biochem. Biophys. 75*, 376.
36. King, M. E., Godman, G. C. & King, D. W. (1972) *J. Cell Biol. 53*, 127.
37. Hallman, M. (1971) *Biochem. Pharmacol. 20*, 1797.
38. Coote, J. L. & Work, T. S. (1971) *Eur. J. Biochem. 23*, 564.
39. Beattie, D. S., Patton, G. M. & Stuchell, R. N. (1970) *J. Biol. Chem. 245*, 2177.
40. Birkmayer, G. D. (1971) *Eur. J. Biochem. 21*, 258.
41. Weiss, H. (1972) *Abstr. Commun. 8th Meet. Fed. Eur. Biochem. Soc.* 656.
42. Jackl, G. & Sebald, W. (1972) *Abstr. Commun. 8th Meet. Fed. Eur. Biochem. Soc.* 658.
43. Sebald, W. & Jackl, G. (1972) *Abstr. Commun. 8th Meet. Fed. Eur. Biochem. Soc.* 659.
44. Koldovsky, O., Herfst, J. J., Burker, J. & Sunshine, P. (1970) *Growth 34*, 359.
45. Galjaard, H. & Bootsma, D. (1969) *Exp. Cell. Res. 58*, 79.
46. James, W. P. T., Alpers, D. H., Gerber, J. E. & Isselbacher, K. J. (1971) *Biochim. Biophys. Acta, 230*, 194.
47. Imondi, A. R., Lipkin, M. & Balis, M. E. (1970) *J. Biol. Chem. 254*, 2194.
48. Yeh, S. D. J. & Shils, M. E. (1966) *Proc. Soc. Exp. Biol. Med. 121*, 729.
49. Franklin, T. J. (1964) *Biochem. J. 90*, 624.
50. Bottaner, E. & Vazques, D. (1971) *Biochim. Biophys. Acta, 254*, 316.

H. R. de Jonge and W. C. Hülsmann
Afdeling Biochemie I, Medische Faculteit Rotterdam
Postbus 1738, Rotterdam, The Netherlands

Biochemical Pharmacology, Vol. 22, pp. 2659–2677. Pergamon Press, 1973. Printed in Great Britain.

TOXICITY OF TETRACYCLINES IN RAT-SMALL-INTESTINAL EPITHELIUM AND LIVER

HUGO R. DE JONGE

Department of Biochemistry I, Rotterdam Medical School, Rotterdam, The Netherlands

(*Received* 6 *October* 1972; *accepted* 16 *April* 1973)

Abstract—Rats, treated for 3 days or longer with high concentrations of oxytetracycline (OTC) suffered from a severe damage of structure and function of the small intestinal epithelium and showed an excessive accumulation of fat in the liver. In an attempt to explain these toxic effects, several possible modes of action of this drug were investigated. No direct correlation was found between the strong inhibitory effect on mitochondrial protein synthesis, leading to drastically lowered mitochondrial cytochrome levels in the rapidly dividing small intestinal epithelial cells, and a disturbance of mitochondrial energy production. After chloramphenicol treatment, producing a similar decrease of cytochrome contents, no epithelial cell damage was observed. At a stage of the OTC-treatment in which minimal cytochrome levels were reached but still no structural damage of the epithelium was observed, the energy-stores in the epithelial cells and the rate of oxidative phosphorylation in isolated intestinal mitochondria appeared to be unaffected. After 3–4 days, when morphological alterations of the villus were clearly observed histologically, the rate of proliferation of the crypt cell population was not appreciably affected, suggesting again a lack of influence on energy metabolism in the crypt cells. During short incubations of isolated villous cells, the cellular energy charge and the rate of aminoacid incorporation into protein was strongly decreased after anaerobiosis and addition of KCN, oligomycin or 2,4-DNP, conditions which impair the mitochondrial energetic function. However, incubation with 100–500 μg/ml of several tetracyclines, of which doxycycline showed the strongest effect, led to a significant inhibition of cytoplasmic protein synthesis without affecting the rate of glycolysis and respiration or the energy charge in the epithelial cells. These observations tend to support the view that the toxic effects of OTC are mainly based on its interaction with cytoplasmic protein synthesis under conditions of drug accumulation in blood and tissues. The lowering of energy charge in liver and intestine after prolonged treatment may be interpreted as secondary effects of this action, namely of fatty infiltration in the liver and structural damage of the villous epithelium respectively.

A NUMBER of antibiotics which inhibit protein synthesis in bacterial 70 S ribosomes also interfere with the mitochondrial protein synthesis in eucaryotic cells such as yeast, Neurospora crassa and mammalian systems.[1–3] The consequences of this inhibitory action for mitochondrial structure and function can be studied most successfully in tissues with a rapid and continuous *de novo* synthesis of mitochondrial components. In our previous work, the small intestinal epithelium of adult rats had been used as a model system because of its unequalled high cellular turnover rate.[4] Treatment of rats with chloramphenicol (CAP) or oxytetracycline (OTC) during about 48 hr led to drastically decreased levels of the mitchondrial cytochromes aa_3 and b in the intestinal villous and crypt cells without significantly affecting the contents of a variety of other mitochondrial- and extramitochondrial enzymes.[4,5] Prolongation of the OTC treatment beyond 2 days caused a considerable loss of body weight, lethargy, diarrhea and severe structural damage of the intestinal epithelium, resulting in a

lowered protein yield of the villous cells after isolation. No similar toxic effects were observed with CAP even after 96 hr of treatment although both antibiotics evoked an equal decrease of cytochrome levels.[4-6] These results suggested that the toxicity of OTC was not based on its interference with mitochondrial protein synthesis but that the antibiotic had another site of action in mammalian cells, appearing after accumulation of the drug during prolonged treatment at serum concentrations above 50 μg/ml.[4] High-concentration effects of CAP, e.g. a direct inhibition of cellular respiration,[7,8] were not expected because the intramuscular administration guaranteed serum levels not surpassing 50 μg/ml during the whole course of the experiment.[4]

In the present study, more information was obtained about the energy metabolism of the intestinal mitochondria *in vivo* and *in vitro* under conditions of greatly depressed cytochrome levels (about 2 days after the start of the injection series) and after prolonged treatment with OTC when villous cell loss occurred. In addition, attemps were made to localize other targets of OTC in the intestinal cells. The villous and crypt cell preparations proved to be useful models for a study of the action of tetracycline antibiotics at subcellular levels in mammalian systems.

EXPERIMENTAL

Animals. Male, specific pathogen free, Wistar rats, weighing 200–230 g, were used throughout. They were maintained on a normal laboratory diet and water *ad lib.* Rats were sacrificed by cervical dislocation.

Chemicals. Enzymes and cofactors were purchased from Boehringer and Sons (Mannheim, W. Germany), 2,4-dinitrophenol and oligomycin from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.), fatty acid poor bovine serum albumin from Pentex (Kaukakee, Ill., U.S.A.) and TMPD (*N*, *N*, *N'*, *N'*-tetramethyl 1,4-phenylenediaminedihydrochloride) from Fluka, A. G. (Buchs, Switzerland). All other chemicals were analytical grade.

Antibiotics. Chloramphenicol (sodium succinate) was obtained from Nogepha, Amsterdam, Holland. Oxytetracycline (Terramycin), obtained from Chas. Pfizer & Co., Inc., New York and Tetracycline (Achromycin), purchased from R.I.T., Genval, Belgium, were used in the form of their hydrochlorides. Doxycycline (Vibromycin), obtained from Pfizer, was used as the hyclate salt (hydrochloride, hemihydrate, hemi-ethanolate).

Administration of the antibiotics in vivo. Serum levels of D(−)-threo chloramphenicol (CAP) between 10 and 50 μg/ml were continuously maintained by intramuscular injection of 280 mg CAP-succinate/kg body wt every 4 hr. Oxytetracycline was injected intramuscularly over 8 hr at a dose of 300 mg/kg body wt per 24 hr. After 48 hr of treatment, serum levels fluctuated between 50 and 120 μg/ml. Further details are reported elsewhere.

Assays. The conditions for the determination of protein and alkaline phosphatase (EC 3.1.3.1) are reported by Iemhoff *et al.*[9] Esterase activity was measured as described by Higgins and Lapides.[10]

Preparation of epithelial cell suspensions, homogenates and mitochondrial fractions. Villous- and crypt cells originating from the whole length of the small intestine were harvested separately according to the high-frequency vibration technique of Harrison and Webster[4,11] in a medium containing 0·01 M Tris–HCl buffer, 0·13 M NaCl and

5 mM EDTA (final pH 7·6). Cells and cell sheets were collected by centrifugation for 15 sec at 800 g_{max}. Homogenates of villous cells were prepared in a medium, containing 0·25 M sucrose, 20 mM Tris–HCl buffer, 1 mM EDTA and I mg/ml bovine serum albumin (final pH 7·4), by treatment of the suspension for 60 sec with a small Polytron homogenizer (type PT_{10}, Kinematica, Luzern, Switzerland) set at position 4·5 (8000 rev/min). Subfractionation of the homogenate was carried out according to the scheme, extensively described by Hülsmann *et al.*[12] The gentle homogenization procedure used gave a small yield of mitochondria. Their quality however, was better than of those obtained by earlier methods[13] where higher yields were obtained.

Preparation of rat liver mitochondria. Rat liver mitochondria were isolated according to Schneider[14] in a medium containing 0·25 M sucrose, 0·01 M Tris–HCl and 1 mM EDTA (pH 7·4). After removing the fluffy layer on top of the mitochondrial pellet by washing with sucrose, the mitochondria were washed four times by resuspension in the isolation medium and resedimenting at 6500 *g* for 10 min. The final pellet was suspended in the isolation medium to a final concentration of about 30 mg protein/ml.

Polarographic experiments. Oxygen uptake was recorded polarographically with a Clark oxygen electrode (Yellow Springs Instrument Co.) at 30° in an air-saturated medium in a final volume of 1·6 ml. Villous cells were incubated in a Ca^{2+}-free Krebs Ringer bicarbonate buffer, pH = 7·4. The incubation medium for cell homogenates and mitochondrial preparations contained 250 mM sucrose, 20 mM Tris–HCl, 1 mg/ml bovine serum albumin, 5 mM potassium phosphate and 1 mM EDTA. The final pH was 7·2. The measurements were started after addition of 1–4 mg of cellular or mitochondrial protein. For calculation of P/O ratios and respiratory control indices (RCI) from the stimulation of respiration rates caused by the addition of 200 nmoles ADP, the method of Chance and Williams[15] was applied, except in the experiments described in Table 4 with intestinal mitochondria. In that case, the basic reaction medium in the polarographic vessel was supplemented with 2 mM $MgCl_2$, 16 mM glucose, 10 IU of dialyzed hexokinase (EC 2.7.1.1) and 200 nmoles ADP. After determination of the oxygen uptake following the addition of the mitochondria in the presence or absence of OTC, the reaction was terminated by addition of $HClO_4$ (final concentration 4 per cent). After deproteinization and neutralization with KOH, the samples were assayed for hexosemonophosphate. Further details are reported elsewhere.[16]

Determination of adenine nucleotides and creatine phosphate in intestinal segments, epithelial cells and liver. Rats were anaesthetized with Nembutal. After opening their abdomen through a midline incision and cannulation of the lumen at the proximal part of the duodenum, the terminal ileum was tied off and cut near the ligature. The lumen contents of the small intestine were carefully removed by rinsing with saline (temperature: 38°). The jejunal part of the intestine was quickly frozen by means of a Wollenberg clamp, precooled in liquid nitrogen. The frozen tissue was pulverized in a porcelain mortar, kept cold with liquid nitrogen, mixed with 5% $HClO_4$, thawed and centrifuged. The pellet was used for the determination of DNA.[17] After filtering the supernatant through "Millipore" filter (pore size 0·4 μm) it was neutralized with KOH and centrifuged. ATP,[18] ADP,[19] AMP[19] and creatinephosphate[20] (CP) were determined enzymically within 3 hr from the start of the operation.

Freeze-clamping of the right lobe of the liver was performed after elevation by

means of a loose ligature conducted through the organ with a needle. Determinations of DNA and adenine nucleotides were carried out as described for intestine.

Levels of adenine nucleotides and DNA in freshly isolated villous cells were determined after gentle shaking of 1·5 ml of a cell suspension (15–20 mg protein) in a Ca^{2+}-free Krebs–phosphate buffer, containing 10 mM glucose, for 20 min in polyethylene vials at 37°. The cells were rapidly disrupted by the addition of 1·5 ml 10% $HClO_4$, immediately followed by an ultrasonic desintegration procedure. Hereafter the same procedure was followed as described for freeze-clamped samples.

Glucose metabolism in isolated intestinal villous cells and human erythrocytes. Glucose utilization was measured in 2 ml of a Ca^{2+}-free Krebs Ringer–phosphate buffer (pH 7·4) containing 10 mM [1—^{14}C]glucose (20 μCi/m-mole). Villous cell suspensions were incubated in polyethylene vials for 30 min at 37°, as described in the previous section. The cells were preincubated with the drugs for 10 min before the addition of glucose. Glucose consumption, lactate production and the production of CO_2, originating from the 1-position of glucose, were measured according to Iemhoff *et al.*[9] Human erythrocytes were collected from heparinized blood samples and washed three times with 8 volumes of saline. The top layer and buffy coat were discarded and the packed cells were suspended in an equal volume of the incubation medium. For measurements of glucose metabolism, the same procedure was applied as described for villous cells.

Incorporation of ^{14}C labeled amino acids into protein of isolated villous and crypt cells. Isolated villous and crypt cells were suspended in a Ca^{2+}-free Krebs Ringer–phosphate buffer, pH 7·4, fortified with a mixture of L-amino acids of the same concentration and composition as used in the Basal Medium, described by Eagle *et al.*[21] Incubations of 1·1 ml aliquots (protein content 5-20 mg) were carried out in polyethylene vials at 37° as described before. After preincubation in the presence of the antibiotics for 5 min, the incorporation of labeled amino acids was started by addition of 0·25 μCi[^{14}C]amino acid mixture (sp. act. 52 mCi/mAtC; code CFB.25, Radiochemical Centre, Amersham) and was stopped after 15 min by the addition of 6 ml ice-cold 5% trichloroacetic acid (TCA). The precipitate was centrifuged and resuspended in TCA (5% w/v) by ultrasonic homogenization. After standing at 90° for 30 min the suspension was centrifuged again and the pellet was washed once with TCA and once with 5 ml ethanol–diethyl ether (2:1, v/v). The final pellet was taken up in 0·5 ml NCS solubilizer (Amersham, Searle) and sonified. This solution was transferred to counting vials, 10 ml toluene liquifluor was added and counting was performed in a liquid scintillation counter with automatic standardization.

Measurement of ^{3}H thymidine incorporation into DNA of villous and crypt cells after in vivo *administration of the label.* Control rats and rats treated for 80 and 60 hr with OTC, were injected intraperitoneally with [^{3}H]-thymidine ([^{3}H]methyl-thymidine, specific activity 37·8 Ci/m-mole, Radiochemical Centre, Amersham). Treatment with OTC was continued for 20 and 40 hr respectively. The animals were then sacrificed and their guts were removed. A duodenal segment (5 cm below the pylorus) was processed for autoradiography. According to Harrison and Webster,[11] different villous cell fractions were harvested during the vibration procedure, as judged from the specific activity of alkaline phosphatase and esterase (Table 1). The first fraction (vibration time 0–10 min, amplitude 0·2 cm) mainly contained cells from the apical areas of the villi and the third fraction (18–27 min) appeared to be rich in basal

TABLE 1. DISTRIBUTION OF LABELED DNA IN CRYPT AND VILLOUS CELL FRACTIONS 20 AND 40 hr AFTER [^{3}H]METHYL-THYMIDINE LABELING

Cell fraction	Total protein (mg)		mg protein / mg DNA		Esterase (nmoles × min^{-1} × mg^{-1} protein)		Alkaline phosphatase (nmoles × min^{-1} × mg^{-1} protein)		Specific incorporation (10^{-3} × dis/min × μg^{-1} DNA) 20 hr†		40 hr†	
	Control	OTC*	Control	OTC	Control	OTC	Control	OTC	Control	OTC	Control	OTC
Villous fraction 1	29	18	16·9	16·7	2900	3110	390	175	0·4	0·6	1·4	1·2
Villous fraction 2	37	12	16·4	15·1	2600	2780	165	83	1·1	1·0	1·3	1·3
Villous fraction 3	24	15	15·2	13·9	1960	2040	141	55	1·4	1·3	1·0	1·2
Crypt fraction	30	27	9·7	10·5	1020	1010	32	29	1·3	1·4	1·1	1·0
Total fractions	120	72										

* Period of OTC-treatment: 100 hr.

† Period after pulse-labeling with [^{3}H]thymidine.

For details concerning the isolation of cells, the antibiotic treatment and the determinations see Experimental. Each value represents an average of six animals, except for the calculations of specific incorporation, where values derived from three animals were used.

villous cells. After isolation of the crypt cells by means of dilatation and vibration, the fractions were centrifuged and the cells resuspended in isolation medium, from which samples were drawn for the assay of protein and enzymes. The cells were then precipitated with ice-cold TCA (5% w/v) and centrifuged again. The precipitates were washed four times with 10 ml 5% TCA and twice with 10 ml ethanol–diethylether (2:1, v/v) and were finally taken up in 5% TCA. Part of this suspension was used for determination of DNA.[17] The other part was centrifuged again. The final pellet was taken up in 0·5 ml NCS and counted, as described for the protein samples in the previous section.

Autoradiography. Duodenal segments were fixed in neutral formalin. Paraffin sections were cut at 6 μm and coated with Ilford K-2 emulsion. After an exposure time of 4 weeks the sections were poststained with hematoxylin and eosin. The average distance covered by labeled cells was determined from micrographs of perfect longitudinal cut crypts and villi. For each animal 10–15 sections were examined and 20–25 crypts and villi were measured.

RESULTS AND DISCUSSION

Turnover rate of the intestinal cell population after OTC-treatment. As shown in previous reports,[4,5] after treatment of rats for 48 hr with OTC or CAP a minimal value of cytochrome aa_3 content in the epithelial cells was reached corresponding with about 25–30 per cent of the control value, whereas cytochrome b showed a residual activity of about 40 per cent. No further decrease was observed after prolonged treatment. The residual activities of the cytochromes may be explained by an incomplete inhibition of mitochondrial protein synthesis by OTC or CAP under the *in vivo* conditions or, alternatively, by a strong reduction of the turnover rate of the intestinal cell population. According to Firkin and Linnane,[8] long-term treatment of Hela cell cultures with CAP caused an 80 per cent decrease of cytochrome aa_3 content resulting in a loss of dividing activity. Since a similar inhibition of the proliferation of intestinal crypt cells should provide a reasonable explanation for the structural alterations of the villous epithelium after prolonged treatment with OTC, this hypothesis was tested by comparing the proliferative activity of crypt cells from normal rats with those from OTC rats during a period between 60 and 100 hr after the first injection of the antibiotic. For that purpose, the incorporation of [^{3}H]thymidine in the crypt cells and the migration of the label from the crypt to the villous top was measured as described in the Experimental section.

Table 1 shows that 20 and 40 hr after the injection of [^{3}H]thymidine the specific activity of DNA in the three villous fractions and in the crypt of the OTC-rats does not differ significantly from the control. Incorporation of [^{3}H]thymidine into the DNA of the crypt cells did not seem to be impaired by the antibiotic treatment for 60 or 80 hr. The data also suggest that the migration of label to the villus top had not been delayed by OTC-treatment for 100 hr. However, because of the OTC-induced morphological changes, especially of the villus, examined histologically,[6] and the incomplete separation of cells from different regions of the villus, indicated by the presence of label in villous fraction 1 20 hr after the pulse, autoradiography was considered as a more accurate technique to trace a possible change in turnover time after prolonged antibiotic treatment.

Table 1 also shows that the protein/DNA ratio in the fractions was not significantly changed, indicating that the diminished yield of villous cell protein (cf. also Ref. 4) was accompanied by a loss of DNA, which is most easily explained by an increased extrusion of villous cells during the antibiotic treatment. The loss of villous cells contributes to the sharp decrease in villus length, observed histologically (Fig. 1). Finally, the specific activity of the marker enzyme alkaline phosphatase, in contrast with esterase, was decreased after OTC-treatment for 100 hr (Table 1). A similar specific depression of this enzyme has been observed following *in vivo* administration of cycloheximide, a potent inhibitor of protein synthesis, at concentrations not inhibiting cell migration.[22]

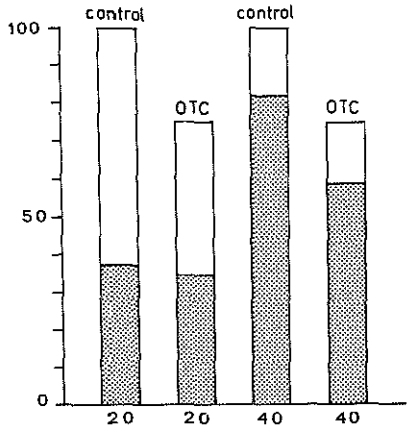

FIG. 1. Abscissa: hours after [^{3}H]thymidine pulse. Ordinate: average distance of migration (arbitrary units of length) of [^{3}H]thymidine-labeled cells (shaded) on the duodenal villi (open). 0 = crypt bottom, 100 = villus length of control rats. Period of OTC-treatment = 100 hr.

Autoradiographic results, given in Fig. 1, show that the distance travelled by the label 20 hr after the [^{3}H]thymidine pulse was not significantly changed by OTC-treatment. In both cases, the boundary between labeled and unlabeled cells was located 8-12 cell positions above the villus-crypt junction. Apparently OTC-treatment did not drastically alter the rate of proliferation of the crypt cells. After another 20 hr the label was at about the same space from the villus top in control and OTC rats. This means, however, that the migration rate of the villous cells was reduced, because the villus length had decreased markedly (Fig. 1). This retardation may be explained by a partial loss of cells during their migration to the villus top, in addition to a reduction of cell size, due to a general dehydration of the animal, as is occasionally observed during treatment with high doses of OTC.[23]

Influence of OTC and CAP administration in vivo *on respiration and energy production of intestinal mitochondria* in vitro. From the lack of influence of the OTC-administration on the proliferative activity of the crypt cells it might be concluded that the energy production in these cells had not been drastically reduced by the antibiotic. However, this does not exclude a diminished mitochondrial energy production, because, as in beating heart cells,[24] the glycolytic capacity of the intestinal tissue might conpensate for such a decrease. A deficiency of oxidative phosphorylation may result from two factors: a lowered rate of electron transport, induced by critical levels of the affected cytochromes and/or a partially uncoupled state of the mito-

chondria, as might arise under conditions of a decreased rate of synthesis of some of the coupling factors (cf. Refs. 8, 24, 25). These two possibilities were investigated in experiments with isolated villous cells, homogenates and mitochondrial fractions derived from 48 hr OTC- or CAP-treated rats, when minimal levels of the cytochromes aa_3 and b were obtained. The results are presented in Table 2.

Villous cell suspensions in Krebs–phosphate buffer maintained a high rate of endogenous respiration for at least 3 hr after the isolation. A KCN-sensitivity of 90–95 per cent was found. In most of the control preparations the Q_{O_2} value was not influenced to an appreciable extent by the addition of glucose, pyruvate and cofactors such as ADP and NAD^+. Succinate, an amino acid mixture or 2,4-dinitrophenol gave a slight and variable stimulation. After gentle homogenization of a cell suspension in sucrose medium in the presence of ADP, P_i and bovine serum albumin, the respiration decreased to a low level, but could be greatly enhanced by the addition of the substrates indicated in Table 2. No stimulation by cytochrome *c* or NADH was observed. Polarographic measurements of oxidative phosphorylation in homogenates and mitochondrial preparations were only successful in a Mg^{2+}-free sucrose-medium in the presence of 1 mM EDTA in order to suppress the hydrolysis of ATP and ADP by the Zn^{2+}-dependent enzyme alkaline phosphatase.[26] Addition of 1·5–3 mM Mg^{2+} to the medium caused a similar stimulation of state 4 respiration as could be induced by the uncoupler 2,4-DNP (results not shown). According to preliminary experiments, Mg^{2+} stimulates the mitochondrial-bound hexokinase in the preparations. The glucose arises from the sucrase activity present in contaminating brushborder fragments. Furthermore, the preparations contain a specific oligomycin-sensitive Mg^{2+}-ATPase, which has been noticed also in mitochondrial preparations of heart- and skeletal muscle.[27,28] Under our conditions we obtained P:O and RCI values, which exceeded those measured by Clark and Sherrat[29] in mitochondrial preparations from guinea-pig mucosa. These authors described a defective oxidative phosphorylation in small intestinal mitochondria isolated from the rat. As shown in Table 2, the only significant differences in maximal Q_{O_2}, observed between control and 48 hr OTC- or CAP-treated rats, appeared with sonicated preparations in the presence of cytochrome *c* and TMPD plus ascorbate as substrate (sonication caused a ten-fold stimulation of respiration). In that case, the decrease of Q_{O_2} to about 35 per cent of the control value, agrees with the strongly reduced activity of cytochrome *c* oxidase mentioned before.[4,5] However, since the lowered level of TMPD-ascorbate oxidation always exceeded the respiration rates found with the other substrates, the electron transport process via cytochrome aa_3 need not be rate-limiting for the overall oxidation with these compounds, neither in the control, nor in the treated rats. The results of the measurements of state 3 oxidation rates, P:O ratios and RCI values with succinate and NAD^+-linked substrates, summarized in Table 2, indicate that neither an influence on the rates of electron transport, nor on the phosphorylating efficiency could be detected in the cytochrome-depleted mitochondria. Similar results for heart mitochondria were obtained by Hallman[3] after treatment of neonatal rats for 4 days with CAP. Furthermore, Firkin and Linnane[30] failed to detect an effect of CAP on the respiration and P:O ratios of mitochondria from regenerating liver. Apparently, even in isolated mitochondria lacking 60–70 per cent of their content of cytochrome b and aa_3, state 3 oxidation rates are not limited by the turnover rates of these cytochromes. However, these experiments do not entirely exclude the possibility that under

TABLE 2. RESPIRATION RATES AND OXIDATIVE PHOSPHORYLATION IN VILLOUS CELLS AND HOMOGENATES FROM CONTROL RATS AND RATS TREATED FOR 48 hr WITH OTC OR CAP

Substrate added	Cells		Total homogenates					
	Q_{O_2}		Q_{O_2}		P:O		RCI	
	Control $n = 8$	Treated $n = 16$	Control $n = 8$	Treated $n = 6$	Control $n = 8$	Treated $n = 6$	Control $n = 8$	Treated $n = 6$
—	19·2 ±0·7	17·5 ±1·0	3·1 ±0·4	3·6 ±0·5				
Glucose (30 mM)	20·5 ±1·2	17·9 ±0·9						
Succinate (5 mM)	24·0 ±1·5	20·5 ±1·7	31·2 ±2·7	27·8 ±2·7	2·14 ±0·12	2·05 ±0·13	2·47 ±0·16	2·21 ±0·41
Glutamate (5 mM)			15·2 ±1·8	15·7 ±2·0	3·20 ±0·14	3·09 ±0·17	3·15 ±0·13	3·03 ±0·16
3-DL-Hydroxybutyrate (5 mM)			15·3 ±0·9	14·1 ±1·2	2·84 ±0·10	2·80 ±0·11	2·24 ±0·15	2·16 ±0·16
Pyruvate (10 mM) plus L-malate (2 mM)			15·1 ±1·3	13·6 ±1·3	3·02 ±0·13	2·94 ±0·18	2·21 ±0·12	2·35 ±0·14
IMPD (0·5 mM) plus ascorbate (10 mM) plus cytochrome *c*			106 ±6·2	44·6 ±8·1 P < 0·01				

The Q_{O_2} values, measured polarographically as described in Experimental, are expressed as nmoles O_2 consumed/min/mg protein followed by their standard errors. P-values were calculated using the Student *t*-test. The Q_{O_2} of homogenates is given for state 3 respiration rates. Final substrate concentrations are indicated in parenthesis. All substrates were added in the form of their sodium salt. Calculations of respiration rates with TMPD plus ascorbate, measured with sonicated preparations, were corrected for auto-oxidation. Because the results with CAP- and OTC-rats showed no significant differences, all data obtained after both kinds of treatment, were pooled for the calculation of mean values and standard errors. With preparations of isolated mitochondria (results not shown), P: O-ratios and RCI-values were essentially similar to those measured with total homogenates, the Q_{O_2}-values increased about twice.

certain *in vivo* conditions higher rates of respiration are required, which cannot be sustained by the low levels of the affected cytochromes (cf. Ref. 31). Therefore we felt that additional information concerning the energy metabolism in the intestine *in vivo* was needed.

Levels of adenine nucleotides and creatine phosphate in small intestinal tissue and liver of the rat in vivo *after OTC- or CAP-treatment.* A depression of oxidative phosphorylation *in vivo* was expected to evoke similar alterations as occur under hypoxic conditions, e.g. a decrease of the energy stores in the epithelial cells, concomitant with an increased rate of glycolysis.[32] Such changes can only be measured accurately with the aid of the freeze-clamping technique, because a preliminary isolation of epithelial cells leads to a rapid and irreversible fall in ATP content of the tissue.[9] However, the contribution of the muscle layer to the energy reserves in the freeze-clamped samples cannot be measured directly. Assuming an equal nucleotide content per mg of dry matter in intestinal epithelial cells and muscle of control rats, about 40 per cent of the estimated nucleotides is derived from the muscle layer.[33] An unequal distribution of creatine phosphate (CP), between epithelial cells and muscle cells is unlikely, as we have found that about 60 per cent of the total activity of creatine kinase in small intestinal tissue was recovered in isolated villous- and crypt cells.* Therefore, a specific influence of the antibiotic treatment on the energy metabolism of the epithelial cells should manifest itself in a significant change in the content of adenine nucleotides, as well as of CP in total intestinal tissue. For comparison, levels of nucleotides were measured in liver of rats subjected to the same OTC regime.

Table 3 shows that intramuscular treatment of the rats for 2 days with OTC or for 3 days with CAP had no significant influence on the levels of ATP, ADP, AMP and CP in intestine and liver, as compared with saline-injected rats. This indicates that, in accordance with the *in vitro* results with isolated mitochondria, the cytochrome-poor mitochondria in intestine kept their normal ATP-generating capacity. However, after 3 days of OTC-treatment, the energy charge of the adenylate system in liver and intestine, proposed by Atkinson[34] as an important metabolic regulatory parameter, and the CP-content of intestine showed a significant decrease. Histochemical investigations showed an excessive accumulation of fat in the liver (unpublished results), in agreement with many earlier reports about the effects of high doses of OTC or tetracycline (TC) on liver,[35–37] and a structural damage of the intestinal villus,[6] in which no accumulation of fat was found.

Both observations may explain the substantial fall of phosphate potential in these organs or, alternatively, both phenomena are the consequences of a decreased ATP-content of the cells. In the latter case, the energy deficiency is most probably caused by a direct effect of OTC, accumulated in liver and intestine, on energy providing processes, e.g. oxidative phosphorylation or glycolysis. This hypothesis was further tested using isolated intestinal cells and mitochondria from intestine and liver as model systems.

Influence of OTC on glucose utilization in intestinal epithelial cells and erythrocytes. According to a recent report of Carević and Čerlek,[38] erythrocytes of patients treated with OTC (1 g/day for 2 days) and human erythrocytes incubated in the presence of 100 μg/ml of this antibiotic, showed a strongly reduced rate of glucose utilization. However, our results apparently conflict with this result. Under similar conditions

* H. R. de Jonge, unpublished results.

TABLE 3. LEVELS OF ADENINE NUCLEOTIDES AND CREATINE PHOSPHATE IN JEJUNUM AND LIVER OF THE RAT *in vivo*

Drug	Dose/injection (mg/kg body wt)	Number of injections per 24 hr	Duration of treatment (hr)	Metabolite concentrations (nmoles/mg DNA)* ATP I	ATP L	ADP I	ADP L	AMP I	AMP L	CP I	Energy charge*,† I	Energy charge*,† L	ATP* ADP + AMP I	ATP* ADP + AMP L
Saline ($n = 5$)	— (I.M.)	3–6‡	48–72‡	230 ±9	1470 ±52	41 ±2	356 ±18	9 ±2	42 ±5	220 ±15	0·89 ±0·02	0·88 ±0·01	4·56 ±0·30	3·66 ±0·20
OTC ($n = 4$)	100 (I.M.)	3	48	223 ±14	1312 ±39	44 ±3	370 ±21	7 ±2	39 ±7	210 ±21	0·89 ±0·03	0·87 ±0·01	4·48 ±0·25	3·21 ±0·24
OTC ($n = 4$)	100 (I.M.)	3	72	167 ±10 P<0·05	810 ±62 P<0·05	70 ±6 P<0·05	758 ±40 P<0·05	16 ±4 P<0·05	86 ±11 P<0·05	132 ±9 P<0·05	0·80 ±0·02 P<0·05	0·72 ±0·02 P<0·05	2·00 ±0·29 P<0·05	0·96 ±0·17 P<0·05
CAP ($n = 3$)	200 (I.M.)	6	48	250 ±12		45 ±5		12 ±5		234 ±19	0·88 ±0·03		4·40 ±0·33	
CAP ($n = 3$)	200 (I.M.)	6	72	233 ±8		38 ±6		10 ±4		250 ±16	0·89 ±0·03		4·85 ±0·30	

* Values are given as means ±S.E.

† The energy charge of the adenylate system is defined as the ratio (ATP + ½ADP)/(ATP + ADP + AMP).[34]

‡ No significant differences were found between saline-injected rats after different methods of treatment. Therefore, all values were pooled for calculation of mean values and standard errors.

Abbreviations used: I = intestine, L = liver, I.M. = intramuscular, n = number of rats, P = probability value, calculated by the Student t-test.

in vitro, incubation with 100–300 μg/ml of oxytetracycline, tetracycline or doxycycline for 30 min did not affect glucose consumption of intestinal epithelial cells (mean value: 15·8 nmoles $\times$ min^{-1} $\times$ mg^{-1} protein glucose used) or human erythrocytes (2·07 μmoles $\times$ hr^{-1} $\times$ ml^{-1} packed cells). Also the lactate production (27·5 and 3·9 respectively) and the production of CO_2 originating from the 1-position of glucose (2·4 nmoles $\times$ min^{-1} $\times$ mg^{-1} protein for villous cells) were not appreciably affected. If an extremely high dose of OTC (400 mg/kg body wt) was given to rats by means of a stomach tube or intraperitoneal injection, essentially the same results (not shown) were obtained with villous cells isolated 4 hr later. Since a direct action of the tetracyclines on glycolysis could not be confirmed by our experiments, we tend to believe that the changes in carbohydrate metabolism observed after tetracycline-treatment[39,40] mainly result from an impaired function of the fatty liver.

Influence of oxytetracycline on oxidative phosphorylation in vitro. As suggested by Linnane *et al.*,[41,42] considering mitochondrial ribosomes as an integral part of the mitochondrial membrane, inhibitors of mitochondrial protein synthesis may interact with other membrane functions, e.g. oxidative phosphorylation and electron transport. Indeed, it has been established that high concentrations of CAP,[7,8] carbomycin, oleandomycin, paromycin[41] and mikamycin[43] may interfere with respiration. Brody *et al.*[44] described an inhibitory action of 5×10^{-4} M OTC (300 μg/ml) on oxidative phosphorylation and octanoate oxidation by isolated Mg^{2+}-poor mitochondria from rat liver and brain. This inhibition could be reversed in the presence of excess Mg^{2+}-ions.

Reinvestigation of these effects (Table 4, Fig. 2) revealed a rather complex inter-

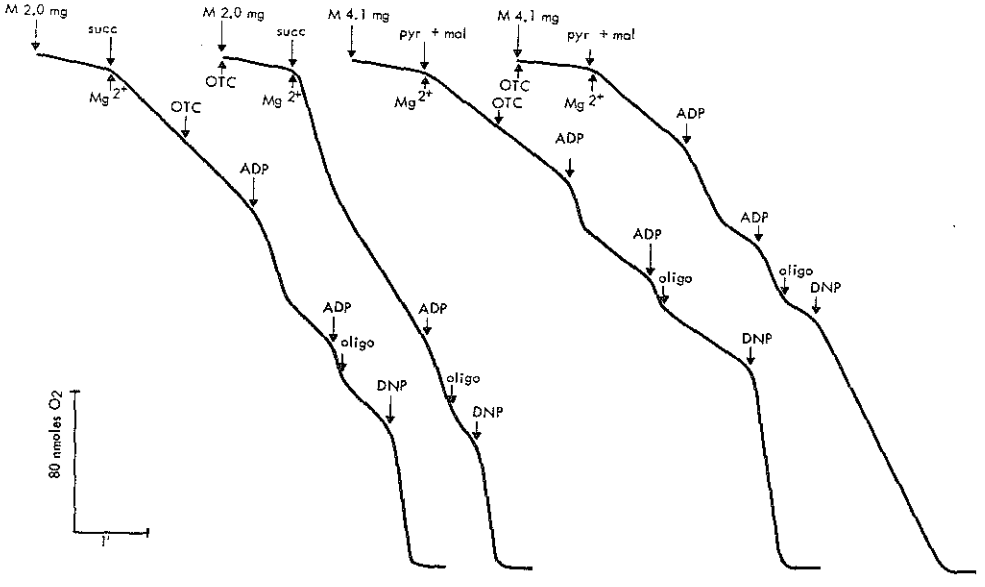

FIG. 2. Effect of oxytetracycline on mitochondrial respiration. Oxygen uptake was determined polarographically at 30° in a medium containing 250 mM sucrose, 20 mM Tris–HCl, 1 mM EDTA, 5 mM potassium phosphate buffer and 1 mg/ml BSA, pH 7·2. Additions: M = liver mitochondria (mg of protein); 2 mM Mg^{2+} as $MgCl_2$; 200 μg/ml OTC (0·33 mM); 200 nmoles ADP; 10 μg oligomycin; 10^{-4} M 2, 4-dinitrophenol; substrate concentrations: see Table 4.

TABLE 4. EFFECT OF OXYTETRACYCLINE ON OXIDATIVE PHOSPHORYLATION OF MITOCHONDRIA ISOLATED FROM RAT SMALL INTESTINAL EPITHELIUM AND LIVER

		Intestinal mitochondria					Liver mitochondria							
		Mg^{2+} omitted			2mM Mg^{2+}		2 mM Mg^{2+}							
Substrate	OTC (μg/ml)	Q_{O_2} (state 4)	Q_{O_2} (state 3)	Q_{O_2} (uncoupled)*	P:O† A‡	P:O† B§	Q_{O_2} (state 4) A	Q_{O_2} (state 4) B	Q_{O_2} (state 3) A	Q_{O_2} (state 3) B	Q_{O_2} (uncoupled) A	Q_{O_2} (uncoupled) B	P:O A	P:O B
Succinate (5 mM)	0	28	70	75	2·3		21		117		124		2·1	
	200	41	48	76	1·2	2·2	50¶	19	65	115	118	120	0·6	1·9
Pyruvate (10 mM)	0	11	35	37	3·0		7		44		46		2.9	
+ L-malate (2 mM)	200	13	17	26	1·4	2·6	9	7	20	39	27	40	1·4	2·4

Q_{O_2} values are expressed as nmoles O_2 consumed/min/mg of protein. The polarographic experiments were carried out six times with different mitochondrial preparations. All results were quite reproducible.

* The uncoupled state was induced by 10^{-4} M dinitrophenol (DNP) added to ADP supplemented mitochondria.

† P:O ratios were determined using the glucose hexokinase assay described in Experimental. No inhibition of HK-activity was observed under these conditions (200 μg/ml OTC, 10 I.U. HK).

‡ Columns A represent P:O or Q_{O_2} values measured if OTC was added 1 min prior to Mg^{2+}.

§ Columns B refer to the experiments in which preincubation with 2 mM $MgCl_2$ for 1 min preceded the addition of OTC.

¶ Higher values were obtained during a transitory period immediately after the addition of succinate (Fig. 2).

action of the antibiotic with the respiratory chain of Mg^{2+}-depleted mitochondria from rat liver and intestine, isolated in sucrose–Tris–EDTA medium. At concentrations of 150–200 μg/ml (175–250 nmoles/mg of protein), OTC caused a stimulation of succinate respiration in the energy-controlled state 4,[15] but only slightly affected state 4 oxidation rates with NADH-linked substrates (glutamate, pyruvate plus malate). Furthermore, an inhibitory effect of OTC on state 3 respiration was observed with all substrates tested. Addition of the uncoupler DNP released the inhibition of succinate oxidation but only partly restored the respiration with NADH-linked substrates. Concomitantly, P:O ratios, measured with the glucose-hexokinase assay, showed a strong decrease if OTC was added prior to the addition of Mg^{2+}-ions (Table 4, column A). Where this influence on the phosphorylation process could not be released by subsequent addition of excess magnesium (2–8 mM), it could be prevented to a large extent by preincubation of the mitochondria with 2–3 mM Mg^{2+} (Table 4, column B).

The interaction of the drug with succinate respiration may be summarized as a uncoupling effect, resulting in a lowering of the P:O ratio and stimulation of controlled respiration, superimposed on a partial oligomycin-like inhibition of the process of energy transfer, implying a depression of state 3 respiration rates. The electron transfer between succinate and oxygen appeared unobstructed, since DNP was able to release this depression. If NADH-linked substrates are offered, the uncoupling effect of OTC, traced by a strong decline of the P:O ratio, does not manifest itself clearly in a stimulated rate of state 4 respiration. This may be explained by an additional influence of the antibiotic on the rate of transfer of reducing equivalents from these substrates to cytochrome b, suggested as well by a decrease of the Q_{O_2} values in the uncoupled state.

The behaviour of Mg^{2+}-poor mitochondria in the presence of OTC and other tetracyclines is similar, in certain aspects, to the detrimental action of 25–100 mM Na^+ on oxidative phosphorylation, described by Gómez-Puyou *et al.*[45] for liver mitochondria, incubated in a Mg^{2+}-free sucrose–EDTA medium. Here too, the respiratory control could be restored by including Mg^{2+} in the incubation mixture. This similarity and the insensitivity of the tetracycline action to subsequent addition of Mg^{2+} suggest that tetracyclines exercise a rather aspecific and readily irreversible influence on the structure of the Mg^{2+}-depleted mitochondrial innermembrane, where the respiratory chain and the enzymes involved in β-oxidation of fatty acids are localized. An explanation of this action, merely based on the Mg^{2+}-chelating properties of tetracyclines, as proposed by Brody *et al.*[44] does not agree with the low binding constant of the Mg^{2+}-tetracycline complex[46] and the inability of excess Mg^{2+} to cancel the action of these drugs.

Whereas a direct inhibition of oxidative phosphorylation by high concentrations of tetracyclines is well established by these *in vitro* experiments, a similar action *in vivo* can be expected only if the concentration of Mg^{2+}-ions in the mitochondria of the cells is very low and if tetracyclines accumulate in this region. However, radiolabeling techniques showed that tetracycline is selectively bound to the microsomal fraction of liver, kidney and muscle cells of the rat.[47] Stronger arguments against a disturbance of oxidative phosphorylation by these antibiotics in the intact cell could be derived from measurements of respiration rates and adenine nucleotide levels of isolated villous cells.

TABLE 5. ADENINE NUCLEOTIDE LEVELS IN ISOLATED VILLOUS CELLS

Additions	Concn.	Σ (ATP + ADP + AMP)	Adenine nucleotide levels (nmoles/mg DNA)			ATP + ½ADP	ATP
			ATP	ADP	AMP	ATP + ADP + AMP	ADP + AMP
—		260	99	83	78	0·54	0·61
Nitrogen*		254	24	90	140	0·27	0·10
2,4-DNP	0·2 mM	243	12	80	151	0·21	0·05
Oligomycin	35 μg/ml	258	22	100	136	0·28	0·09
KCN	0·7 mM	239	10	59	170	0·17	0·04
OTC	500 μg/ml	281	105	106	70	0·56	0·60
TC	500 μg/ml	254	95	71	88	0·51	0·60
Doxy	300 μg/ml	268	103	98	67	0·57	0·62

Incubations were carried out as described in Experimental. Mean values are shown, calculated from three different experiments. For further details see also Table 2.

* Anaerobiosis was maintained by a stream of nitrogen gas through two needles in rubber-capped incubation vials during the whole course of the incubation.

The Q_{O_2} of villous cells, incubated for 10 min in a glucose containing Krebs-Ringer–bicarbonate buffer, did not change after addition of 100–300 μg/ml OTC, TC or doxycycline (results not shown).

Table 5 shows that the total nucleotide content per mg of DNA did not differ significantly from the value obtained with freeze-clamped samples of jejunum (Table 3). This confirms the intact state of the isolated villous cells (cf. also Ref. 12). The energy charge in these cells appeared to be highly dependent on a proper functioning of the intestinal mitochondria. Incubation of villous cells in a glucose-containing medium under anaerobic conditions or in the presence of the uncoupler 2,4-DNP, oligomycin, an inhibitor of oxidative phosphorylation, or KCN, led to a remarkable lowering of the cellular energy charge. However, high concentrations of OTC, TC or doxycycline had no effect on this parameter, although penetration into the cells was indicated by the influence of these antibiotics, especially of doxycycline, on the aminoacid incorporation into cellular protein under comparable experimental conditions (see Table 6).

Table 6. Inhibitory effect of tetracyclines on the incorporation of [^{14}C]-labeled amino acids into protein of isolated small intestinal villous- and crypt cells

Additions	Time of incubation (min)	Incorporation activity (dis/min × mg^{-1}protein)		Percentage of inhibition of extramitochondrial protein synthesis	
		Villous cells	Crypt cells	Villous cells	Crypt cells
None	7·5	260	1299		
None	15	482	2575		
CAP (50 μg/ml)	15	469	2510		
Puromycin (300 μg/ml)	15	37	126	92 (89–96)	95 (92–98)
OTC (100 μg/ml)	15	419	2270	10 (8–12)	11 (8–13)
OTC (300 μg/ml)	15	355	1832	24 (23–26)	27 (24–31)
TC (100 μg/ml)	15	389	2083	17 (14–19)	17 (13–19)
TC (300 μg/ml)	15	290	1581	38 (34–41)	37 (35–38)
Doxy (100 μg/ml)	15	368	1810	21 (19–22)	28 (25–30)
Doxy (300 μg/ml)	15	184	890	60 (50–70)	65 (60–70)
KCN (0·7 mM)	15	45	237	90 (88–93)	90 (86–94)
Nitrogen*	15	56	291	88 (85–91)	88 (82–93)
Oligomycin (35 μg/ml)	15	97	529	80 (77–84)	79 (76–83)
2,4-DNP (0·2 mM)	15	83	424	83 (80–86)	84 (79–89)

Each value of the incorporation activity represents the average of four determinations. The incorporation activities after 7·5 or 15 min were diminished with the small values found at zero min incubation. The extent of inhibition of cytoplasmic protein synthesis was obtained after correction for the diminished incorporation activity, caused by the action of 50 μg/ml CAP on mitochondrial- and bacterial protein synthesis.

* See the legend of Table 5.

Inhibition of cytoplasmic protein synthesis by tetracyclines. Experiments with cell free systems from rat liver[48] and yeast,[49] with intestinal slices[50] or rings[51] and *in vivo* studies of incorporation of labeled amino acids into various organs of the rat[52] indicate that tetracyclines at high concentrations not only inhibit bacterial or mitochondrial protein synthesis, but in addition act on the 80 S-ribosomes of eucaryotic cells. Therefore, cytoplasmic protein synthesis is also partially inhibited. The results

of our studies with isolated villous and crypt cells, presented in Table 6, are in agreement with this concept. The incorporation of labeled aminoacids into villous and crypt cell protein, followed during an incubation period of 7·5–15 min, showed a linear rate. The capacity of the crypt cells for protein synthesis, expressed on a protein base, surpassed that of the villous cells about five times, probably reflecting the *in vivo* situation.[53] During these short-term incubations with a mixture of labeled amino acids the extent of inhibition of cytoplasmic protein synthesis by 100–300 μg/ml of the antibiotics varied from 10–25 per cent for OTC to 28–65 per cent for doxycycline. Puromycin, a strong inhibitor of cytoplasmic- and mitochondrial protein synthesis, inhibited the incorporation by about 95 per cent. Although it was found that the rate of protein synthesis in isolated intestinal cells was very sensitive to an impairment of oxidative phosphorylation induced by KCN or anaerobiosis (Tables 5 and 6), even under conditions of a high aerobic glycolysis, this could not explain the inhibitory effects of the tetracyclines, because the adenine nucleotide levels in the isolated villous cells were unaltered under similar experimental conditions (Table 5). According to Ling and Morin,[51] inhibition of amino acid transport by these drugs may only be expected as an effect, possibly secondary to the inhibition of synthesis of a transport protein possessing a rapid turnover rate. It is unlikely that this action interfered with our studies in which very short incubation periods were used.

The much stronger inhibitory action of doxycycline as compared with OTC or TC may be due to its higher lipophilicity,[54] which possibly enables it to penetrate the intestinal cell much easier than the other tetracyclines. Despite the potential danger of the use of doxycycline suggested by our incorporation studies, this antibiotic is specially recommended for patients with renal insufficiency. However, as shown by the work of Schach von Wittenau *et al.*,[54] it has little chance to become toxic because of the low maintenance dose and the enhanced elimination of this compound from the blood by diffusion into the intestinal lumen, which prevents its accumulation in blood and tissues.

In the liver the action of high concentrations of OTC on cytoplasmic protein synthesis may be responsible for a disturbance of lipoprotein synthesis and/or transport resulting in fatty infiltration, also observed after puromycin treatment[55] which might cause the decrease of the energy charge in this organ after 3 days of antibiotic treatment.

In intestine, the unchanged rate of proliferation of the epithelial cells in the OTC rats does not necessarily conflict with a partial inhibitory action of OTC on overall protein synthesis, because a degree of inhibition, much less than 80 per cent, does not significantly affect the number of proliferating crypt cells.[56] However, in the villus region it may influence the synthesis of some protein components of the brushborder, e.g. alkaline phosphatase,[22] transport proteins[51] or glycoproteins of the glycocalix. The latter might increase the lability of cell membranes, suggested by the increased loss of soluble enzymes from the cells during the isolation procedure,[4] and induce a loosening of cells from the connective tissue. Also chelating of calcium by the tetracyclines might play a role in the displacement of the villous cells (cf. Ref. 11). Moreover, an additional effect of the drug on glycoprotein synthesis and release by the mucus-producing Goblet cells is conceivable in the light of a recent report of Tucker and Webster,[57] indicating an impaired transport and secretion of pancreatic proteins after administration of high doses of tetracycline. In our view the decrease of energy

charge in the intestinal tissue might be a consequence of the structural damage of the epithelium, resulting from the proposed actions of OTC mentioned above. Finally, inhibition of protein synthesis in other organs such as liver, kidney and pancreas may severely impair their function and exert secundary effects on the gastrointestinal morphology (e.g. dehydration[23]) or function (e.g. decreased absorption of fat[58]). Also, alterations of the intestinal microflora may affect absorption of some nutritients, such as iron.[59]

In conclusion it may be stated that the toxicity of OTC under our experimental conditions is most probably based on a primary action of this antibiotic on cytoplasmic protein synthesis rather than on an interaction of the drug with cellular energy metabolism either directly, or indirectly via depletion of mitochondrial cytochromes in rapidly proliferating tissues.

Acknowledgements—The author is indebted to Prof. Dr. W. C. Hülsmann for his suggestions and advice and to Mr. L. M. Scheek for expert technical assistance. He also thanks Mr. W. J. Visser (Department of Cell Biology, Erasmus University, Rotterdam, head: Prof. Dr. H. Galjaard) for doing the autoradiography.

REFERENCES

1. M. ASHWELL and T. S. WORK, *Ann. Rev. Biochem.* **39,** 251 (1970).
2. A. M. KROOM, in *Inhibitors in Cell Research* (Eds. TH. BÜCHER and H. SIES), pp. 159–166. Springer, Berlin (1969).
3. M. HALLMAN, *Biochem. Pharmac.* **20,** 1797 (1971).
4. H. R. DE JONGE and W. C. HÜLSMANN, *Eur. J. Biochem.* **32,** 356 (1973).
5. H. R. DE JONGE and W. C. HÜLSMANN, *Abstr. Commun. Meet. Fed. Eur. Biochem. Soc.* 8, No. 663, Amsterdam (1972).
6. A. M. DE PIJPER and W. C. HÜLSMANN, *Histochemie* **33,** 181 (1973).
7. K. B. FREEMAN and D. HALDAR, *Biochem. biophys. Res. Commun.* **28,** 8 (1967).
8. F. C. FIRKIN and A. W. LINNANE, *Biochem. biophys. Res. Commun.* **32,** 398 (1968).
9. W. G. J. IEMHOFF, J. W. O. VAN DEN BERG, A. M. DE PIJPER and W. C. HÜLSMANN, *Biochim. biophys. Acta* **215,** 229 (1970).
10. C. HIGGINS and J. LAPIDES, *J. biol. Chem.* **170,** 467 (1947).
11. D. D. HARRISON and H. L. WEBSTER, *Exp. Cell Res.* **55,** 257 (1969).
12. W. C. HÜLSMANN, J. W. O. VAN DEN BERG and H. R. DE JONGE, *Methods in Enzymology*, in press.
13. W. G. J. IEMHOFF and W. C. HÜLSMANN, *Eur. J. Biochem.* **23,** 429 (1971).
14. W. C. SCHNEIDER, *J. biol. Chem.* **176,** 259 (1948).
15. B. CHANCE and G. R. WILLIAMS, *Adv. Enzymol.* **17,** 65 (1956).
16. W. C. HÜLSMANN, J. W. DE JONG and A. VAN TOL, *Biochem. biophys. Acta* **162,** 292 (1968).
17. D. N. CROFT and M. LUBRAN, *Biochem. J.* **95,** 612 (1965).
18. W. LAMPRECHT and I. TRAUTSCHOLD, in *Methoden der Enzymatischen Analyse* (Ed. H. U. BERMEYER), p. 2024. Verlag Chemie, Weinheim (1970).
19. D. JAIVOREK, W. GRUBER and H. U. BERGMEYER, in *Methoden der Enzymatischen Analyse* (Ed. H. U. BERMEYER), p. 2051. Verlag Chemie, Weinheim (1970).
20. W. LAMPRECHT, P. STEIN, F. HEINZ and H. WEISSER, in *Methoden der Enzymatschen Analyse* (Ed. H. U. BERMEYER), p. 1729. Verlag Chemie, Weinheim (1970).
21. H. EAGLE, V. I. OYAMA, M. LEVY and A. E. FREEMAN, *J. biol. Chem.* **226,** 191 (1956).
22. A. M. SPIELVOGEL, R. D. FARLEY and A. W. NORMAN, *Exptl. Cell Res.* **74,** 359 (1972).
23. M. E. SHILS, *Clin. pharmac. Ther.* **3,** 321 (1962).
24. A. M. KROON and R. J. JANSEN, *Biochim. biophys. Acta* **155,** 629 (1968).
25. A. M. KROON, in *Atti del Semenario di Studi Biologi* (Ed. E. QUAGLIARIELLO), Vol. 4, pp. 9–26. Adriata Editrice, Bari (1969).
26. G. HÜBSCHER and G. R. WEST, *Nature, Lond.* **205,** 799 (1965).
27. D. D. TYLER and J. GONZE, *Methods in Enzymology* Vol. X, pp. 76–77. Academic Press, New York (1967).
28. L. E. ERNSTER and K. NORDENBRAND, *Methods in Enzymology* Vol. X, p. 93. Academic Press, New York (1967).
29. B. CLARK and H. S. A. SHERRAT, *Comp. Biochem. Physiol.* **20,** 223 (1967).

30. F. C. Firkin and A. W. Linnane, *Exptl. Cell Res.* **55,** 68 (1969).
31. M. Hallman, *Scand. J. clin. Invest.* **25,** Suppl. 113, 80 (1970).
32. J. M. J. Lamers and W. C. Hülsmann, *Biochim. Biophys. acta* **275,** 491 (1972).
33. H. S. A. Sherrat, *Comp. Biochem. Physiol.* **24,** 745 (1968).
34. D. E. A. Atkinson and G. M. Walton, *J. biol. Chem.* **242,** 3239 (1967).
35. J. T. Seto and M. H. Lepper, *Antibiot. Chemother.* **4,** 666 (1954).
36. L. D. Wrubble, A. J. Ladman, L. G. Britt and A. J. Cummins, JAMA **192,** 92 (1965).
37. K. Breen, S. Schenker and M. Heimberg, *Biochim. biophys. Acta* **270,** 74 (1972).
38. O. Carević and N. Čerlec, *Enzymologia* **41,** 53 (1971).
39. S. Banerjee, K. S. Kumar and A. Bandyopadhyay, *Proc. Soc. exp. Biol. Med.* **125,** 618 (1967).
40. V. Y. Kudryavtseva, *Antibiotiki* **3,** 65 (1958).
41. A. W. Linnane and J. M. Haslam, *Current Topics in Cellular Regulation*, Vol. 2, p. 101. Academic Press, New York (1970).
42. C. L. Bann, C. H. Mitchell, H. B. Lukins and A. W. Linnane, *Proc. natn. Acad. Sci., U.S.A.* **67,** 1233 (1970).
43. H. Dixon, G. M. Kellerman, C. H. Mitchell, N. H. Towers and A. W. Linnane, *Biochem. biophys. Res. Commun.* **43,** 780 (1971)
44. T. M. Brody, R. Hurwitz and J. A. Bain, *Antibiot. Chemother.* **4,** 864 (1954).
45. A. Gómez-Puyou, F. Sandoval, A. Peña, E. Chávez and M. Tuena, *J. biol. Chem.* **244,** 533 (1969).
46. R. H. Connamacher and H. G. Mandel, *Biochim. biophys. Acta* **166,** 475 (1968).
47. E. Dale, J. B. Baxley and F. D. Scutchfield, *Experientia* **24,** 164 (1967).
48. T. J. Franklin, *Biochem. J.* **90,** 624 (1964).
49. E. Bottaner and D. Vazques, *Biochim. biophys. Acta* **254,** 316 (1971).
50. N. J. Greenberger, *Nature, Lond.* **214,** 702 (1967).
51. V. Ling and C. L. Morin, *Biochim. biophys. Acta* **249,** 252 (1971).
52. S. D. J. Yeh and M. E. Shils, *Proc. Soc. exp. Biol. Med.* **121,** 729 (1966).
53. B. C. Das and G. M. Gray, *Biochim. biophys. Acta* **195,** 255 (1969).
54. M. Schach von Wittenau, T. M. Twomey and A. C. Swindell, *Chemotherapy* **17,** 26 (1972).
55. D. S. Robinson and A. Seakins, *Biochim. biophys. Acta* **62,** 163 (1962).
56. R. S. Verbin, H. Liang, L. M. Saez, G. Diluiso, P. J. Goldblatt and E. Farber, *Exptl. Cell Res.* **65,** 81 (1971).
57. P. C. Tucker and P. D. Webster, *Am. J. Dig. Diseases* **17,** 675 (1972).
58. S. D. J. Yeh and M. E. Shils, *Proc. Soc. exp. Biol. Med.* **123,** 367 (1966).
59. B. S. Reddy, J. R. Pleasants, D. R. Zimmerman and B. S. Wostmann, *J. Nutr.* **87,** 189 (1965).

Adenylate Cyclase Activities and Adenosine 3′:5′-Cyclic Monophosphate Concentrations in Villus and Crypt Cells of Rat Small-Intestinal Epithelium: Response to Cholera Toxin, Prostaglandin E_1 and Hormones

H. R. DE JONGE and W. C. HÜLSMANN

Department of Biochemistry I, Erasmus University, P.O. Box 1738, *Rotterdam, The Netherlands*

The regulatory function of cyclic AMP in intestinal processes, e.g. fluid and electrolyte secretion (Schafer *et al.*, 1970; Pierce *et al.*, 1971; Chen *et al.*, 1972; Parkinson *et al.*, 1972; Guerrant *et al.*, 1973; Grand *et al.*, 1973; Kimberg *et al.*, 1971, 1973) and glycoprotein synthesis (Forstner *et al.*, 1973), has commonly been studied by use of intestinal biopsies, mucosal scrapings or stripped mucosa. These preparations consist of a heterogeneous population of epithelial cells, fragments of lamina propria and (in the latter case) fragments of muscularis mucosae. Information more specifically related to well-defined cell types can be obtained by measuring adenylate cyclase activities and cyclic AMP concentrations in relatively homogeneous preparations of villus and crypt epithelial cells, isolated separately by the vibration technique of Harrison & Webster (1969), as described by Iemhoff *et al.* (1970) and De Jonge (1973). During the isolation procedure only a small percentage of the cells is damaged, as judged by leakage of soluble enzymes and measurements of protein and DNA synthesis (Iemhoff *et al.*, 1970; De Jonge, 1973).

As shown in Table 1, the cyclic AMP content of crypt cells, incubated in Krebs–Ringer phosphate buffer, pH 7.4, containing glucose, expressed on a protein base, is about three times that of the villus cells. The cyclic AMP concentration in the isolated proliferative crypt cells therefore exceeds that in the differentiated villus cells about 1.5–2-fold (see De Jonge & Hülsmann, 1973). Extrapolation of these results to the situation *in vivo* suggests that apparently no reverse relationship exists between cyclic AMP content and proliferative activity of intestinal epithelial cells. The mean concentration of cyclic AMP determined in freeze-clamped samples of rat small intestine (including muscle layers and lamina propria) was found to be 4.7 ± 0.6 ($n = 6$) and showed no significant variation along the length of the intestine. Therefore this method leads to values resembling those obtained with isolated villus cells.

Table 1 also shows a stimulatory effect of prostaglandin E_1, theophylline and adrenaline on cyclic AMP concentrations. No appreciable qualitative or quantitative differences were found in the response of either type of cells to these agents. The β-adrenergic stimulation of cyclic AMP concentrations by adrenaline may explain the increased rate of glycoprotein synthesis measured in intestinal slices (Forstner *et al.*, 1973). However,

Table 1. *Cyclic AMP concentrations in villus and crypt cells of rat small intestine*

Cells, transferred to plastic vials and suspended in Krebs–Ringer phosphate buffer, pH 7.4, containing 0.1 mM-$CaCl_2$ and 20 mM-glucose, were incubated for 15 min in a water bath at 37°C with gentle agitation. Cyclic AMP contents were measured in trichloroacetic acid-precipitated samples by a slight modification of the method of Brown *et al.* (1971), as described by Cooke *et al.* (1972). The results are expressed as means ±S.E.M., with the numbers of animals in parentheses.

Conditions	Cyclic AMP content (pmol/mg of protein)	
	Villus cells	Crypt cells
Control	4.3±0.7 (21)	12.1±1.3 (21)
Prostaglandin E_1 (1 μM)	7.5±0.9 (14)	15.3±2.0 (14)
Prostaglandin E_1 (10 μM)	13.1±0.9 (11)	27.8±5.2 (8)
Prostaglandin E_1 (0.1 mM)	16.6±1.3 (6)	38.0±4.5 (4)
Theophylline (10 mM)	14.5±2.4 (4)	20.9±3.0 (4)
Adrenaline (1 μM)	7.2±0.8 (12)	17.5±2.9 (12)
Adrenaline (10 μM)	8.7±1.6 (10)	19.5±2.3 (7)
Adrenaline (0.1 mM)	10.4±0.9 (6)	17.3±1.9 (4)
Insulin (20 μunits/ml)	4.0 (2)	11.7±2.1 (3)
Insulin (200 μunits/ml)	6.0±1.2 (6)	9.3±2.0 (6)
Insulin (1 munit/ml)	5.1±0.7 (6)	10.8±1.7 (6)
Prostaglandin E_1 (0.1 mM)+ insulin (20–200 μunits/ml)	14.4±2.1 (5)	34.1±4.2 (5)

the possibility cannot be excluded that this effect is limited to the mucus-secreting goblet cells present in our preparations.

Insulin and glucagon, tested over a broad concentration range, were without effect on cyclic AMP concentrations during the short incubation period used (H. R. De Jonge & W. C. Hülsmann, unpublished work). Although intestinal metabolism is generally considered to be insensitive to insulin under physiological conditions, the brush borders of the villus cells may contain insulin receptors responding to insulin added to the intestinal lumen. Further investigations *in vivo*, with prolonged exposure times and low insulin concentrations, to test this hypothesis, might have important implications in view of a possible therapeutic value of luminal-administrated insulin in cases of *Vibrio cholera* infections (compare Cuatrecasas, 1973).

No effects of cholera toxin (0.1–5 μg/ml) on cyclic AMP concentrations in villus and crypt cells were detected during a 15–60 min period of incubation. After 1 h the cyclic AMP concentrations measured *in vivo* begin to rise (Schafer *et al.*, 1970; H. R. De Jonge, unpublished work), as a result of a stimulatory effect on adenylate cyclase activity in the intestinal cells (Kimberg *et al.*, 1971). In our system longer periods of incubation were not tested because of the occurrence of severe cellular damage.

In order to study long-term effects of cholera toxin and hormones on intestinal cells we measured adenylate cyclase activities in freshly isolated villus and crypt cells from treated and control rats. Table 2 gives the results of control experiments.

Basal and fluoride-stimulated values did not vary appreciably from duodenum to ileum. In agreement with cyclic AMP measurements, both villus-cell and crypt-cell adenylate cyclase activity showed a rapid, stimulated, response to prostaglandin E_1 and relatively high concentrations of adrenaline. The villus-cell/crypt-cell ratio of enzyme activity per mg of protein in the fluoride-stimulated state varied between 0.7 and 0.9, indicating an approximately equal enzyme content in upper villus and crypt cells. Because only the mature villus cells possess a well-developed brush border, this result argues strongly against a localization of the enzyme in the brush-border region and is in

Table 2. *Adenylate cyclase activities in epithelial cells from different segments of rat small intestine*

Cells from the upper part of the villus were obtained by short vibration of the everted intestine (0–15 min), as described by De Jonge (1973). Enzyme activities in cell homogenates, obtained by Polytron treatment (De Jonge, 1973), were measured in a volume of

50μl at 30°C by the method of Krishna *et al.* (1968), with 0.1mM-[α-^{32}P]ATP, 10mM-theophylline and 0.1mM-cyclic AMP. Protein samples contained 10–40μg. The incubation period was 10min; preincubation without ATP was for 5min. The results are expressed as means ± S.E.M. (n = number of animals).

	Adenylate cyclase activity (pmol of cyclic AMP/10min per mg of protein)					
	Duodenum (n = 3)		Jejunum (n = 3)		Ileum (n = 6)	
	Upper-villus cells	Crypt cells	Upper-villus cells	Crypt cells	Upper-villus cells	Crypt cells
Basal	32±5	39±4	30±4	43±4	31±3	38±4
NaF (10mM)	240±11	270±14	241±9	332±7	266±6	324±8
Prostaglandin E_1 (20μM)	60±3	83±3	58±4	78±3	59±3	70±4
Adrenaline (20μM)	—	—	—	—	42±3	57±5

agreement with studies in rabbit small intestine (Parkinson *et al.*, 1972) indicating a preferential localization in basal and lateral plasma membranes.

Insulin (1–100ng/ml) and cholera toxin (1–10μg/ml) did not influence the basal activity of adenylate cyclase to a significant extent during the 15min incubation procedure, in agreement with our studies of the cyclic AMP concentrations in isolated cells.

Further work is required to localize the effects of cholera toxin on intestinal adenylate cyclase more precisely during the course of the infection period, in order to verify the hypothesis that only the crypt-cell population reacts, on stimulation of adenylate cyclase activity, with a hypersecretion of fluid and electrolytes (compare Roggin *et al.*, 1972; Serebro *et al.*, 1969).

We gratefully acknowledge the technical assistance of Mr. L. M. Scheek. Purified cholera toxin was a generous gift from Dr. R. A. Finkelstein, University of Texas, Southwestern Medical School, Dallas, Tex., U.S.A., and Prostaglandin E_1 from the Unilever Research Laboratories, Vlaardingen, The Netherlands.

Brown, B. L., Albano, J. D. M., Ekins, R. P. & Sgherzi, A. M. (1971) *Biochem. J.* **121**, 561–562
Chen, L. C., Rohde, J. E. & Sharp, W. G. (1972) *J. Clin. Invest.* **61**, 731–740
Cooke, B. A., Van Beurden, W. M. O., Rommerts, F. F. G. & Van der Molen, H. J. (1972) *FEBS Lett.* **25**, 83–86
Cuatrecasas, P. (1973) *Biochemistry* **12**, 3547–3581
De Jonge, H. R. (1973) *Biochem. Pharmacol.* **22**, 2659–2677
De Jonge, H. R. & Hülsmann, W. C. (1973) *Eur. J. Biochem.* **32**, 356–364
Forstner, G., Shik, M. & Lukie, B. (1973) *Can. J. Physiol. Pharmacol.* **51**, 122–129
Grand, R. J., Rorti, F. M. & Jaksina, S. (1973) *J. Clin. Invest.* **52**, 2053–2059
Guerrant, R. L., Ganguly, U., Casper, A. G. T., Moore, E. J., Pierce, N. F. & Carpenter, C. C. J. (1973) *J. Clin. Invest.* **52**, 1707–1714
Harrison, D. D. & Webster, H. L. (1969) *Exp. Cell Res.* **56**, 245–253
Isemhoff, W. G. J., Van den Berg, J. W. O., de Pijper, A. M. & Hülsmann, W. C. (1970) *Biochim. Biophys. Acta* **215**, 229–241
Kimberg, D. V., Field, M., Johnson, J., Henderson, A. & Gershon, E. (1971) *J. Clin. Invest.* **50**, 1281–1230
Kimberg, D. V., Field, M., Gershon, E., Schooley, R. T. & Henderson, A. (1973) *J. Clin. Invest.* **52**, 1376–1383
Krishna, G., Weiss, B. & Brodie, B. B. (1968) *J. Pharmacol. Exp. Ther.* **163**, 379–385
Parkinson, D. K., Ebel, H., DiBona, D. R. & Sharp, G. W. G. (1972) *J. Clin. Invest.* **51**, 2292–2298

Pierce, N. F., Carpenter, C. C. J., Elliott, H. L. & Greenough, W. B. (1971) *Gastroenterology* **60**, 22–32
Roggin, G. M., Banwell, J. G., Yardley, J. H. & Hendrix, T. R. (1972) *Gastroenterology* **63**, 981–989
Schafer, D. E., Lust, W. D., Sircar, B. & Goldberg, N. D. (1970) *Proc. Nat. Acad. Sci. U.S.* **67**, 851–856
Serebro, H. A., Iber, F. L., Yardley, J. H. & Hendrix, T. R. (1969) *Gastroenterology* **56**, 506–511

Reprinted from

Biochimica et Biophysica Acta, 381 (1975) 128—143

BBA 27562

THE RESPONSE OF SMALL INTESTINAL VILLOUS AND CRYPT EPITHELIUM TO CHOLERATOXIN IN RAT AND GUINEA PIG

EVIDENCE AGAINST A SPECIFIC ROLE OF THE CRYPT CELLS IN CHOLERAGEN-INDUCED SECRETION

H.R. DE JONGE

Department of Biochemistry I, Erasmus University, P.O. Box 1738, Rotterdam (The Netherlands)

(Received July 9th, 1974)

Summary

1. Some kinetic properties of adenylate cyclase in separately isolated upper villous and crypt cells from rat and guinea pig small intestine were compared. An apparent K_m of 0.4 mM was found for both enzymes in the rat. The slight difference between the V-values measured in the fluoride-stimulated state (132 and 165 pmoles cyclic AMP formed per min per mg protein respectively) indicated an approximately equal enzyme content of both cell populations and argues strongly against a preferential localization in the brushborder region of the epithelial cell.

2. Prolonged contact of the small intestine with luminally administered choleragen led to an irreversible activation of adenylate cyclase in both villous and crypt compartments. The maximal stimulation of the upper villous enzyme (4—7 times) exceeded the maximal effect on the crypt enzyme by two-fold.

3. A lag phase of at least 30 min was found between the first luminal contact with the purified choleragen and a significant activation of the adenylate cyclase associated with isolated intestinal brushborders from the upper villous region.

4. By using a short exposure time (2 min) of the luminal surface to high amounts of choleragen, adenylate cyclase activity in the upper villus could be optimally stimulated in the absence of any alteration of crypt cell activity.

5. By comparing, in vivo, the effects of short and prolonged contact with choleratoxin on the unidirectional and net flux of ions and water in ileal and jejunal segments, it was concluded that both villous and crypt regions contribute to the secretion of water and electrolytes (sodium, chloride and bicarbonate ions) during cholera. The serosal to mucosal flux of sodium and chloride ions increased without a significant alteration of the opposite flux. These

results imply that absorptive and secretory processes occur within the same epithelial compartment.

6. The view that the crypt epithelium fulfills a specific role during the choleragen-induced secretion of ions and water is incompatible with the results of the present study.

Introduction

The enterotoxin of *Vibrio cholerae* has been found to elevate cyclic AMP levels by stimulating the adenylate cyclase activity in a variety of mammalian cell types such as small intestinal epithelium [1], platelets [2], liver [3], adrenal cell [4], adipocytes [5], fibroblasts [6], leucocytes [7] and thymocytes [8,9]. This action of choleratoxin appears to be dependent on a highly specific interaction of the large subunit of the toxin molecule with G_{M_1} ganglioside receptors of the cytoplasmic membrane [9—15]. During clinical cholera, the receptors exposed to choleragen are located exclusively at the surface of the microvillous membrane of the small intestinal epithelial cells. Active immunization against cholera is probably due to inhibition of attachment of choleratoxin to these binding sites by intestinal antibodies [16].

The epithelium mainly consists of two distinct cell types localized in different compartments called villus and crypt. The absorptive villous cells arise by division, differentiation and migration of the crypt population [17]. Immunohistochemical and autoradiographic studies showed that both villous and crypt epithelium are able to bind choleratoxin to their surface [18]. The binding of labeled choleragen is finished within a few minutes and cannot be reversed by subsequent washings with excess of unlabeled choleratoxin or toxin-free solutions [13,16]. The activation of adenylate cyclase needs the presence of both subunits of the toxin molecule [9,12,14,19]. It is preceeded by a characteristic lag phase of 30—60 min [13] which is not found with *Escherichia coli* enterotoxin [20], prostaglandins [21—23] or hormones [23]. Activation even persists in vitro in a solubilized form of the enzyme [24]. Also this irreversible character forms a clearcut difference with the reversible action of the fore-mentioned substances [20—23]. Stimulation of adenylate cyclase leads to elevated tissue levels of cyclic AMP, for phosphodiesterase activities are not influenced by choleragen [25].

In small intestine, the increase of adenylate cyclase activity and the change of water and ion fluxes occur simultaneously [26]. Moreover, various agents known to raise cyclic AMP levels (dibutyryl-cyclic AMP, theophylline, prostaglandins, *E. coli* enterotoxin) mimic the effect of choleragen on fluid secretion in vivo as well as in vitro [27]. Finally, stimulation of adenylate cyclase activity seems to be the only common factor in the response of different mammalian cell types to choleragen. Therefore, there are many reasons to assume a strong relationship between this stimulation and the diarrheal response of small intestinal epithelium. However, the epithelium is composed of a variety of differentiated cell types with possibly a different response to elevated levels of cyclic AMP. For instance, the mucus-secreting Goblet cells, constituting about fifteen percent of the total epithelial cell population in rat villus and crypt, react on contact with choleragen by an increased outpouring

of mucus [28] and, most probably, de novo synthesis of mucin-glycoproteins [29]. The absence of any alteration in mucosal to serosal glucose and sodium fluxes in vivo, in spite of reversal of net fluid movement from absorption to secretion, led to the assumption of an anatomical separation of absorption and secretion sites [27,30]. Indeed, since cycloheximide-inhibited cholera secretion is not accompanied by impaired glucose absorption through the villus [31], fluid secretion might be a specific function of the crypts of Lieberkühn. Moreover, damage to the villous surface by hypertonic sodium sulfate impaired glucose absorption without altering the secretory response to choleratoxin [32]. Also morphological observations suggest a specific secretory function of the crypt [33].

Other studies consider the possibility that the secretory function is limited to the Goblet cells of the epithelium [28].

According to a third model, the whole villous as well as the crypt cell population contribute to the secretion [27]. This implies that absorption and secretion may occur within the same epithelial region, either across the mucosal cell membrane or via interepithelial pathways. Also in this model, the characteristics of ion fluxes through villous and crypt region may differ in qualitative or quantitative respect.

Our study was especially designed to make a decision between the presented models by determining the relative contribution of villous and crypt epithelium to the choleragen-induced secretion in vivo.

Experimental procedure

Materials

Choleratoxin (lot 0172), purified by the method of Finkelstein and Lo Spalluto [34], a gift from Dr R.S. Northrup, SEATO Cholera Research Program, was prepared under contract for the National Institute of Allergy and Infectious Diseases by Dr R.A. Finkelstein, the University of Texas, Southwestern Medical School, Dallas, Texas, U.S.A. [α-^{32}P]ATP (adenosine 5′-triphosphate, sodium salt (740—1530 Ci/mole)), $^{3}H_2O$ (tritiated water (5 Ci/ml)), $^{36}Cl^-$(sodium chloride (73 mCi/g Cl^-)) and cyclic [^{3}H]AMP (cyclic 3′,5′-[G-^{3}H]adenosine monophosphate)—ammonium salt (24.1 Ci/mmole) were obtained from the Radiochemical Centre, Amersham, Great Britain. $^{24}Na^+$ (sodium chloride) was a gift from IRI, Delft, The Netherlands. Cyclic AMP (ammonium salt), ATP (sodium salt), 2-phosphoenolpyruvate (tricyclohexylammonium salt), pyruvate kinase (EC 2.7.1.40) and myokinase (EC 2.7.4.3) from rabbit muscle were products from Boehringer, Mannheim, Western Germany. Dowex (50Wx8, 200-400 mesh) was purchased from Fluka A.G., Buchs, Switzerland; theophylline (1,3-dimethylxanthine) was from Sigma, St. Louis, Mo., U.S.A. Fatty acid poor serum albumin was obtained from Pentex, Kaukakee, Ill., U.S.A. Instagel was obtained from Packard S.A., Brussels, Belgium. All other reagents were Analytical Grade.

Animals

Male Wistar rats and guinea pigs, weighing 250—300 g were maintained on a regular laboratory chow and water ad libitum.

Transport of water and electrolytes

Rats or guinea pigs were anaesthetized with ether during the operation procedures. Their abdomen was opened by midline incision and a segment of small intestine (60—70 cm) comprising the ileum and the distal part of the jejunum was tied off. The intestinal contents were removed through a small distal incision by gently rinsing the lumen with prewarmed isotonic saline introduced through a needle attached to a syringe, stuck into the proximal end of the segment. Any extension of the intestinal wall was avoided. Residual saline was largely removed by gently stripping the segment between the fingertips. The volume of the remaining fluid was determined by means of an air insufflation procedure in six rats and appeared remarkably constant (0.16 ± 0.02 ml per segment). The segment was then closed by a distal ligature. Net transport of water was measured by introducing through the needle a fixed amount of isotonic saline, closing the abdomen with woundclips, removing the intestine from the animal exactly 1 h later and measuring the volume of the residual fluid by gently squeezing it out into a calibrated tube. The intestinal segment was further processed for measurements of adenylate cyclase activity as described below. In our hands, these discontinuous measurements of net absorption or secretion rates in these small animals were found more satisfactory and reproducible than the commonly applied continuous perfusion technique using an unabsorbable marker as indicator for volume changes [35]. The latter method needs permanent narcotized animals, a relatively large perfusion volume and causes some dilatation of the gut and serious enzyme or cell loss from the tips of the intestinal villi as judged from a decrease of alkaline phosphatase and sucrose activity in upper villous cells from control rats, especially after prolonged perfusion times (2—5 h). Moreover, the markers tested by us ([^{3}H]inuline and phenol red) tend to adsorb to the mucus and require equilibration periods of 0.5—1 h (unpublished observations).

Concentrations of ions were determined in the secretion fluid, accumulated during 1 h in initially emptied ileal—jejunal segments. Fluid samples were immediately covered with paraffine until analysis of their ion composition. Sodium and potassium were determined on a flame photometer with internal lithium standard (Instrumentation Lab. Inc., Lexington, Mass., U.S.A.), chloride was determined by volumetric titration with mercurinitrate using diphenylcarbazone as indicator [36] and bicarbonate was determined by measuring the original sample-pH, addition of a standardized portion of HCl in excess of the amount of $HClO_3$ and backtitration with alkali until the original pH had been reached. This method, recently published by den Boer et al. [37], is insensitive to interference by high protein concentrations in the sample.

Unidirectional ion and water flux in the rat

After emptying and closing the ileo—jejunal segment as described in the foregoing, the segment was divided into two parts by placing a ligature approx. 50 cm from its distal end. The proximal jejunal part (nearly 20 cm) was used to obtain internal control values for flux measurement in rats, treated with choleragen in the ileo—jejunal segment. Flux studies were started by rapidly introducing 3.0 ml of tracer-containing isotonic saline into the distal part and 1.5 ml into the proximal part of the segment. Tracer activities were 4 μCi/ml ^{24}Na, 1

μCi/ml ^3H_2O and 2 μCi/ml ^{36}Cl respectively. Temperature was maintained at 37 ± 2°C by means of a heating lamp and by continuously bathing the intestine in saline brought at 39°C. At 5 min intervals (in the case of ^3H_2O: 2 min intervals), 20 μl samples were removed from the luminal fluid by means of a Hamilton syringe and taken up in Instagel for subsequent counting. After 30 min, the intraluminal fluid volume and sodium or chloride content were measured as described previously. The small volume of the residual fluid was determined by air insufflation and used for calculation of the true initial fluid volume. From this value and the calculated net transport rate of fluid, the luminal fluid volume could be calculated for each time point desired. ^{36}Cl and ^3H_2O activities were determined in a liquid scintillation spectrometer (Packard, Tri-Carb 3380), the γ activity of ^{24}Na in an Auto-Gamma Counter (Packard, 5120). For calculation of the mucosal to serosal flux rate (J_{ms}) the equations derived by Curran and Solomon, originally applied for flux studies in rat ileum [35], were used. The final formula is identical to the formulation of Berger and Steele [38] if the initial and final ion concentrations of Na and Cl are replaced by a mean value over the time period studied. This was allowed in our rat studies where the change of Cl^- and Na^+ concentrations during the first 15 min appeared extremely small. This also held for cholera segments which secreted only very low amounts of bicarbonate (< 10 mM).

Application of the formula for various time intervals between 0 and 30 min showed that the calculated flux rates for sodium and chloride ions were constant between 5 and 15 min but slowly decreased afterwards, apparently caused by a backflow of label from blood to lumen. Therefore, the mean of the values obtained over three different time periods (5—10 min, 10—15 min and 5—15 min) was considered as the best approximation of the J_{ms} for these ions. The rapid outflux of ^3H_2O from the lumen already slowed down after the first 5 min. In this case, the mean mucosal to serosal flux rate over the 0—2, 2—4 and 0—4 time intervals was calculated.

Administration of choleragen

In one type of experiment, short contact of choleragen with the epithelial villi was ensured by gently filling the ileo—jejunal segment with 3 ml of isotonic saline supplied with 20 μg/ml of purified choleragen and removal of the unabsorbed toxin 2 min later by thorough flushing of the lumen with 60 ml saline.

In the alternative experiments, both villous and crypt regions were exposed to choleratoxin by incubating the segment for 1 h with 3 ml of saline containing 5 μg/ml choleragen. The control animals went through the same procedures in the absence of choleratoxin.

Isolation of intestinal cells and brushborders

Upper villous, lower villous and crypt epithelial cells were isolated separately by application of the high-frequency vibration technique of Harrison and Webster [39], as described by Iemhoff et al. [40]. Short vibration (0—15 min, frequency 50 Hz, amplitude 2 mm) mainly delivered cells from the apical areas of the villi. In the next phase (15—30 min) the lower villous cells were shed off. Finally, crypt cells were isolated after dilatation of the gut. This method guarantees rather homogeneous cell preparations, relatively free of lamina propria fragments, plasma cells and muscle tissue.

The origin of the cells was routineously verified by measuring alkaline phosphatase (EC 3.1.3.1) as a marker enzyme for the intestinal brushborder and in some experiments also by measuring [^{3}H]thymidine incorporation as a typical crypt cell function (results not shown). If the specific activity of alkaline phosphatase in the upper villous fraction was less than six times the activity in the crypt fraction, the separation was considered to be insufficient and the preparation discarded.

Brushborders principally derived from the upper villous cells were isolated as described by Harrison and Webster [41] by vibration (50 Hz, amplitude 1.5 mm) for 10 min in 2.5 mM EDTA containing 1 mM dithiothreitol (pH 7.4), followed by removal of nuclear aggregates and a washing procedure.

Assay of adenylate cyclase activity

Freshly isolated epithelial cells or brushborders were taken up in 15 ml of a homogenization buffer (30 mM Tris—HCl, 5 mM $MgCl_2$, pH 7.3) and gently homogenized by Polytron treatment as described earlier [42]. Samples of the homogenate (20 µl, containing 10—40 µg of protein) were directly used for the assay of adenylate cyclase activity in principle according to Krishna et al. [43]. The incubation mixture (final volume: 50 µl) usually contained 30 mM Tris—HCl (pH 7.5), 0.1 mM cyclic AMP, 0.1 mM ATP, 5 mM $MgCl_2$, 5 mM phosphoenolpyruvate, 40 µg/ml pyruvate kinase, 20 µg/ml myokinase, 2 mM theophylline, 0.02% bovine serum albumin and 1 µCi [α-^{32}P]ATP. Occasionally, 10 mM NaF was included in the mixture. For determinations of Lineweaver—Burk plots, the concentration of ATP was varied between 0.01 and 1.0 mM. Samples of the homogenate were preincubated during 5 min prior to the addition of radioactive substrate. During the incubation period (10 min at 30°C) cyclic AMP was produced linearly with time. Reaction blanks were measured in the absence of homogenate. Termination of the reaction and purification of the product using cyclic [^{3}H]AMP as internal standard were carried out exactly according to Krishna et al. [43]. The amount of cyclic AMP formed was calculated from the final specific activity of [α-^{32}P]ATP and the amount of cyclic AMP recovered (40—60 percent of the amount produced) minus the reaction blank. The variation between duplicate determinations was less than five percent. At a concentration of 0.1 mM cyclic AMP, 10—15 percent of this product was hydrolyzed by phosphodiesterase during the adenylate cyclase assay in the absence of theophylline. Addition of 2 mM theophylline inhibited the phosphodiesterase activity for about 60 percent (unpublished results). Accordingly, adenylate cyclase activities increased 5—10 percent in the presence of theophylline.

Assay of protein concentration.

Protein was determined according to Lowry et al. [44] using bovine serum albumin as a standard.

Results

Villous and crypt activities of adenylate cyclase in ileo-jejunal segments

Fig. 1 compares the kinetic properties of fluoride-stimulated adenylate

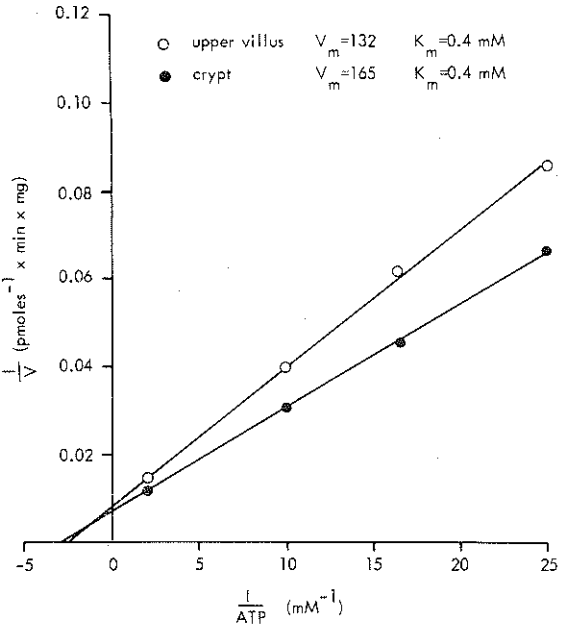

Fig. 1. Lineweaver—Burk plots for fluoride-stimulated adenylate cyclase in isolated upper villous and crypt cells from rat small intestinal epithelium. The V is expressed as pmoles cyclic AMP formed per min per mg protein.

cyclase measured in upper villous and crypt cell homogenates from rat ileo—jejunal segments. The apparent K_m of both enzymes was found to be 0.4 mM. The slight difference found between the K_m values defined on a protein base disappears if expressed on a cellular base, taking into account the higher amount of proteins in the villous cell as compared to the crypt cell [45]. Also the basal activities of adenylate cyclase in control animals, measured at a sub-optimal substrate concentration (0.1 mM), remained remarkably constant from crypt to upper villus (Table I). These activities appeared insensitive to repeated sham-operation (results not shown).

Exposure of the ileo—jejunal lumen for 1 h to 15 μg of purified choleragen evoked a gradual rise of basal enzyme activity in the upper villous cells during the next 4 h (Fig. 2A and Table I). A much smaller rise, following approximately the same time course, was found in basal villous and crypt cells. Also the fluoride-stimulated activity appeared to be slightly but significantly increased after prolonged exposure to choleragen, in agreement with an earlier report of Sharp et al. [46].

A significant increase of adenylate cyclase activity in the upper villous cells, in first contact with the choleragen, was detected as early as 45 min after toxin-administration. This time lag could be shortened to 30 min by measuring enzyme activities in isolated brushborders from villous top cells. The specific activity of this enzyme in the non-activated state (30.1 ± 1.3 pmoles cyclic AMP formed per 10 min per mg protein; n = 5) approached the value measured in the whole cells. On the other hand, the brushborder marker alkaline phosphatase appeared six times enriched in these brushborder preparations (result not shown).

TABLE I

ADENYLATE CYCLASE ACTIVITIES IN UPPER VILLOUS AND CRYPT CELLS FROM RATS AND GUINEA PIGS FOLLOWING 5 h OF TREATMENT

Enzyme activity is reported as pmoles cyclic AMP formed per 10 min per mg protein ± S.E. n represents the number of animals.

	Adenylate cyclase activity							
	Upper villus				Crypt			
	Basal		Fluoride		Basal		Fluoride	
	Rat	Guinea pig	Rat	Guinea pig	Rat	Guinea pig	Rat	Guinea pig
Saline	30.1	35.2	258	300	39.6	62.3	330	361
$n = 5$	± 3.4	± 4.3	± 11	± 18	± 2.9	± 7.0	± 10	± 22
Choleragen								
(2 min, 20 μg/ml)	123*	240*	289**	329**	41.7	69.2	336	352
$n = 5$	± 14	± 12	± 15	± 24	± 3.4	± 11.4	± 12	± 26
Choleragen								
(1 h, 5 μg/ml)	136*	268*	287**	406*	103*	191*	387*	409**
$n = 4$	± 9	± 16	± 13	± 17	± 8	± 13	± 16	± 21

* $P < 0.05$ (one-tailed Student's t-test).
** $0.05 < P < 0.10$.

Quite a different picture was obtained by raising the amount of toxin up to 60 μg but drastically reducing simultaneously the period of contact from 60 to 2 min (Fig. 2B, Table I). Whereas the rise of adenylate cyclase localized in the upper villous cells was of the same magnitude as shown in Fig. 2A, the response of the lower villus appeared strongly diminished and the crypt cell population did not respond to the choleragen at all. Apparently, these conditions permitted saturation of binding sites at the villous tip but prevented the penetration of toxin molecules into the depth of the intestinal crypts. This selectivity of binding often diminished after mechanical dilatation of the gut.

Unidirectional and net flux rates of water and electrolytes

Fig. 2A (upper graph) shows that the gradual fall of net absorption rate of isotonic saline closely followed the rise of adenylate cyclase activity in the villous cells of cholera rats. Within 2.5 h net absorption changed to net secretion that became constant 3 h afterwards. Net absorption in control rats was not clearly influenced by the operation procedures. A very short contact with choleratoxin (Fig. 2B), restricting its action to the villous region, also led to a gradual decrease of fluid absorption rates during the first 3 h. However, in this case the reversal of net absorption into net secretion rarely occurred. These results prove that the net fluid movement from mucosa to serosa (J_{net}) through the intestinal villi in vivo has been strongly depressed during cholera but do not decide whether the decrease is due to a diminished mucosal to serosal influx (J_{ms}), an increased serosal to mucosal outflux (J_{sm}) or to both. This question was further approached by measuring unidirectional flux rates of sodium and chloride ions and water in intestinal segments of control rats and

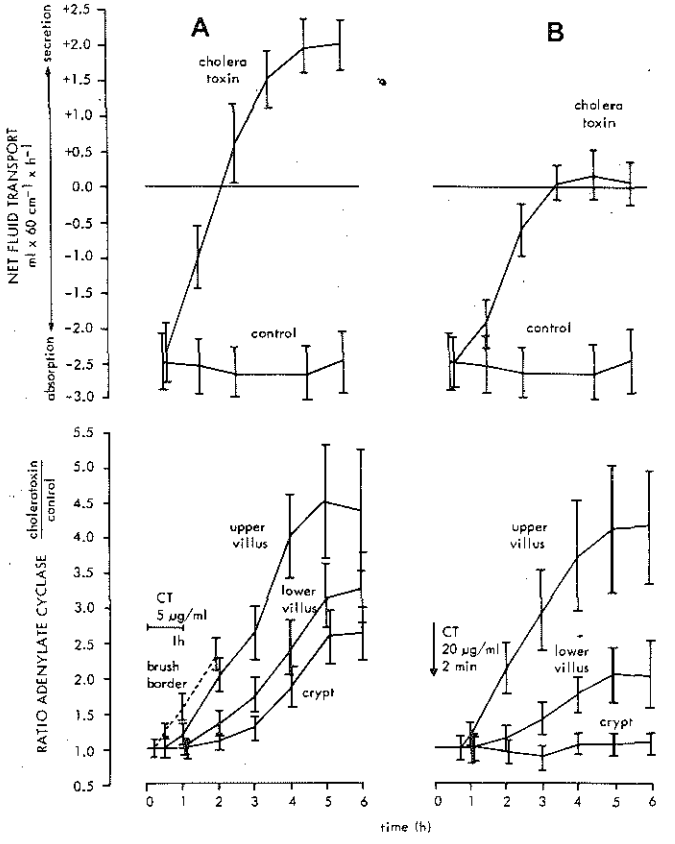

Fig. 2. Upper graph: time course of the effect of choleratoxin (CT) on net fluid transport by rat ileo—jejunal segments. Vertical bars represent S.E. Fluid transport was measured at hourly intervals in five control and six choleragen-treated animals. A = prolonged contact with choleratoxin (1 h, 5 µg/ml), B = short contact with choleratoxin (2 min, 20 µg/ml). Lower graph: corresponding increase of basal adenylate cyclase activity in upper and lower villous cells, crypt cells and brushborders. Each bar represents the ratio ± SE of the mean specific enzyme activity in cells isolated from five choleragen-treated and four control rats at the time points indicated on the abscis.

rats, treated for 2 or 60 min with choleragen instilled exclusively into the ileo—jejunal part of the segments.

As indicated in Fig. 3, the flux rates of sodium and chloride ions in sham-operated rats increased from the distal to the proximal end of the small intestine. To avoid this source of variation, care was taken to use completely corresponding regions of the gut for the flux studies in control and choleragen-treated animals. Under this condition pretreatment with choleratoxin had no significant influence on the unidirectional mucosal to serosal flux of sodium and chloride ions (Fig. 3). The decrease of net absorption was apparently due to an increase of J_{sm} solely in the villus (2 min choleragen) or in both villous and crypt compartments (1 h choleragen). In contrast, the flux rates in the adjacent jejunal segments of cholera rats appeared to be unaltered as compared to sham-operated animals.

The bidirectional flux of ^3H_2O in ileo—jejunal segments of rat intestine

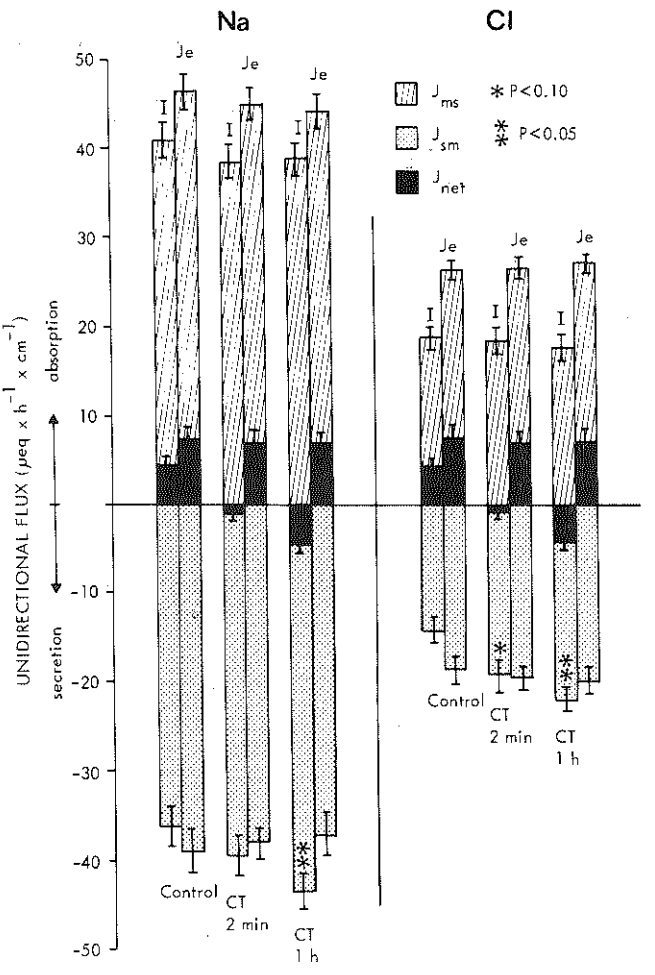

Fig. 3. Unidirectional transmucosal flux of sodium and chloride in rat ileum and jejunum. Flux rates of sodium and chloride ions, measured in separate experiments after 3 h of treatment, are expressed as μequivalents transported per h per cm of small intestine. A + sign denotes net absorption. Mean values are based on four experiments. Vertical bars represent S.E. I = ileo—jejunal segment, Je = jejunal segment. Choleragen was added exclusively to the ileo—jejunal segments. Jejunal uptake was measured as internal reference. *P* values according to Student's *t*-test.

(0.91 ml/h · cm; n = 4) nearly equalled the unidirectional flux rates (J_{ms} = 0.94 ml/h · cm). For this reason, it could not be established with certainty whether $J_{sm}^{H_2O}$, $J_{ms}^{H_2O}$ or both had been changed in experimental cholera in the rat. However, it is generally accepted that the water flux follows movement of electrolytes approximately isoosmotically also during cholera [47].

In summary, an increased secretion of fluid exclusively derived from the villus may entirely account for the diminished uptake of water shown in Fig. 2B. By elevating the cyclic AMP levels in basal villous and crypt cells, these cell populations further contribute to the secretion component J_{sm} which finally exceeds the J_{ms}, resulting in net secretion of water and electrolytes (Fig. 2A).

TABLE II

VOLUME AND CONTENT OF ELECTROLYTES OF FLUID SECRETED BY GUINEA PIG SMALL INTESTINE

Secretion was measured in ileo—jejunal segments between 4 and 5 h after luminal administration of choleragen. *n* represents the number of animals. Mean values are given followed by their S.E.

	Net secretion	Concentration of electrolytes (mM)			
	ml/h·60 cm	Na	K	Cl	HCO_3
Sham-operated	0.4	153	6.8	76	82
$n = 4$	±0.2	± 3	±0.5	± 3	± 6
Choleragen (2 min, 20 μg/ml)	4.8	156	6.4	79	86
$n = 5$	±1.0	± 4	±0.6	± 5	± 8
Choleragen (1 h, 5 μg/ml)	6.7	149	7.0	77	76
$n = 4$	±1.2	± 4	±0.5	± 5	± 7

Ion composition of fluid secreted during cholera

The previous section showed that during prolonged exposure of rat intestine to choleragen the sodium and chloride ions in the accumulated fluid originate from both villous and crypt regions. However, the origin of the bicarbonate, contributing only a very small part ($\leqslant 7\%$) of the total anion concentration in the rat ileo—jejunal segments in our experiments, could not be easily established because no significant accumulation of fluid into the lumen occurred after short contact with choleragen (Fig. 2B). The guinea pig seemed more suitable as a model to solve this question. Even under our mild conditions (minimal dilatation of the gut, short anaesthesia, no mechanical damage) the small intestine of this animal spontaneously secreted appreciable quantities of fluid containing high amounts of bicarbonate (Table II). Stimulation of adenylate cyclase activity exclusively in the villous cells (Table I) was accompanied by a large increase of net fluid secretion which could be further stimulated by additionally affecting the adenylate cyclase in the crypt area by means of choleragen. The ion composition of the luminal fluid appeared unaltered after short or prolonged contact with the toxin (Table II).

Discussion

The kinetic data for adenylate cyclase, measured in isolated villous and crypt cells from control intestine, indicated a similar enzyme content and properties in both cell types. This result argues strongly against a preferential localization of the enzyme in the brushborder region of the mature villous cell. This structure is far less developed in the undifferentiated crypt cell. Using a different approach, other investigators also agree that adenylate cyclase must be localized mainly in the basal and lateral plasma membranes of the epithelial cell [48,49]. Although we detected some adenylate cyclase activity in purified brushborders of upper villous cells, the enzyme was not enriched in this compartment as was shown for alkaline phosphatase. It is conceivable that the adenylate cyclase is not integrated into the brushborder mosaic but resides in the adhering tags of lateral plasma membranes contaminating the preparations.

The brushborder-associated enzyme from the villous top is apparently affected first during cholera (Fig. 2A). Even in this preparation, a significant stimulation of its basal activity requires a lag time of at least 30 min after exposure to high amounts of choleragen. This lag phase is not unique for intestinal epithelium and seems therefore not related to a possible different localization of toxin receptor sites and the target enzyme in this tissue. Binding of the choleragen to its receptors proceeds extremely rapidly (compare Fig. 2B) and cannot be responsible for this time delay. Neither can it be explained by a need for protein, RNA or prostaglandin synthesis because enzyme activation also occurs in the presence of specific inhibitors of these processes [8,13,50,51]. Up till now it is not clear whether the lag time is needed for a chemical or physical modification of the toxin molecule into an active state (e.g. splitting into its two major subunits [9,12—15], compare diphtheriatoxin [52]), a slow change of membrane structure causing the irreversible activation of adenylate cyclase [12,13], a slow diffusion of a small fraction of the toxin molecules or molecular fragments into the cell interior, or a combination of some of these factors. The action of choleragen seems to depend critically on an intact state of the cellular structure, for the activation of adenylate cyclase has never been reproducibly demonstrated in isolated cell membranes or broken cell preparations [13,23].

Prolonged exposure of the intestinal lumen to choleragen caused a gradual rise of basal adenylate cyclase activity in both villous and crypt compartments. This result agrees with the uniform distribution of choleragen from the tips of the villi to the crypt area detected by immunofluorescense in mice intestine [18] and additionally proves that both villous and crypt cells may functionally respond to the toxin—receptor interaction. The smaller response of the basal villous and crypt adenylate cyclase as compared to the upper villous enzyme may be explained by incomplete saturation of binding sites, a decreased number of microvillous receptor sites (compare [13]), or a lower sensitivity of the enzyme to the choleragen—receptor interaction. The small but significant increase of the fluoride-stimulated activity of adenylate cyclase during cholera, shown in Table I, is probably due to alteration of its kinetic properties rather than to de novo synthesis [13].

The lack of knowledge concerning the origin of fluid and electrolyte secretion during cholera [47,53] mainly arises from the extreme difficulty to measure the secretory response of villous and crypt cells separately under conditions that all epithelial cell populations are affected by the choleragen. At best, only indirect evidence may be obtained in vitro based on measurements of electrical profiles by impalement of cells by microelectrodes [54]. Ion fluxes may be easily disturbed by these manipulations and the in vitro results usually do not apply to the in vivo situation [27]. To avoid these problems, we looked for a method to limit the action of choleragen to the villous epithelium without disturbing the normal crypt functions. We found these conditions by restricting the contact between intestinal lumen and choleragen to a very brief period, concomitantly avoiding any mechanical extension of the gut. Unabsorbed toxin was thoroughly washed away after 2 min. Obviously, the time of contact is now too short for the toxin molecules to diffuse into the depth of the intestinal crypts. Preferential localization of luminal administered proteins along the

tips of the villi, especially during the first period of contact, has also been demonstrated in horseradish peroxidase tracer studies [55] and immunohistochemical studies with impure choleragen [56].

The irreversible increase of basal adenylate cyclase activity in the cell was taken as a criterion for a functional response to choleragen. This activation is still present in vitro and can be easily detected after the separate isolation and homogenization of the epithelial cells. As shown in Fig. 2B, our conditions ensured the complete absence of any stimulatory effect on crypt cell adenylate cyclase in short-contact experiments. Hypothetically, it may be argued that a hormone-like substance, possibly released by the cholera-affected cells, should evoke water and ion secretion from the choleragen-free crypts. Indeed, studies in rabbits indicated a fluid secretion in adjacent intestinal segments, not in direct luminal contact with the choleragen [57,58]. If such a compound should act via an increase of adenylate cyclase, this might be missed in in vitro enzyme measurements due to its supposed reversible action. Our unidirectional flux studies however, simultaneously performed in choleragen-affected and toxin-free segments of rat intestine, made this hypothesis highly improbable. We never detected any change of flux rate in the toxin-free segments when compared with control animals. Therefore, alterations of net fluid movements and ion flux rates after 2 min exposure to choleragen will represent exclusively the response of the villus.

From the gradual decrease of net fluid absorption under these conditions the conclusion has been drawn that choleragen primarily affects net movement of water and electrolytes through the villous epithelium. After prolonged exposure to the toxin, also the basal villous and crypt region contribute to the flux changes.

Comparison of unidirectional flux rates of ions under both conditions enabled us to discriminate between effects on mucosal to serosal flux (J_{ms}), the opposite flux (J_{sm}) or on both flux rates simultaneously. These measurements are complicated by the small size of the rats, soon leading to a significant backflow of labeled ions into the lumen, and the large bidirectional component particularly in the case of $^{3}H_2O$ and $^{24}Na^{+}$. The latter implies that the percentual change of unidirectional flux rates in the rat during cholera will be relatively small. Moreover, the flux rates of water and ions are functions of the pore size in the epithelium and vary along the length of the gut; they reach a maximum in the proximal part of the intestine. For this reason, reproducible measurements need the use of perfectly corresponding intestinal segments. All unidirectional flux studies were done 3 h after toxin administration. Up to then, net fluid loss into the intestinal lumen was negligible and plasma ion concentrations were unaffected. Intravenous infusion of fluid and electrolytes to prevent dehydration seemed therefore unnecessary.

Our results indicate that choleragen-treatment in vivo selectively increased the J_{ms} of sodium and chloride ions through villous as well as crypt epithelium without significantly impairing the opposite flux. Unequivocal results have been reported using rabbits and dogs or in studies of human cholera. According to most in vitro studies with stripped mucosa, choleragen primarily diminishes a glucose-independent neutral influx of sodium and chloride ions across the luminal border of the epithelial cells [27,47,59]. These results suggest that

choleratoxin (via cyclic AMP) unmasks a pre-existing secretion of ions by inhibiting their absorption. In contrast, a recent in vitro study [60] and most of the experiments in vivo [61—63] indicate a stimulatory action of choleragen on a neutral secretion process of NaCl or $NaHCO_3$, in agreement with our conclusions for the rat. Of course, our results give no information about the relative importance of the interepithelial or transepithelial route followed by the secreted ions or about the steps between cyclic AMP increase and augmented serosal to mucosal flux during cholera. Recent work in vitro suggests that the J_{sm} for sodium ions mainly transverses the extracellular route [64]. Possibly the contribution of this pathway is changed by choleragen, e.g. via alteration of serosal membrane potential or membrane permeability or an increased shunt conductance [47].

Bicarbonate is the third important ion in cholera fluid. As pointed out by Hubel [65], its formation is most probably due to an increased movement of hydroxyl ions from the epithelium into the lumen followed by equilibration with the CO_2 principally derived from cellular metabolism in the epithelium. The guinea pig offers a very useful model to study bicarbonate production. A'i sham-operated animals showed a spontaneous secretion of a bicarbonate-rich fluid. This seem to be a general finding in herbivorous animals [66]. Our results demonstrated that choleragen merely stimulates a pre-existing secretory process in the guinea pig without changing the ion composition of the fluid. A further elucidation of the mechanism of choleratoxin-induced secretion should therefore be of much value for the understanding of ion movements in the untreated state. Our experiments also excluded the possibility that the bicarbonate solely originates from the crypt region. The production of this ion normally proceeded in the absence of a choleragen-effect on the crypt.

All results together strongly suggest a functional homogeneity of villous and crypt regions in rat and guinea pig in their secretory response to choleragen. They are conflicting with the concept that fluid secretion is a specific function of the crypt [31,32]. Also the suggestion that choleragen stimulates secretion of anions preferentially in the crypt cells and simultaneously inhibits active absorption of sodium and chloride by villous cells [59] must be critisized in view of our results.

The role of the Goblet cells during fluid secretion remains obscure. The greatly increased outpouring of mucus during cholera, also observed in our experiments, might be accompanied by an increased flux of water and electrolytes into the lumen. Alternatively, the situation should be comparable to that in the pancreas, where the secretion of water and electrolytes is provided by the ductular cells which are morphologically and functionally distinct from the protein-secreting acinar cells [67]. The question should be answered if fluid and mucin production could be studied under conditions in which cyclic AMP levels were stimulated selectively in either Goblet cells or absorptive cells. Adrenalin could be a possible candidate for use in such a study because the hormone is known to stimulate the synthesis of mucin glycoproteins in vitro [29] and to elevate slightly the levels of cyclic AMP in isolated intestinal epithelial cells [23]. Another approach was followed by us by studying the effect of choleragen on the rat colon. Preliminary results indicate a threefold stimulation of adenylate cyclase activity in the epithelial cells of the colon by

10 μg choleragen, concomitantly with a significantly decreased rate of fluid absorption. If the observations of Yardley et al. [28] are valid that the Goblet cells in the colon do not react to choleratoxin with an increased secretion of mucin, our results suggest that, at least in the colon, Goblet cells play no essential role during fluid secretion in experimental cholera.

Acknowledgements

The author is greatly indebted to Professor Dr W.C. Hülsmann for critical reading of the manuscript and to Mr L.M. Scheek for expert technical assistance. Miss A.C. Hanson is thanked for typewriting the manuscript.

References

1 Chen, L.C., Rohde, J.E. and Sharp, G.W. (1972) J. Clin. Invest. 51, 731—740
2 Zieve, P.D., Pierce, N.F. and Greenough, W.B. (1970) Clin. Res. 18, 690
3 Gorman, R.E. and Bitensky, M.W. (1972) Nature 272, 439
4 Donta, S.T. and King, M. (1973) Nat. New Biol. 243, 246—247
5 Greenough, III, W.B., Pierce, N.F. and Vaughn, M. (1970) J. Infect. Dis. 121, S111—114
6 Hollenberg, M.D. and Cuatrecasas, P. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2964—2968
7 Lichtenstein, L.M., Henney, C.S., Bourne, H.R. and Greenough, III, W.B. (1973) J. Clin. Invest. 52, 691—697
8 Boyle, J.M. and Gardner, J.D. (1974) J. Clin. Invest. 53, 1149—1158
9 Holmgren, J., Lindholm, L. and Lönnroth, I. (1974) J. Exp. Med. 139, 801—819
10 Van Heyningen, W.E., Carpenter, C.C.J., Pierce, N.F. and Greenough, III, W.B. (1971) J. Infect. Dis. 124, 415—418
11 King, C.A. and Van Heyningen, W.E.J. (1973) J. Infect. Dis. 127, 639—647
12 Cuatrecasas, P., Parikh, I. and Hollenberg, M.D. (1973) Biochemistry 12, 4253—4263
13 Cuatrecasas, P. (1973) Biochemistry 12, 3547—3581
14 Finkelstein, R.A., Boesman, M., Neoh, S.H., LaRue, M.K. and Delaney, R. (1974) J. Immunol. 113, 145—150
15 Van Heyningen, S. (1974) Science 183, 656—657
16 Walker, W.A., Field, M. and Isselbacher, K.J. (1974) Proc. Natl. Acad. Sci. U.S. 71, 320—324
17 Trier, J.S. (1968) Handbook Physiol. Am. Physiol. Soc., Washington, Section 6, Vol. 3, pp. 1125—1146
18 Peterson, J.W., Lo Spalluto, J.J. and Finkelstein, R.A. (1972) J. Infect. Dis. 126, 617—628
19 Lönnroth, I. and Holmgren, J. (1973) J. Gen. Microbiol. 76, 417—427
20 Guerrant, R.L., Ganguly, U., Casper, A.G.T., Moore, E.J. Pierce, N.F. and Carpenter, C.C.J. (1973) J. Clin. Invest. 52, 1707—1714
21 Matachansky, C. and Bernier, J. (1973) Gastroenterology 64, 1111—1118
22 Pierce, N.F., Carpenter, Jr, C.C., Elliot, H.L., Greenough, III, W.B. (1971) Gastroenterology 60, 22—32
23 de Jonge, H.R. and Hülsmann, W.C. (1974) Biochem. Soc. Transact. 2, 416—419
24 Beckman, B., Flores, J., Witkum, P.A. and Sharp, G.W.G. (1974) J. Clin. Invest. 53, 1202—1205
25 Kimberg, D.V., Field, M., Johnson, J., Henderson, A. and Gershon, E. (1971) J. Clin. Invest. 50, 1218—1230
26 Guerrant, R.L., Chen, L.C. and Sharp, G.W.G. (1972) J. Infect. Dis. 125, 377—381
27 Banwell, J.G. and Sherr, H. (1973) Gastroenterology 65, 467—497
28 Yardley, J.H., Bayless, T.M., Luebbers, E.H., Halsted, C.H. and Hendrix, T.R. (1972) Hopkins Med. J. 131, 1—10
29 Forstner, G., Shih, M. and Lukie, B. (1973) Can. J. Physiol. Pharmacol. 51, 122—129
30 Hendrix, T.R. and Bayless, T.M. (1970) Ann. Rev. Physiol. 32, 139—164
31 Serebro, H.A., Iber, F.L., Yardley, J.H. and Hendrix, T.R. (1969) Gastroenterology 56, 506—511
32 Roggin, G.M., Banwell, J.G., Yardley, J.H. and Hendrix, T.R. (1972) Gastroenterology 63, 981—989
33 Trier, J.S. (1964) Gastroenterology 47, 480—495
34 Finkelstein, R.A. and Lo Spalluto, J.J. (1970) J. Infect. Dis. 121, S63—72
35 Curran, P.F. and Solomon, A.K. (1957) J. Gen. Physiol. 41, 143—168
36 Schales, O. and Schales, S.S. (1941) J. Biol. Chem. 140, 879—885
37 den Boer, N.C., Mantel, P.A. and Leijnse, B. (1974) Clin. Chim. Acta 55, 87—96

38 Berger, E.Y. and Steele, J.M. (1957) J. Gen. Physiol. 41, 1135—1152
39 Harrison, D.D. and Webster, H.L. (1969) Exp. Cell Res. 55, 257—260
40 Iemhoff, W.G., van den Berg, J.W.O., de Pijper, A.M. and Hűlsmann, W.C. (1970) Biochim. Biophys. Acta 215, 229—241
41 Harrison, D.D. and Webster, H.L. (1964) Biochim. Biophys. Acta 93, 662—664
42 de Jonge, H.R. (1973) Biochem. Pharmac. 22, 2659—2677
43 Krishna, G., Weiss, B. and Brodie, B.B. (1968) J. Pharm. Exp. Ther. 163, 379—385
44 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
45 de Jonge, H.R. and Hülsmann, W.C. (1973) Eur. J. Biochem. 32, 356—364
46 Sharp, G.W.G., Hynie, S., Ebel, H., Parkinson, D.K. and Withum. P. (1973) Biochim. Biophys. Acta 309, 339—348
47 Schultz, S.G. and Frizzel, R.A. (1972) Gastroenterology 63, 161—170
48 Parkinson, D.K., Ebel, H., DiBona, D.R. and Sharp, G.W.G. (1972) J. Clin. Invest. 51, 2292—2298
49 Grand, R.J., Torti, F.M. and Jaksina, S. (1973) J. Clin. Invest. 52, 2053—2059
50 Kimberg, D.V., Field, M., Gershon, E., Schooley, R.T. and Henderson, A. (1973) J. Clin. Invest. 52, 1376—1383
51 Kimberg, D.V., Field, M., Gershon, E. and Henderson, A. (1974) J. Clin. Invest. 53, 941—949
52 Gill, D.M., Pappenheimer, Jr, A.M. and Uchida, T. (1973) Fed. Proc. 32, 1508—1515
53 Sladen, G.E. (1973) Gut 14, 671—680
54 Hirschhorn, N. and Frazier, H.S. (1973) Hopkins Med. J. 132, 271—280
55 Yardley, J.H. and Brown, G.D. (1973) Lab. Invest. 28, 482—493
56 Kao, V.C.Y., Sprinz, H. and Burrows, W. (1972) Lab. Invest. 26, 148—153
57 Serebro, H.A., McGonagle, T., Ibers, F.L., Royal, R. and Hendrix, T.R. (1968) Hopkins Med. J. 120, 229—232
58 Williams, E.M.V. and Dohadwalla, A.N. (1969) J. Infect. Dis. 120, 658—663
59 Frizzel, R.A., Nellans, H.N., Rose, R.C., Markscheid-Kaspi, L. and Schultz, S.G. (1973) Am. J. Physiol. 224, 328—337
60 Powell, D.W., Binder, H.J. and Curran, P.F. (1973) Am. J. Physiol. 225, 781—787
61 Love, A.H.G. (1969) Gut 10, 63—67
62 Swallow, J.H., Code, C.F. and Freker, R. (1968) Gastroenterology 54, 35—40
63 Iber, F.L., McGonagle, T., Serebro, H.A., Luebbers, E., Bayless, T.M. and Hendrix, T.R. (1969) Am. J. Med. Sci. 258, 340—350
64 Frizzel, R.A. and Schultz, S.G. (1972) J. Gen. Physiol. 59, 318—346
65 Hubel, K.A. (1974) J. Clin. Invest. 53, 964—970
66 Powell, D.W., Molawer, S.J. and Plotkin, G.R. (1968) Am. J. Physiol. 215, 1226—1233
67 Rutten, W.J., de Pont, J.J.H.M. and Bonting, S.L. (1972) Biochim. Biophys. Acta 274, 201—213

tration was calculated from the total Mn^{2+} concentration by subtracting the amounts of Mn^{2+} sequestrated in creatinephosphate and GTP [10]. Samples of 2–10 μg protein were preincubated at 30°C for 2 min and incubated in the presence of substrate for 10 min. The reaction was stopped by adding 0.1 ml of a solution containing 5 mM GTP, 0.5 mM cyclic [^{3}H]GMP (approx. 20 000 dpm) and 6 mM pyrophosphate, pH 7.6. The final mixture was applied to Al_2O_3 columns (1 g dry Al_2O_3 (Merck) in Pasteur pipettes) and eluted with 2 ml 10 mM Tris–HCl, pH 7.6. The eluate was acidified with 30 μl 5 M HCl and applied to a Dowex 50H$^+$-column (200-400 mesh, 4 × 1 cm). During elution with 0.05 M HCl 80-85 per cent of the applied cyclic [^{3}H]GMP was recovered in the 4th through 8th ml and directly counted in a Packard liquid scintillation spectrometer (Tri-Carb 3380) for measurements of ^{3}H and ^{32}P activities using Dimilume (Packard) as the liquid scintillation mixture. Overall recovery of cyclic [^{3}H]GMP: 75–80%. Reaction blanks (complete system minus enzyme) were approximately 0.01 per cent of the initially added ^{32}P activity. About 95% of the ^{32}P-labelled product could be identified as cyclic GMP by means of a cyclic 3′, 5′-phosphodiesterase degradation procedure followed by rechromatography on Dowex 50H$^+$-columns. Conversion of cyclic GMP during incubation was less than 5% with all preparations tested. In all experiments the production of cyclic GMP was linear with time and protein concentration.

Alkaline phosphatase was determined at 37°C as described by Iemhoff et al. [11], non-specific esterase activity was measured according to Higgins and Lapides [12] at 37°C, sucrase was tested for according to Dahlqvist [13], rotenone-insensitive NADPH-cytochrome *c* reductase, succinate dehydrogenase and adenylate cyclase were determined as described earlier [5,14]. Ouabain-sensitive (Na^+-K^+)-ATPase was measured spectrophotometrically at 30°C according to Ferard et al. [15], using 1 mM ouabain. Protein was determined by the Lowry procedure [16], using bovine serum albumin as standard.

Biochemical products, if not stated, were obtained from Boehringer, Mannheim. Radioactive materials were obtained from the Radiochemical Centre, Amersham.

3. Results

By measuring guanylate cyclase activities in 100 000 *g* supernatants of isolated villous and crypt cells after homogenization, using Potter-Elvehjem (Teflon-glass), Polytron homogenizers or a pressure bomb (table 3) it could be confirmed that over 95% of the total activity was particulate in both cell types. The stability of the enzyme at 0°C as well as at 30°C [6] appeared of much advantage in prolonged fractionation studies.

Table 1 summarizes the specific enzyme activities measured in cell homogenates and brushborders from rat small intestine. On a protein base, sucrase, a marker enzyme of the microvillous membrane [17] is 5.4 times enriched in the purified brushborders as compared to upper villous cells. Alkaline phosphatase, localized in both microvilli and basal-lateral plasma membranes [18] appeared 4.2 times enriched in the brushborder preparations. However, the specific activities of (Na^+-K^+)-ATPase, a plasma membrane marker [19] and NADPH-cytochrome *c* reductase or non-specific esterase, both mainly localized in the endoplasmic reticulum [20,21] are similar or slightly diminished in the brushborders. The same holds for adenylate cyclase, also generally accepted as a basal-lateral plasma membrane marker in small intestinal epithelium [3–5]. In contrast, guanylate cyclase activity, measured at two different Mn^{2+} concentrations, is increased by 4.6-fold in the brushborders as compared to upper villous cells. The strongly increased specific activity is not due to a removal of enzyme inhibitors or release of activators during brushborder purification for combined assays of upper villous cell and brushborder samples gave completely additive results.

The finding of a preferential localization of guanylate cyclase in the brushborder region agrees with the rise of its activity during the development of the epithelial cell from the proliferative stage (represented by the crypt-con A cells) via its differentiating phase (present in total crypt cell preparations) into mature villous cells (table 1). The activities of other brushborder-bound enzymes (sucrase, alkaline phosphatase) also increase together with the development of intestinal microvilli (table 1). However, the presence of an activity jump from

Table 1
Specific enzyme activities in various cell preparations and brushborders isolated from rat small intestinal epithelium

Enzyme	Upper villous cells	Brush-borders	Crypt cells (total)	Crypt cells (con A-selected)
Guanylate cyclase*				
0.5 mM Mn^{2+}_{free}	16.1 ± 1.0 (9)	75.6 ± 15.2 (6)	6.1 ± 0.6 (5)	2.2 ± 0.3 (3)
5.0 mM Mn^{2+}_{free}	48.3 ± 2.4 (9)	218 ± 18 (6)	16.5 ± 1.0 (5)	7.4 ± 0.4 (3)
Adenylate cyclase (fluoride-stimulated)*	26.3 ± 1.2 (4)	24.9 ± 1.5 (4)	34.5 ± 1.0 (4)	–
(Na^+-K^+)-ATPase (ouabain-sensitive)**	70.7 ± 5.5 (4)	83.1 ± 4.8 (4)	28.3 ± 2.7 (3)	–
NADPH-cytochrome *c*-reductase (rotenone-insensitive)**	6.3 ± 0.4 (4)	4.1 ± 0.5 (3)	2.4 ± 0.3 (3)	–
Esterase**	2780 ± 70 (4)	1610 ± 40 (4)	980 ± 30 (4)	570 (2)
Alkaline phosphatase**	410 ± 16 (8)	1710 ± 42 (6)	40 ± 4 (5)	26 ± 2 (3)
Sucrase**	92.7 ± 4.3 (3)	495 ± 24 (4)	12 ± 3 (4)	–

* Specific activity expressed as picomoles cyclic GMP or cyclic AMP formed per min mg of protein.
** Specific activity expressed as nanomoles substrate converted per min per mg of protein.
Number of preparations tested is given in parentheses.

Table 2
Relative specific activities and distribution of enzymes after subfractionation of purified brushborders according to Forstner et al. [8].

Brushborder fractions**	Relative specific activity*				
	Sucrase	Alkaline phosphatase	NADPH-cytochrome *c* reductase (rotenone-insensitive)	Esterase	Guanylate cyclase
Membrane fraction I (microvillous)	1.33 (24)	1.72 (31)	3.70 (65)	3.33 (60)	1.40 (25)
Membrane fraction II (microvillous)	1.54 (63)	1.28 (52)	0.68 (27)	0.84 (34)	1.61 (66)
Fibrillar fraction	0.26 (10)	0.26 (10)	0.13 (5)	0.11 (4)	0.29 (11)
Recovery (%)	97	93	97	98	103

* The relative specific activity represents the ratio of the specific activity of the enzyme in each fraction and its specific activity in the whole brushborder preparation (see table 1).
** Terminology derived from the paper of Forstner et al. [8].
The percentage of total brushborder activity recovered in the subfractions is given in parentheses.
Mean values of two experiments are shown.

crypt to villus does not give sufficient proof for a typical localization in the brushborder region because marker enzymes of the endoplasmic reticulum (e.g. esterase, table 1 and [21]) and of basal-lateral plasma membranes ((Na^+-K^+)-ATPase, table 1 and [22]) also rise in activity from crypt to upper villus.

After separation of the isolated brushborders into microvillous and fibrillar fractions (table 2) the distribution of guanylate cyclase followed that of the microvillous enzymes sucrase and alkaline phosphatase and was clearly different from the endoplasmic reticulum markers. The distribution pattern also excludes a possible fibrillar origin of the enzyme.

Finally, homogenates of isolated villous cells were subfractionated according to the fractionation scheme of Douglas et al. [9] and the distribution of guanylate cyclase and a variety of marker enzymes among these fractions was studied.

The distribution of alkaline phosphatase and guanylate cyclase followed the same pattern and was clearly different from that of the other membrane-bound markers tested (table 3). The highest relative specific activity of both enzymes was found in the so-called M_1 fraction, enriched in the basal-lateral plasma membrane marker (Na^+-K^+)-ATPase and nearly devoid of mitochondrial and microvillous membranes. The conclusions drawn from the previous experiments with isolated brushborders are strengthened by the observation that the major part of the guanylate cyclase activity is recovered in the 1500 *g* pellet (fraction B) that mainly consists of brushborders, nuclei and whole cells. The data from table 3 strongly suggest that most, if not all of the guanylate cyclase outside the brushborder is

Table 3
Relative specific activities and distribution of enzymes in subcellular fractions prepared from rat small intestinal villous cells according to Douglas et al. [9]

Cell fraction**	(Na^+-K^+)-ATPase ouabain-sensitive	Alkaline phosphatase	NADPH-cytochrome *c* reductase (rotenone-insensitive)	Sucrase	Guanylate cyclase	Succinate dehydrogenase
Fraction B	1.37	2.53	0.94	2.35	2.76	0.34
	(38)	(71)	(26)	(66)	(77)	(9)
Sorbitol layer	1.22	0.07	4.06	0.26	0.26	0.68
	(12)	(1)	(41)	(3)	(3)	(7)
Fraction D	3.02	3.00	1.23	1.44	2.32	7.70
	(30)	(30)	(12)	(14)	(23)	(77)
Fraction M_1	7.69	3.69	2.79	0.11	3.93	0.50
	(10)	(6)	(4)	(0)	(5)	(1)
Fraction M_2	2.43	1.26	5.64	0.04	1.60	0.42
	(6)	(3)	(14)	(0)	(4)	(1)
Supernatant	–	0.06	–	–	0.08	–
(105 000 *g*)	(0)	(2)	(0)	(0)	(3)	(0)
Recovery	87	113	97	83	115	95

* The relative specific activity compares the specific activity of each fraction (on a protein base) with that of the whole homogenate obtained by disruption of the villous cells in a pressure bomb.

** Terminology used by Douglas et al. [9].

The M_1 and M_2 fractions were isolated by dextran gradient centrifugation at barrier density of 1.075 exactly as described by Douglas et al. [9].

The percentage of total homogenate activity recovered in the subcellular fractions is given in parentheses.

Fraction B mainly contains brushborders, nuclei and whole cells, the sorbitol layer is enriched in endoplasmic reticulum, fraction D contains a mixture of mitochondria, basal-lateral plasma membranes and some brushborder and endoplasmic reticulum activities, fraction M_1 consists mainly of basal-lateral plasma membranes and fraction M_2 is enriched in endoplasmic reticulm membranes.

localized in the anti-luminal membranes of the villous cell, together with (Na^+-K^+)-ATPase, alkaline phosphatase and adenylate cyclase.

4. Discussion

Our results indicate that the bulk of guanylate cyclase is present in the apical region of the villous cell together with other microvillous enzymes like sucrase and alkaline phosphatase. A much smaller part of the enzyme content is integrated in the basal-lateral plasma membrane as has been shown too for alkaline phosphatase [18]. The different distribution pattern of membrane-bound guanylate cyclase and adenylate cyclase together with their different properties [1,10] argues strongly against the hypothesis [23] that the same enzyme system would be responsible for the synthesis of both cyclic GMP and cyclic AMP.

The preponderance of guanylate cyclase in the brushborder presumably corresponds to the greater surface area of the microvilli. In proliferating crypt cells, lacking the microvillous structure [24], the enzyme activity is about 15% of the upper villous cell activity (table 1). The preferential localization in the brushborder may explain the high specific activity of this particulate enzyme in mucosal scrapings as compared to most other tissues of the rat in which microvillous structures are less well developed or nearly absent (compare [2]). Leakage of cyclic GMP from the brushborder region into the lumen could also account for the considerable amounts of this nucleotide found in the intestinal contents [1].

It is tempting to speculate about the function of this brushborder-bound guanylate cyclase. Regulation of its activity by direct interaction with hormone receptors in the basel-lateral membranes of the epithelial cell seems very unlikely. Regulation by changes of the free Ca^{2+} concentration within the cell [25] seems equally questionable because Ca^{2+} has very little influence on the activity of the particulate enzyme in vitro [10].

Maybe the unique localization of guanylate cyclase in this cell type is related to the transport function of the microvillous membrane. In rabbit ileum in virto, addition of cyclic GMP caused an alteration of ion fluxes in the same direction as evoked by cyclic AMP [26]. A possible role of cyclic nucleotides in regulation of transport processes has been brought forward earlier in studies correlating cyclic AMP levels and transport rates of Na^+, K^+ and HCO_3^- [5,27], Ca^{2+} [28] and amino acids [29] in small intestinal epithelium.

Up till now no physiological conditions are known in which intestinal cyclic GMP levels undergo significant changes [10,27]. This might indicate that constant levels of cyclic GMP are necessary to maintain normal cellular functions and that deviations from this level, as in the case of cyclic AMP accumulation [5,27], soon lead to pathological states. However, physiological alterations of cellular cyclic GMP levels could be very small, especially if confined to a certain compartment within the cell. In other tissues these are usually very rapid and transient phenomena. Such changes may therefore easily be missed in an experimental approach, particularly as long as the role of cyclic GMP in the small intestinal epithelium is a matter of mere speculation.

Acknowledgements

The author wishes to thank Professor Dr W. C. Hülsmann for advice and critical reading of the manuscript and Drs B. J. P. de Jong for advice and help in fractionation studies using the pressure bomb and for providing some of the con A-selected crypt cell preparations. The expert technical assistance of Mr L. M. Scheek is gratefully acknowledged. Miss A. C. Hanson is thanked for typing the manuscript.

References

[1] Ishikawa, E., Ishikawa, S., Davis, J. W. and Sutherland, E. W. (1969) J. Biol. Chem. 244, 6371–6376.
[2] Kimura, H. and Murad, F. (1974) J. Biol. Chem. 249, 6910–6916.
[3] Parkman, D. K., Ebel, H., Dibona, D. R. and Sharp, G. W. G. (1972) J. Clin. Invest. 51, 2292–2298.
[4] Grand, R. J., Torti, F. M. and Jaksina, S. (1973) J. Clin. Invest. 52, 2053–2059.
[5] de Jonge, H. R. (1975) Biochim. Biophys. Acta 381, 128–143.
[6] Webster, H. L. and Harrison, D. D. (1969) Exp. Cell Res. 56, 245–253.

[7] Podolsky, D. K. and Weiser, M. M. (1973) J. Cell Biol. 58, 497–500.
[8] Forstner, G. G., Sabesin, S. M. and Isselbacher, K. J. (1968) Biochem. J. 106, 381–390.
[9] Douglas, A. P., Kerley, R. and Isselbacher, K. J. (1972) Biochem. J. 128, 1329–1338.
[10] de Jonge, H. R., manuscript in preparation.
[11] Iemhoff, W. G., van den Berg, J. W. O., de Pijper, A. M. and Hülsmann, W. C. (1970) Biochim. Biophys. Acta 215, 229–241.
[12] Higgins, C. and Lapides, J. (1947) J. Biol. Chem. 170, 467–482.
[13] Dahlqvist, A. (1964) Anal. Biochem. 7, 18–25.
[14] de Jonge, H. R. and Hülsmann, W. C. (1972) Eur. J. Biochem. 32, 356–364.
[15] Ferrard, G., Sall, I. and Metais, P. (1975) Enzyme 19, 38–47.
[16] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[17] Eichholz, A. (1967) Biochim. Biophys. Acta 135, 475–482.
[18] Clark, S. L. (1961) Amer. J. Anat. 109, 57–83.
[19] Fujita, M., Ohta, H., Kawai, K., Matsui, H. and Nakao, M. (1972) Biochim. Biophys. Acta 274, 336–347.
[20] Clark, M. L., Lanz, H. C. and Senior, J. R. (1969) Biochim. Biophys. Acta 183, 233–235.
[21] Deimling, O. V. and Madreiter, H. (1972) Histochemie 29, 83–96.
[22] Charney, A. N., Gots, R. E. and Giannella, R. A. (1974) Biochim. Biophys. Acta 367, 265–270.
[23] Illiano, G., Tell. G. P. E., Siegel, M. I. and Cuatrecasas, P. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2443–2447.
[24] de Both, N. J., van Dongen, J. M., van Hofwegen, B., Keulemans, J., Visser, W. J. and Galjaard, H. (1974) Dev. Biol. 38, 119–137.
[25] Goldberg, N. D., O'Dea, R. F. and Haddox, M. K. (1973) in: Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G. A. eds) Vol. 3, pp. 155–223. Raven Press, New York.
[26] Field, M. (1971) Amer. J. Physiol. 221, 992–997.
[27] Field, M. (1974) Gastroenterology 66, 1063–1084.
[28] Neville, E. and Holdsworth, E. S. (1969) FEBS Lett. 2, 313–316.
[29] Kinzie, J. L., Ferrendelli, J. A. and Alpers, D. H. (1973) J. Biol. Chem. 248, 7018–7024.

PROPERTIES OF GUANYLATE CYCLASE AND LEVELS OF CYCLIC GMP IN RAT SMALL INTESTINAL VILLOUS AND CRYPT CELLS

H. R. de JONGE

Department of Biochemistry I, Faculty of Medicine, Erasmus University Rotterdam, P.O. Box 1738, Rotterdam, The Netherlands

Received 1 May 1975

1. Introduction

A number of studies on the mechanism of action of bacterial enterotoxins [1,2], prostaglandins [2] and the recently discovered gastrointestinal hormone vasoactive intestinal polypeptide (VIP)* [3] on small intestinal epithelium stress the role of cyclic AMP as a regulator of water and electrolyte transport in this tissue. The function of cyclic GMP in the small intestine is however entirely unknown. Its level as well as the activity of the particulate form of the enzyme guanylate cyclase in rat small intestinal epithelium are relatively high as compared to most other mammalian tissues [4–7]. The soluble form of guanylate cyclase which, at least in rat heart [6] and lung [8], clearly differs from the particulate enzyme in kinetic and physical properties, is nearly absent in small intestinal mucosa [4,6,7]. Our previous work [7] showed that the major part of the intestinal enzyme activity is associated with the microvillous membrane of the brushborder, the remaining part being localized in the basal-lateral plasma membranes. Accordingly, proliferative crypt cells displayed a much lower guanylate cyclase activity than the mature villous cells [7]. Since intestinal brushborders, which are easily obtained in a relatively pure state, provide one of the richest sources of particulate guanylate cyclase in the rat, the kinetic and other properties of this enzyme may be measured accurately even in the absence of an activating detergent used in other studies [6,8].

In the present investigation some characteristics of the brushborder-localized guanylate cyclase are compared with those of microsomal enzyme preparations from isolated villous and crypt cells, mainly representing enzyme bound to basal-lateral plasma membranes. A variety of hormones or hormone-like agents reported to raise cyclic AMP levels in rat small intestine, to elevate cyclic GMP in other tissues or to affect intestinal transport functions, were also tested for their ability to alter the intestinal guanylate cyclase activity in vitro or to regulate cyclic GMP levels in isolated villous and crypt cells and mucosal scrapings. The apparent insensitivity of the particulate enzyme to hormones and the possible function of cyclic GMP in the intestinal mucosa are discussed.

2. Materials and methods

Upper villous and crypt cells from the whole length of rat small intestine were harvested separately as described earlier [9,10]. Brushborders were isolated and purified in principle according to Harrison and Webster [11] as described before [12]. Microsomal preparations from villous and crypt cells were obtained by homogenizing the cells in 0.25 M sucrose–1 mM EDTA–1 mM Tris–HCl (pH=7,6) in a tightly fitting Potter-Elvehjem homogenizer, followed by differential centrifugation [10]. Mucosal scrapings were isolated by rinsing the intestinal lumen with icecold saline followed by gently scraping

* *Abbreviations:* Cyclic GMP, cyclic 3′, 5′-guanosine monophosphate. Cyclic AMP, cyclic 3′, 5′-adenosine monophosphate. GTP, guanosine 5′-triphosphate. PEP, phosphoenolpyruvate, tricyclohexylammonium salt. PTH, parathyroid hormone. VP, vasopressin. CP, creatinephosphate.

the superficial mucosal layer with a microscope slide. The cell sheets were taken up in saline and centrifuged for 2 min at 850 *g*. This procedure was repeated twice. Brushborders, cell preparations and microsomes were taken up in 0.10 M Tris–HCl (pH=7.6; final protein concentration: about 1 mg/ml) and homogenized in a Potter-Elvehjem homogenizer prior to enzyme assay.

Assay of guanylate cyclase activity and purification of the labeled product were done essentially as before [7]. The concentration of free Mn^{2+} was calculated from $[Mn^{2+}_{total}] = [Mn^{2+}_{free}] + [MnCP^{-}] + [MnGTP^{2-}]$. The stability constant of $MnCP^{-}$ used for calculating $[MnCP^{-}]$ was 110 M^{-1} [13]. Conversion of creatine-phosphate during the assay was less than 15 per cent. In view of the high binding constants of Mn^{2+}-nucleoside triphosphates [14] it was assumed that all GTP was present as the $MnGTP^{2-}$ complex. Binding of Mn^{2+} to cyclic GMP and the possible binding of GTP to albumin were neglected.

Cyclic GMP concentration was measured according to Dinnendahl [15], using a cyclic GMP-binding protein from lobster tail muscle [16]. Cyclic AMP concentration was measured according to Tovey et al. [17], using a cyclic AMP-binding protein isolated

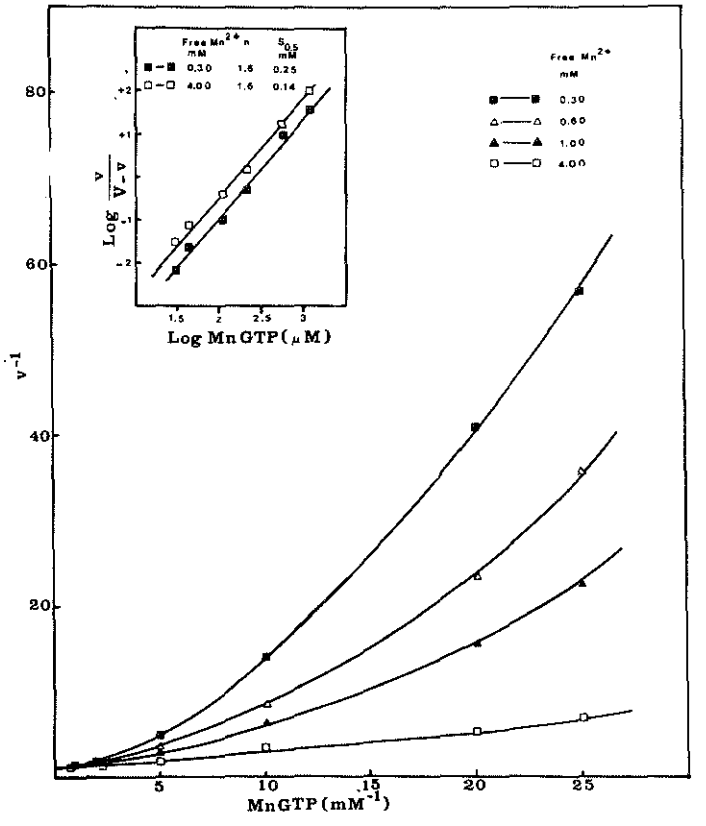

Fig.1. Double reciprocal plots of cyclic GMP formation as a function of the $MnGTP^{2-}$ concentration measured with the brushborder preparation. v is expressed as nmoles cyclic GMP · min^{-1} · mg^{-1} protein. Inset: Hill plots.

from bovine skeletal muscle [18]. After further purification of the cyclic nucleotide samples by thinlayer chromatography on silica plates or chromatography on Dowex 50H$^+$-columns, essentially the same results were obtained.

Protein was determined according to Lowry et al. [19], using bovine serum albumin as a standard.

Biochemicals were usually from Boehringer, Mannheim. Radioactive materials were from the Radiochemical Centre, Amersham. Arginine-vasopressin (synthetic, grade IV) was from Sigma, PTH (TCA-powder) from Inolex Corp. (Chicago), secretin from the Boots Company (Nottingham), concanavalin A (3 times crystallized) from Miles Labs., insulin (pure) from Boehringer, pentagastrin (Peptavlon) from ICI (Maclesfield), bradykinin (synthetic) from Sandoz and glucagon from Lilly (Indianapolis). The ionophore A-23187 was a gift from the Eli Lilly Company; it was dissolved in ethanol prior to use. Prostaglandins were gifts from the Unilever Research Lab. (Vlaardingen). Purified choleratoxin was kindly supplied by Dr R. S. Northrup, SEATO Cholera Research Program, and was prepared under contract for NIAID by R. A. Finkelstein, Ph.D., the University of Texas, Southwestern Medical School, Dallas, Texas.

3. Results

Some kinetic properties of the brushborder-bound guanylate cyclase are shown in fig.1 and 2. Lineweaver-Burk plots for different concentrations of free Mn^{2+} (fig.1) showed a positive co-operative behaviour indicating multiple binding sites for

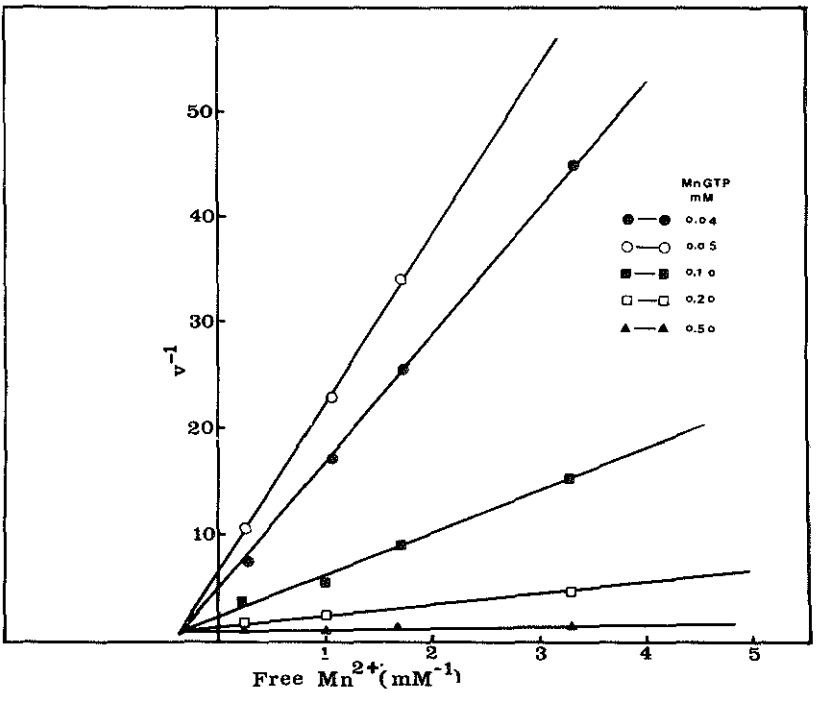

Fig. 2. Double reciprocal plots of cyclic GMP formation as a function of excess Mn^{2+} measured with the brushborder preparation. v is expressed as nmoles cyclic GMP · min^{-1} · mg^{-1} protein.

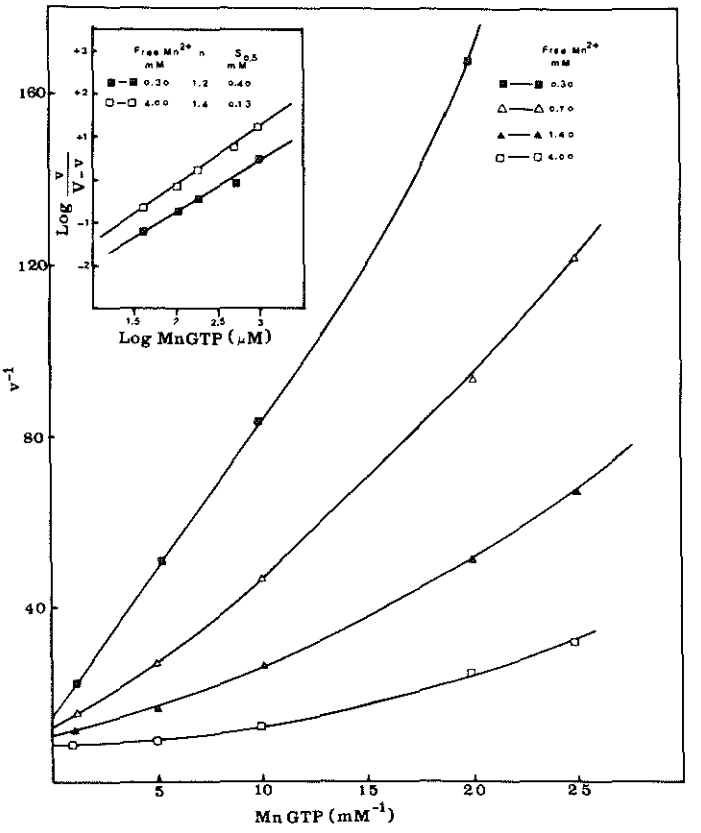

Fig.3. Double reciprocal plots of cyclic GMP formation as a function of $MnGTP^{2-}$ concentrations measured with the microsomal preparation from upper villous cells. v is expressed as nmoles cyclic GMP · min^{-1} · mg^{-1} protein. Inset: Hill plots.

$MnGTP^{2-}$. The Hill plot (fig.1, inset) gave a Hill coefficient (n) of 1.6. The $S_{0.5}$ for $MnGTP^{2-}$ decreased from 0.25 to 0.14 mM when Mn^{2+}_{free} was changed from 0.3 to 4.0 mM. Double reciprocal plots of cyclic GMP formation as a function of excess Mn^{2+} at fixed levels of $MnGTP^{2-}$ gave straight lines with a common point of intersection (fig.2). The dissociation constant for excess Mn^{2+}, calculated from the position of this point, was 2.5 mM. At $[MnGTP^{2-}] > 0.5$ mM the slopes of the plots approached zero, indicating that at saturating substrate concentrations the guanylate cyclase no longer needs excess Mn^{2+} for optimum activity.

The kinetic patterns of particulate guanylate cyclase from upper villous cell microsomes, presumably only slightly contaminated with brushborder material [10], clearly showed different characteristics (figs.3 and 4). There was a marked loss of co-operativity with respect to $MnGTP^{2-}$, especially at low $[Mn^{2+}_{free}]$. At 0.3 mM Mn^{2+}_{free} the Hill coefficient approached 1.2 and the value for $S_{0.5}$ was increased (fig.3). Even at near saturating concentrations of $MnGTP^{2-}$ the

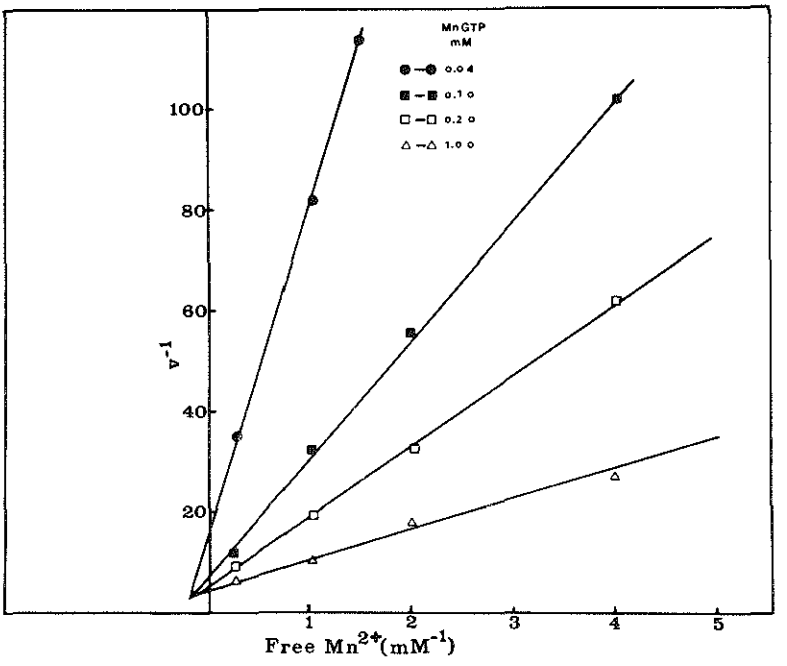

Fig.4. Double reciprocal plots of cyclic GMP formation as a function of excess Mn^{2+} measured with the microsomal preparation from upper villous cells. v is expressed as nmoles cyclic GMP · min^{-1} · mg^{-1} protein.

slope of the reciprocal plots of enzyme activity versus $[Mn^{2+}_{free}]$ did not approach zero. At 0.10 mM $MnGTP^{2-}$ the apparent K_m for Mn^{2+}_{free} was 2.7 mM and decreased to 0.7 mM at 1.0 mM $MnGTP^{2-}$ (fig.4).

Guanylate cyclase from crypt microsomes displayed an intermediate type of kinetic behaviour showing n-values for $MnGTP^{2-}$ between 1.3 and 1.4 (results not shown). These preparations, however, are seriously contaminated with luminal membranes (compare [20]) and thus contain a mixture of guanylate cyclase activities localized in basal-lateral membranes and partially developed microvilli.

Some regulatory properties of guanylate in vitro were tested with homogenates of isolated villous and crypt cells, mucosal scrapings, microsomes and brush-borders. Activities were measured at 0.1 mM $MnGTP^{2-}$ and 0.3 or 4.0 mM Mn^{2+}_{free}. Table 1 summarizes our results with the brushborder preparation. In agreement with Ishikawa et al. [4] we found that incubation of the enzyme in the presence of 0.1% Triton X-100 gave a two-fold rise in activity. 1.4 mM $CaCl_2$ slightly inhibited the enzyme at 0.3 mM Mn^{2+}_{free} but stimulated 1.2 fold at 4 mM Mn^{2+}_{free}. Strong inhibitory effects were found with PEP, oxaloacetate and ATP or ADP. Similar effects of metabolites have been reported earlier for the soluble enzyme from rat lung [21]. Alloxan, a strong inhibitor of intestinal adenylate cyclase (H. R. de Jonge, unpublished results), slightly depressed guanylate cyclase activity, possibly due to an in vitro oxidation of SH-groups (compare [22]). Aminoacids in physiological concentrations had a small stimulatory effect (table 1). D-isomers were ineffective in this respect. No significant differences from control values were obtained by addition of NaF, glucose, choleragen, prostaglandin E_1, $F_{2\alpha}$ and A_1, concanavalin A and a number of catechol-

Table 1
Guanylate cyclase activity in brushborder preparations from rat small intestinal epithelium

Agents	Cyclic GMP formation at 0.1 mM GTP (% of basal rate)	
	0.3 mM Mn^{2+}_{free}	4.0 mM Mn^{2+}_{free}
–	100	100
Triton X-100 (0.1%)	190	210
NaF (10 mM)	100	100
D (+)glucose (1–50 mM)	100	100
$CaCl_2$ (1.4 mM)	71	117
PEP (1 mM)	74	82
PEP (10 mM)	56	62
Oxaloacetate (0.5 mM)	52	61
ATP (1 mM)	26	29
ADP (1 mM)	32	36
D-alanine (1 mM)	100	99
L-alanine (1 mM)	119	120
L-aspartate (1 mM)	116	114
L-arginine (1 mM)	118	115
Glycine (1 mM)	109	110
Alloxan (5 mM)	70	75

The following agents, tested in the presence of 1.4 mM $CaCl_2$, had no effect as compared to $CaCl_2$ alone: Concanavalin A (0.2–1 μM), choleratoxin (10–100 μg/ml), prostaglandin E_1, A_1 and $F_2\alpha$ (1–50 μM), the catecholamines adrenalin, noradrenalin and serotonin-creatinesulphate (10–100 μM), the cholinergic agents carbamylcholine, acetylcholine and pilocarpine (0.5–50 μM) and the peptide hormones insulin (1–100 μg/ml), glucagon (1–10 μM) ± pentagastrin (10–50 μM), VP (4–50 mU/ml), PTH (0.04–2 U/ml) and bradykinin (50 μM).

amines, cholinergic agents and peptide hormones specified in the legend of table 1. These agents have been reported to alter intestinal transport of water and electrolytes (Na^+, $K^+$and HCO_3^- or Ca^{2+}) [2,3], to stimulate the soluble form of guanylate cyclase in liver in vitro (secretin, [23]) or to raise cyclic GMP levels in other tissues (compare [24]). Similar negative results were obtained using villous and crypt cell homogenates, microsomes or mucosal scrapings. Also after addition of Mg^{2+} (1 mM) or Ca^{2+} (1.4 mM), the insensitivity of the guanylate cyclase for these agents was maintained.

Because in other tissues cyclic GMP levels may be raised by hormones that are unable to stimulate the guanylate cyclase activity in vitro, our studies were extended with measurements of cyclic nucleotide levels in isolated villous and crypt cells during incubation at 37°C. Because hormone action may be disturbed by isolation of cells in EDTA-containing media, these studies were repeated with mucosal scrapings, isolated in saline.

As shown in table 2, the basal levels of cyclic GMP, like the cyclic AMP levels [25], decreased from crypt to villous cells. In contrast, the guanylate cyclase activity of cell homogenates increased about three-fold [7]. This apparent discrepancy may indicate a concomitantly raised cyclic GMP degradation rate in the villous cells. Moreover the assay conditions in vitro (concentrations of substrates and inhibitors) may considerably differ from the enzyme environment in the intact cells. The cyclic AMP/cyclic GMP ratio was relatively low as compared to a number of other tissues [4,26]. $CaCl_2$ (1.4 mM) alone caused a slight but statistically insignificant depression of basal cyclic GMP levels. Also the ionophore A-23187, known to mimic hormone actions in other tissues by stimulating Ca^{2+} transport through the plasma

Table 2
Levels of cyclic GMP and cyclic AMP in upper villous and crypt cells and mucosal scrapings from rat small intestinal epithelium

Additions	1.4 mM $CaCl_2$	pmoles/mg protein* Cyclic GMP			Cyclic AMP			cyclic AMP / cyclic GMP ratio
		Villous cells	Crypt cells	Scrapings	Villous cells	Crypt cells	Scrapings	Villous cells
–		0.97 ± 0.20 (5)	1.90 ± 0.36 (4)	0.80 ± 0.15 (4)	4.3 ± 0.7 (7)	12.8 ± 1.1 (5)	5.1 ± 0.8 (6)	4.3
–	+	0.75 ± 0.10 (8)	1.35 ± 0.22 (6)	0.70 ± 0.10 (5)	5.0 ± 0.6 (7)	11.9 ± 1.0 (5)	5.0 ± 0.7 (5)	6.7
Ionophore A-23187 (1 μg/ml)	+	0.79 ± 0.21 (5)	1.78 ± 0.41 (4)	0.88 ± 0.17 (4)	5.2 ± 0.9 (3)	–	4.8 ± 1.0 (3)	6.6
Theophylline (5 mM)	+	0.76 ± 0.25 (7)	–	0.76 ± 0.20 (5)	8.5 ± 1.3 (6)***	–	9.6 ± 1.7 (4)***	11.2
Alloxan (5 mM)	+	2.20 ± 0.36 (4)***	–	2.05 ± 0.40 (4)***	5.5 ± 0.9 (4)	–	4.5 ± 1.2 (3)	2.5
PGE_1 (20 μM)	+		no effect		10.7 ± 1.1 (6)***	–	–	
$PGF_{1\alpha}$ (20 μM)	+		no effect		7.1 ± 0.9 (3)***	–	–	
Adrenalin (20 μM)	+		no effect		7.8 ± 0.7 (4)***	–	–	
Cholinergic agents **								
Choleratoxin	+		no effect		no effect			
Peptide hormones								

Freshly isolated villous and crypt cells or mucosal scrapings were gently dispersed in a Krebs-Ringer HCO_3^- buffer (pH=7.4) containg 20 mM glucose to a final protein concentration of 10 - 20 mg/ml. 1 ml portions of the suspension were preincubated in plastic vials for 5 min at 37°C, with gentle agitation, followed by a further incubation period of 1 min in the presence of the additions shown. $CaCl_2$, ionophore and theophylline were already present during preincubation. The incubation was rapidly stopped with 1 ml trichloroacetic acid (20% v/v) containing 0.01 μCi [^{3}H]cyclic AMP (0.5 pmol) or [^{3}H]cyclic GMP (0.7 pmol) immediately followed by ultrasonic disruption. Trichloroacetic acid was removed by ether extraction and most of the non-cyclic nucleotides were bound to Al_2O_3-columns as described previously [7]. The eluate was concentrated under a stream of N_2 and the residue was taken up in water prior to measurements of recovery and cyclic nucleotide-binding assays as described in Materials and methods. Overall recovery cyclic GMP: 60 - 80%, cyclic AMP: 50 - 70%.

* Cyclic nucleotide levels are expressed as means ± S.E.M., with the number of animals in parentheses. Similar results (not shown) were obtained after 5 min incubation (10 min in the presence of A-23187).

** A specification of these agents and their concentration range tested is given in the legend of table 1.

*** $p < 0.05$, p-values were calculated from the Student t-test.

membrane or releasing Ca^{2+} from intracellular stores, had no effect of intestinal cyclic GMP concentrations. Again, no significant changes of cyclic GMP were found in a Ca^{2+}-containing medium in the presence of choleratoxin, prostaglandins, cholinergic agents, concanavalin A or the hormones mentioned above.

Unexpectedly, incubation for 6–10 min in the presence of the phosphodiesterase inhibitor theophylline (5 mM) with or without hormones, did not raise the cyclic GMP content whereas cyclic AMP was significantly elevated (table 2, [25]). A possible depression of net formation of cyclic GMP by cyclic-AMP-elevating agents would not be unique for intestinal epithelium (compare [26]). It is also possible that theophylline induced a selective leakage of cyclic GMP to the medium where it may be rapidly metabolized by the residual activity of phosphodiesterases released from damaged cells.

Alloxan (5 mM) strongly elevated cyclic GMP in the isolated cells but had no influence on cyclic AMP levels (table 2). Apparently, the interaction of alloxan with cyclases in the intact cell is opposite to its in vitro effect, or else alloxan possesses an additional inhibitory effect on intracellular cyclic nucleotide degradation. In either case this compound could be a useful tool in future studies of the relationship between elevated cyclic GMP levels and intestinal functions.

4. Discussion

A clear difference was found between the kinetic behaviour of guanylate cyclase in brushborder and microsomal preparations from rat small intestinal villous cells. Whereas the brushborder enzyme had many characteristics in common with the particulate form of guanylate cyclase from sea urchin sperm [27] and rat lung [8] (positive substrate co-operativity, independency from free Mn^{2+} at saturating $MnGTP^{2-}$ concentrations), the kinetics of the microsomal enzyme, mainly localized in the basal-lateral plasma membranes, resembled more the soluble form of the enzyme in rat heart [6] and lung [8] (loss of co-operativity, activation by free Mn^{2+} at high $MnGTP^{2-}$ levels). Maybe the nearly complete absence of a soluble guanylate cyclase in rat small intestine is due to a high affinity of this type of enzyme for the basal-lateral plasma membranes. An alternative interpretation, assuming a rapid release of guanylate cyclase from membranes in other tissues during homogenization, has been suggested previously [26]. Also an alteration of kinetic behaviour during preparation of the microsomes cannot be entirely excluded yet.

The activity of the particulate guanylate cyclase in vitro or the levels of cyclic GMP in isolated small intestinal epithelial cells appeared insensitive to a large number of hormones or hormone-like agents under our experimental conditions. This insensitivity may be interpreted in different ways:

1) The epithelium lacks receptors for the hormones. Consequently the reported hormonal effects on transport rates should be ascribed to effects on intestinal smooth muscle or blood vessels.

2) Hormone effects are mediated by factors different from cyclic GMP.

3) Hormone receptors or coupling systems between receptors and guanylate cyclase are damaged during cell isolation or homogenization. Moreover, 'permissive' serum factors needed for full expression of hormone action may be lost in the in vitro system. The determination of cyclic GMP in samples of intestine, obtained by freeze-clamping the tissue in situ, would better reflect the physiological situation but lacks any tissue specificity.

4) Cyclic GMP levels are indeed altered by some hormones but the changes were too small, transient or delayed to be measured in our experiments. Small effects may be expected particularly if only the guanylate cyclase outside the brushborder region responds to hormones.

5) The reported action of cholinergic agents [24, 26], insulin [24] and the ionophore A-23187 [28, 29] on cyclic GMP levels in other tissues could be mediated by the soluble form of guanylate cyclase which is almost completely missing from the intestinal epithelium.

To our knowledge, a hormone effect on particulate guanylate cyclase is only well-established in fibroblasts where cyclic GMP levels may be elevated by a specific growth factor, a peptide hormone secreted by the pituitary gland [30]. It is not known if a similar growth factor for proliferative crypt cells, regulating the guanylate cyclase activity in their basal-lateral plasma membranes exists. However, in mature villous cells, at least 70 per cent of the total

guanylate cyclase activity has been found in the microvillous structure of the brushborder, making a direct coupling to hormone receptors at the antiluminal plasma membranes highly unlikely [7]. Therefore, a possible role of cyclic GMP, generated by this enzyme, as a second messenger of hormone action must be seriously questioned.

The universal occurrence of specific cyclic GMP-dependent protein kinases in rat tissues and the high levels of these enzymes in arthropod tissues, where the particulate guanylate cylase is usually very active [31], strongly suggest that the biological effects of cyclic GMP are mediated by this class of proteins. In membranes from intestinal smooth muscle, and endogenous cyclic GMP-dependent protein kinase has been detected in association with two distinct substrate proteins [32]. It would be interesting to look for a similar system in the intestinal brushborder. Should the endogenous substrates be transport proteins, the slight stimulation of gunaylate cyclase activity in the brushborder by aminoacids (table 1) might be of physiological significance. The striking resemblance between the tubular structure of microvilli and flagellae [33], the high activity of guanylate cyclase in flagellar membranes from sea urchin sperm [27,34] and in platelets [35], and the possibility that the microfilaments within the core of the intestinal microvilli are actin-like [33,36], lend some support to the idea that cyclic GMP has a role in the assembly or regulation of contractile systems.

Acknowledgements

The author wishes to thank Professor Dr W. C. Hülsmann for advice and critical reading of the manuscript and Mr L. M. Scheek for expert technical assistance. Miss A. C. Hanson is thanked for typing the manuscript.

References

[1] Banwell, J. G. and Sherr, H. (1973) Gastroenterology 65, 467–497.
[2] Kimberg, D. V. (1974) Gastroenterology 67, 1023–1064.
[3] Schwartz, C. J., Kimberg, D. V., Sheerin, H. E., Field, M. and Said, S. I. (1974) J. Clin. Invest. 54, 536–544.
[4] Ishikawa, E., Ishikawa, S., Davis, J. W. and Sutherland, E. W. (1969) J. Biol. Chem. 244, 6371–6376.
[5] White, A. A. and Aurbach, G. D. (1969) Biochim. Biophys. Acta 191, 686–697.
[6] Kimura, H. and Murad, F. (1974) J. Biol. Chem. 249, 6910–6916.
[7] de Jonge, H. R. (1975) FEBS Lett., 53, 237–242.
[8] Chrisman, T. D., Garbers, D. L., Parks, M. A. and Hardman, J. G. (1975) J. Biol. Chem. 250, 374–381.
[9] Lemhoff, W. G., van den Berg, J. W. O., de Pijper, A. M. and Hülsmann, W. C. (1970) Biochim. Biophys. Acta 215, 229–241.
[10] Hülsmann, W. C., van den Berg, J. W. O. and de Jonge, H. R. (1974) in: Methods in Enzym. (Fleischer, S. and Packer, L. eds.) Academic Press, New York, Vol. XXXII, pp. 665–673.
[11] Harrison, D. D. and Webster, N. L. (1964) Biochim. Biophys. Acta 93, 662–664.
[12] de Jonge, H. R. (1975) Biochim. Biophys. Acta 381, 128–143.
[13] Smith, R. M. and Alberty, R. A. (1956) J. Amer. Soc. 78, 2376–2380.
[14] O'Sullivan, W. J. and Perrin, D. D. (1964) Biochemistry 3, 18–26.
[15] Dinnendahl, V. (1974) Naunyn-Schmiedebergs Arch. Pharm. 284, 55–61.
[16] Kuo, J. F. and Greengard, P. (1970) J. Biol. Chem. 245, 2493–2498.
[17] Tovey, K. C., Oldham, K. G. and Whelan, J. A. M. (1974) Clin. Chim. Acta 56, 221–234.
[18] Gilman, A. G. (1970) Proc. Natl. Acad. Sci. U.S. 67, 305–312.
[19] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[20] Parkinson, D. K., Ebel, H., DiBona, D. R. and Sharp, G. W. G. (1972) J. Clin. Invest. 51, 2292–2298.
[21] Hardman, J. G. and Sutherland, E. W. (1969) J. Biol. Chem. 244, 6363–6370.
[22] Cohen, K. L. and Bitensky, M. W. (1969) J. Pharm. Exp. Ther. 169, 80–86.
[23] Thompson, W. J., Williams, R. H. and Little, S. A. (1973) Biochim. Biophys. Acta 302, 329–337.
[24] Illiano, G., Tell, G. P. E., Siegel, M. I. and Cuatrecasas, P. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2443–2447.
[25] de Jonge, H. R. and Hülsmann, W. C. (1974) Biochem. Soc. Transact. 2, 416–419.
[26] Goldberg, N. D., O'Dea, R. F. and Haddox, M. K. (1973) in: Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G. A. eds.) Raven Press, New York, Vol. 3, pp. 155–223.
[27] Garbers, D. L., Hardman, J. G. and Rudolph, F. B. (1974) Biochemistry 13, 4166–4171.
[28] Van Sande, J., Decoster, C. and Dumont, J. E. (1975) Biochem. Biophys. Res. Commun. 62, 168–175.
[29] Ferrendelli, J. A., Rubin, E. H. and Kinscherf, D. A. (1975) Fed. Proc. 34, 231.
[30] Rudland, P. S., Gospodarowicz, D. and Seifert, W. (1974) Nature 250, 741–742 and 773.

[31] Kuo, J. F. (1974) Proc. Natl. Acad. Sci. U.S. 71, 4037–4041.
[32] Camellie, J. E. and Greengard, P. (1974) Proc. Natl. Acad. Sci. U.S. 71, 1891–1895.
[33] Millington, P. F. and Finean, J. B. (1962) J. Cell Biol. 14, 125–139.
[34] Gray, J. P. and Drummond, G. I. (1973) Proc. Can. Fed. Biol. Soc. 16, 79.
[35] Böhme, E., Jung, R. and Mechler, I. (1974) in: Methods in Enzym. (Hardman, J. G. and O'Malley, B. W. eds.) Academic Press, New York, Vol. XXXVIII, pp. 199–202.
[36] Tilney, L. G. and Mooseker, M. (1971) Proc. Natl. Acad. Sci. U.S. 68, 2611–2615.