Induction of Human Choriogonadotropin in HeLa-Cell Cultures by Aliphatic Monocarboxylates and Inhibitors of Deoxyribonucleic Acid Synthesis

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The ectopic production of the glycopeptide hormone human placental choriogonadotropin by HeLa₆₅ cells was measured by radioimmunoassay with antiserum against the β -subunit of choriogonadotropin and with the ¹²⁵I-labelled β -subunit as a tracer antigen. Choriogonadotropin synthesis was markedly (500-fold) stimulated by sodium butyrate. Kinetic studies and the use of an inhibitor of protein synthesis, cycloheximide, indicated that protein synthesis was required for this induction. Investigation of the efficiency of 22 aliphatic short-chain fatty acids and derivatives in causing increased choriogonadotropin synthesis by HeLa cells showed stringent structural requirements. Induction of choriogonadotropin synthesis in HeLa cells was not restricted to butyrate. Other aliphatic acids (propionate, isobutyrate, valerate and hexanoate) were also capable of inducing choriogonadotropin synthesis at 10–50% of the efficiency of butyrate. Hydroxy derivatives of monocarboxylate inducers, related mono- and di-carboxylic acids, alcohols, amines, ketones, esters and sulphoxide were ineffective in increasing choriogonadotropin production by HeLa cells. A saturated C₄ straight-chain acid without substituent hydroxyl groups but with a methyl group at one end and a carboxyl moiety at the other appeared to be most efficient in activating choriogonadotropin production. A second clonal line of HeLa cells, HeLa₇₁, showed a higher constitutive synthesis of choriogonadotropin than HeLa₆₅ cells, which was also markedly increased by butyrate. Butyrate and other aliphatic monocarboxylate inducers of choriogonadotropin synthesis inhibited HeLa-cell growth and DNA synthesis. This inhibition of DNA replication may be related to the mechanism of choriogonadotropin synthesis, since two well-characterized anti-neoplastic inhibitors of DNA synthesis, hydroxyurea and 1- β -D-arabinofuranosylcytosine, also stimulated a 300-fold increase in choriogonadotropin synthesis in HeLa cells and were synergistic with butyrate in promoting choriogonadotropin synthesis. Thus activation in tumour cells of genes normally expressed by foetal tissue and the consequent ectopic synthesis of polypeptide hormones may require neither cell division nor DNA synthesis.

The ectopic production of protein hormones by malignant tumours derived from non-endocrine tissues provides a system for investigating gene expression and for studying agents that modulate the synthesis of these hormones. The glycoprotein tropic hormones are heteropolymers composed of two dissimilar subunits, an α -subunit that is common to four of these hormones (follicle-stimulating hormone, luteinizing hormone, thyrotrophic hormone and choriogonadotropin), and a β -subunit that is unique to each hormone and confers biological specificity. In a preliminary report we described the production of human choriogonadotropin, measured by immunoreactivity for its β -chain, by HeLa cells, a tissue-culture line derived from a cancer of the uterine cervix, and showed that sodium butyrate markedly stimulated the production of this glycoprotein tropic peptide (Ghosh & Cox, 1976a). In the present study the kinetics, specificity and structure-

activity relation of the induction of choriogonadotropin synthesis by sodium butyrate was investigated by comparing this C₄ aliphatic monocarboxylate with other normal and substituted aliphatic acids of different chain length and with closely related organic compounds.

Differentiated gene expression in eukaryotic cells appears, in certain instances, to be associated with cessation of cell multiplication (Leder *et al.*, 1975). Butyrate inhibits HeLa-cell proliferation and decreases DNA synthesis. Since this inhibition of DNA synthesis may be related to the induction of increased choriogonadotropin production in HeLa cells, the effects of other well-characterized inhibitors of DNA synthesis were investigated. Hydroxyurea and 1- β -D-arabinofuranosylcytosine, two drugs that block the entry of cells into the S-phase of the cell cycle, induced increased choriogonadotropin production by HeLa

cells, and both agents acted synergistically with butyrate to promote even greater choriogonadotropin synthesis. This work has been presented, in part, at the 10th International Congress of Biochemistry, Hamburg (Ghosh & Cox, 1976b), and the American Society of Biological Chemists, 61st Annual National Meeting, Federation of American Societies of Experimental Biology, Chicago (Ghosh *et al.*, 1977).

Experimental

Materials

Aliphatic acids and their derivatives were of the highest purity available commercially and were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., Fisher Scientific Co., Springfield, NJ, U.S.A. and Eastman Kodak Co., Rochester, NY, U.S.A. Hydroxyurea was obtained from Fisher Scientific Co., and 1- β -D-arabinofuranosylcytosine was purchased from Upjohn, Kalamazoo, MI, U.S.A. Free mono- and di-carboxylic acids and their hydroxy derivatives were neutralized with stoichiometric amounts of NaOH to prepare the corresponding sodium salts. Solutions were sterilized by filtration through 0.45 μ m Millipore filters.

Cell culture

Two clonal lines of HeLa cells, HeLa₆₅ and HeLa₇₁, with modal chromosome numbers of 65 and 71 respectively were grown in monolayer cultures in Waymouth's media (Waymouth, 1959) containing foetal calf serum (10%, v/v), glucose (5 g/litre) and antibiotics (penicillin, 30 μ g/ml, streptomycin, 50 μ g/ml, and kanamycin, 30 μ g/ml). Fatty acids and inhibitors of DNA synthesis were added to the complete medium. All tissue-culture media and materials were purchased from Grand Island Biological Co., Grand Island, NY, U.S.A. The methods of maintaining cultures and subculturing have been previously described (Cox *et al.*, 1971). In all experiments replicate cultures were inoculated with equal numbers of cells, and incubated overnight to permit cell attachment and initiation of growth before aliphatic acids or derivatives were added to the medium. The medium was not replenished during cell growth. For determination of choriogonadotropin production, the medium was decanted, freed from suspended cells by centrifugation at 1200g for 15 min and stored at -20°C until assayed. The cells were removed from the glass surfaces with 0.02% (w/v) EDTA and 0.05% (w/v) trypsin in Puck's saline A (Marcus *et al.*, 1956). The cells were counted in a Neubauer haemocytometer, washed twice by centrifuging at 1200g for 15 min in 0.15M-NaCl and frozen as a cell pellet at -20°C. Before assay the

thawed cells were suspended in 2 ml of 0.15M-NaCl with an operating frequency of 20 kHz for 30 s and disrupted by ultrasonication in a model W185 Sonifier Cell Disrupter (Heat System-Ultrasonics, Plainview, NY, U.S.A.), with a micro-tip [0.3 cm $\times$ 14 cm ($\frac{1}{8}$ in $\times$ 5.5 in)].

Radioimmunoassay for choriogonadotropin

Choriogonadotropin was measured by radioimmunoassay (Midgley, 1966; Vaitukaitis *et al.*, 1972) by using antiserum against the β -subunit to control for cross-reactivities with free α -chains and other glycoprotein hormones, particularly lutropin. The antiserum, purchased from the Institute of Bio-Endocrinology, Montreal, Que., Canada, was prepared by immunizing rabbit with purified β -subunit of choriogonadotropin. The cross-reactivities of this antiserum with human polypeptides were 0.01% for albumin, <2% with follitropin, <5% with lutropin, <0.1% with thyrotropin and <0.1% with growth hormone (somatotropin). The antiserum did not cross-react with human α -chains and was capable of measuring in culture medium 0.6 ng of choriogonadotropin/ml in the presence of 1078 ng of free- α -chain equivalents for glycoprotein tropic hormones/ml (J. M. Lieblisch, personal communication). The antiserum used in this study at dilutions 1:12000 produced 30–50% binding of 40 pg of ¹²⁵I-labelled β -chain of human choriogonadotropin (specific radioactivity 200 μ Ci/ μ g), prepared by the chloramine-T method and purified on polyacrylamide gel. The antiserum was also calibrated against complete choriogonadotropin by radioimmunoassay by using the 2nd International Standard of human choriogonadotropin at seven dilutions (0–50 m-i.u./ml). In each experiment the above standard in duplicate at seven dilutions and a human serum with known concentrations of choriogonadotropin were assayed concurrently with unknowns to ensure accuracy and reproducibility of the technique. The coefficient of variation in the β -subunit assay was less than 15%. The sensitivity of the assay with the β -subunit antiserum was 0.15 m-i.u. as determined by the concentration of antiserum that decreased maximal binding of tracer antigen (¹²⁵I-labelled β -chain) by 5%. The average recovery of known amounts of complete choriogonadotropin added to culture medium was 99.6%.

Unless otherwise specified antigen-antibody complexes were precipitated with antiserum against rabbit γ -globulin purchased from the Institute of Bio-Endocrinology. The radioactive precipitate was collected by centrifugation at 5000g in a Sorvall RC2-B refrigerated (2°C) centrifuge and counted for radioactivity in a Nuclear-Chicago Gamma-Counter. The concentration of choriogonadotropin in ultrasonicated preparations of HeLa cells and in media was calculated from standard calibration curves made by

plotting percentages of binding of ^{125}I -labelled β -subunits against corresponding concentrations of unlabelled highly purified choriogonadotropin in semilogarithmic or logit-log scales. All assays were carried out in duplicates.

Assay for protein

Total cell protein was measured in ultrasonicated HeLa-cell suspensions diluted 1:50 (v/v) in 0.15M-NaCl by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Incorporation of $[^{14}\text{C}]$ thymidine

DNA synthesis by HeLa-cell cultures was determined by incubating cells for 5 h in complete Way-

mouth's medium containing 0.1 μCi of $[\text{Me-}^{14}\text{C}]$ -thymidine/ml (specific radioactivity 53.5 mCi/mmol; New England Nuclear Corp., Boston, MA, U.S.A.). Cultures were washed with $3 \times 15 \text{ ml}$ of 0.15M-NaCl and lysed with 0.5% deoxycholate. Cell counts were made on trypsin-treated cell suspensions from replicate cultures. A 1 ml portion of the deoxycholate lysate was treated with 4 ml of 5% (w/v) trichloroacetic acid at 5°C. The acid-insoluble precipitate was separated from the supernatant by centrifugation at 1100g. The acid-insoluble material was digested in 0.2 ml of 4M-NaOH at 60°C for 2 h. The digest was added to 15 ml of Hyamine scintillant (Dancis *et al.*, 1973), and counted for radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer, model 2420. Protein content of the deoxycholate lysate was also determined by the method of Lowry *et al.* (1951).

Fig. 1. Kinetics of choriogonadotropin induction by sodium butyrate and its inhibition by cycloheximide. Human choriogonadotropin was measured by radioimmunoassay by using antiserum directed against the β -chain. (a) Intracellular choriogonadotropin concentrations (m.i.u./10⁶ cells): ○, cells grown without butyrate (controls); △, cells grown with 1 mM-sodium butyrate; □, cells grown with 1 mM-sodium butyrate for 48 h and 1 μg of cycloheximide/ml for the subsequent 24 h. The arrow indicates the time of addition of cycloheximide. (b) Extracellular choriogonadotropin concentrations (m.i.u./10⁶ cells): ●, secreted into medium by cells grown without butyrate (controls); △, secreted into medium by cells grown with 1 mM-sodium butyrate; ■, secreted into medium by cells grown with 1 mM-sodium butyrate for 48 h and 1 μg of cycloheximide/ml for the next 24 h. The arrow shows the time of cycloheximide addition. Choriogonadotropin concentrations were measured by the poly(ethylene glycol) method, by using the latter in place of anti-(γ -globulin) antibody as a precipitant for ^{125}I -labelled antigen-antibody complexes. Poly(ethylene glycol), antiserum against choriogonadotropin β -subunit and other materials for radioimmunoassay of choriogonadotropin were purchased from Sero Laboratories, Boston, MA, U.S.A.

[^{14}C]Thymidine incorporation was expressed as c.p.m./ 10^6 cells.

Results

Kinetics of choriogonadotropin induction by HeLa cells grown with and without sodium butyrate

As shown in Fig. 1, the concentration of choriogonadotropin in cultures grown without added butyrate varies depending on the duration of growth and confluence of the culture. Cells 18h after subculturing from confluent cultures (zero time) showed relatively high concentrations of choriogonadotropin, intracellularly and in the medium. As control cultures entered the exponential growth phase, the concentration of choriogonadotropin fell and then rose again as the cells became confluent. Differences in concentration between control cultures and cells grown with sodium butyrate in different experiments were largely the result of variation in the control values of choriogonadotropin, which depended on the rate of proliferation of the culture as regulated by the duration of growth and number of cells in the initial inoculum of the culture.

Fig. 1 also shows the effects of growth in medium containing 1 mM-sodium butyrate on choriogonado-

tropin production by HeLa cells. In contrast with control cultures, the initial intracellular and extracellular choriogonadotropin concentrations were maintained in butyrate-treated cells for the first 48h and then increased dramatically. Addition of cycloheximide, an inhibitor of protein synthesis, at 48h inhibited the subsequent increase in choriogonadotropin mediated by sodium butyrate, as shown in Fig. 1. Inhibition of protein synthesis in HeLa₆₅-cell cultures by incubation with 1.0 μg of cycloheximide/ml for 24h was previously shown to be reversible (Cox *et al.*, 1971).

Within an experiment there was some variation in intracellular and extracellular concentrations of choriogonadotropin between replicate cultures; however, these differences were small compared with the marked statistically significant effect of sodium butyrate on choriogonadotropin production (Table 1).

Effect of chain length of monocarboxylates on choriogonadotropin production

Fig. 2(a) shows the intracellular and Fig. 2(b) the extracellular concentrations of choriogonadotropin in HeLa cells grown for 120h in media containing 1 mM and 5 mM concentrations of sodium salts of

Table 1. *Choriogonadotropin concentrations in HeLa-cell cultures grown in the absence and presence of 1 mM-sodium butyrate*
Choriogonadotropin concentration was measured by the poly(ethylene glycol) method, which uses poly(ethylene glycol) to precipitate radiolabelled antigen-antibody complexes. The results with this technique are similar to those obtained by precipitation with anti-(γ -globulin) as described in the Experimental section. Poly(ethylene glycol) and other materials for radioimmunoassay of choriogonadotropin were obtained from Sero Laboratories. The test of significance (t) and probability (P) are given for the difference between Mean A and Mean B. Numbers 1-6 are replicate cultures grown without sodium butyrate for 144h and 7-12 are cultured in the presence of 1 mM-sodium butyrate for the same time.

Replicate HeLa-cell cultures	Choriogonadotropin concentration		
	Intracellular		Extracellular (m-i.u. secreted by 10^6 cells)
	(m-i.u./ 10^6 cells)	(m-i.u./mg of protein)	
1	0.18	0.60	5.7
2	0.18	0.58	5.4
3	0.21	0.76	5.0
4	0.17	0.61	5.3
5	0.16	0.43	5.4
6	0.16	0.50	9.5
Mean A (for 1-6) $\pm$ S.D.	0.18 ± 0.02	0.58 ± 0.11	6.1 ± 1.7
7	1.9	5.80	29.5
8	2.7	10.33	37.2
9	2.0	6.07	58.9
10	1.54	4.81	50.5
11	1.24	3.11	51.4
12	1.08	3.00	43.0
Mean B (for 7-12) $\pm$ S.D.	1.74 ± 0.59	5.52 ± 2.6	45.1 ± 10.7
t (Mean B versus Mean A)	6.5	4.7	8.8
P (Mean B versus Mean A)	0.001	0.001	0.001

Fig. 2. *Choriogonadotropin induction by aliphatic monocarboxylates of different chain length*
Choriogonadotropin was measured by radioimmunoassay by using antiserum directed against the β -chain. (a) Intracellular choriogonadotropin concentrations induced by (○) 1 mM- and (△) 5 mM-monocarboxylates. (b) Extracellular concentrations of choriogonadotropin secreted into media in response to (●) 1 mM- and (▲) 5 mM-monocarboxylates.

straight-chain monocarboxylic acids with different numbers of carbon atoms. Butyrate (C_4) was by far the most effective inducer of choriogonadotropin synthesis. A 500-fold induction was observed when HeLa cells were grown in the presence of 5 mM-sodium butyrate for 120 h. However, choriogonadotropin induction was not restricted to butyrate. Propionate (C_3), valerate (C_5) and hexanoate (C_6) also increased choriogonadotropin synthesis, but were much less effective than butyrate (Fig. 2). When choriogonadotropin concentrations were expressed per mg of cell protein, the extent of induction was similar (results not shown).

Effect of normal and substituted mono- and dicarboxylates and related organic compounds on choriogonadotropin production

Table 2 shows the structural specificity for the increased synthesis of choriogonadotropin in HeLa-cell cultures by short-chain organic compounds. The C_4 straight-chain acid butyrate was the most effective inducer. Replacement of the terminal methyl group of butyrate by an aromatic phenyl ring or conversion of the straight-chain butyrate into the branched-chain isobutyrate markedly decreased the induction of choriogonadotropin. The substitution of a terminal

amino group or the introduction of a double bond between the second and third carbon atom abolished the capacity of the C_4 monocarboxylates to cause increased choriogonadotropin production. Short-chain dicarboxylates, alcohols, amines, ketones and esters were ineffective as inducers of choriogonadotropin (Table 2). Dimethyl sulphoxide, a well-characterized effector of erythroid differentiation in mouse leukaemia cells (Leder & Leder, 1975; Tanaka *et al.*, 1975), was not an inducer at 1 mM and caused only a slight (1.8-fold) increase in choriogonadotropin production by HeLa cells at concentrations as high as 286 mM.

Table 2 shows that the introduction of hydroxyl groups into C_3 , C_4 , C_5 and C_6 monocarboxylates abolishes their ability to induce choriogonadotropin in HeLa-cell cultures and further emphasizes the structural specificity of the straight-chain aliphatic acids in mediating these effects. There was no apparent competition between hydroxylated aliphatic acids and butyrate, as was shown by the inability of β -hydroxybutyrate to inhibit butyrate-mediated induction when both aliphatic monocarboxylates were present in the medium. As shown in Table 2, those compounds that inhibit HeLa-cell growth were effective in promoting choriogonadotropin production. The relative degree of induction of choriogonadotropin correlated fairly

Table 2. *Effects of various organic compounds on choriogonadotropin production by HeLa₆₅ cells*

Cells were grown for 120h in medium supplemented with the appropriate compound as described in the Experimental section.

Compound	Molecular structure	Concentration (mM)	Inhibition of cell growth (%)	Choriogonadotropin concentration (m-i.u./10 ⁶ cells)			
				Intracellular		Extracellular*	
				Amount	Proportional increase	Amount	Proportional increase
None (control)	—	—	—	0.05	—	2.5	—
Monocarboxylates and substituted derivatives:							
Propionate	CH ₃ -CH ₂ -CO ₂ Na	1	0	0.12	2.4	5.5	2.2
		5	28	1.08	21.6	45.0	17.8
Lactate	CH ₃ -CH(OH)-CO ₂ Na	1	0	0.01	0.2	2.5	1.0
		5	0	0.01	0.2	3.3	1.3
α-Methyl-lactate	CH ₃ -C(CH ₃)(OH)-CO ₂ Na	1	6	0.05	1.0	2.0	0.8
		5	8	0.04	0.8	3.3	1.3
Butyrate	CH ₃ -CH ₂ -CH ₂ -CO ₂ Na	1	54	1.08	21.6	55.6	21.9
		5	83	9.87	197.0	424.0	167.0
β-Hydroxybutyrate	CH ₃ -CH(OH)-CH ₂ -CO ₂ Na	1	0	0.04	0.8	2.7	1.1
		5	8	0.06	1.2	3.4	1.3
γ-Hydroxybutyrate	CH ₂ (OH)-CH ₂ -CH ₂ -CO ₂ Na	1	†	0.04	0.8	3.2	1.3
		5	0	0.02	0.4	2.2	0.9
Phenylpropionate	C ₆ H ₅ -CH ₂ -CH ₂ -CO ₂ Na	1	0	0.03	0.6	12.6	5.0
Isobutyrate	CH ₃ -CH(CH ₃)-CO ₂ Na	1	2	0.25	5.0	17.2	6.6
Crotonate	CH ₃ -CH=CH-CO ₂ Na	1	1	‡	‡	1.8	0.7
γ-Aminobutyrate	NH ₂ -CH ₂ -CH ₂ -CH ₂ -CO ₂ Na	1	†	0.03	0.6	1.4	0.5
Valerate	CH ₃ -[CH ₂] ₃ -CO ₂ Na	1	0	0.19	3.8	16.5	6.5
		5	14	2.38	47.6	238.0	94.1
α-Hydroxyvalerate	CH ₃ -[CH ₂] ₂ -CH(OH)-CO ₂ Na	1	†	0.05	1.0	3.6	1.4
		5	6	0.02	0.4	2.3	0.9
Hexanoate	CH ₃ -[CH ₂] ₄ -CO ₂ Na	1	6	0.04	0.8	1.4	0.6
		5	46	0.10	2.0	63.7	25.5
α-Hydroxyhexanoate	CH ₃ -[CH ₂] ₃ -CH(OH)-CO ₂ Na	1	0	0.04	0.8	1.3	0.5
		5	†	0.04	0.8	1.5	0.6
Dicarboxylates:							
Succinate	NaO ₂ C-CH ₂ -CH ₂ -CO ₂ Na	1	0	0.02	0.4	2.5	1.0
Adipate	NaO ₂ C-[CH ₂] ₄ -CO ₂ Na	1	0	0.01	0.2	1.4	0.5
Alcohols:							
Butanol	CH ₃ -CH ₂ -CH ₂ -CH ₂ -OH	1	0	0.02	0.4	2.0	1.0
Crotyl alcohol	CH ₃ -CH=CH-CH ₂ -OH	1	1	0.03	0.6	3.0	1.2
Amines:							
Butylamine	CH ₃ -CH ₂ -CH ₂ -CH ₂ -NH ₂	1	1	0.03	0.6	1.8	0.7
Putrescine	H ₂ N-CH ₂ -CH ₂ -CH ₂ -CH ₂ -NH ₂	1	0	0.02	0.4	1.8	0.7
Ketone:							
Ethyl methyl ketone	CH ₃ -CO-CH ₂ -CH ₃	1	1	0.01	0.2	1.6	0.6
Ester:							
Butyl acetate	CH ₃ -CO ₂ CH ₂ -[CH ₂] ₂ -CH ₃	1	0	0.01	0.2	0.9	0.3
Sulphoxide:							
Dimethyl sulphoxide§	(CH ₃) ₂ SO	1	0	0.01	0.2	1.20	0.4

* Secreted into media.

† Inhibition of cell growth could not be accurately quantified in these bottles because of marked variations in cell counts in control cultures.

‡ Cell pellets were lost so that intracellular choriogonadotropin could not be assayed.

§ Dimethyl sulphoxide at concentrations of 286mM increased HeLa-cell choriogonadotropin production only 1.8-fold.

well with the inhibition of cell growth by aliphatic monocarboxylates. However, simply inhibiting HeLa-cell growth did not promote choriogonadotropin

synthesis. For example, 5mM-heptanoate inhibited HeLa-cell growth by 70%, prednisolone (3μM) inhibited growth of HeLa₆₅-cell monolayer cultures

by 40% and dimethyl sulphoxide at high concentration (286mM) inhibited HeLa-cell proliferation by 70%, but none of these compounds induced significant choriogonadotropin synthesis by HeLa₆₅ cells. All of the aliphatic monocarboxylates that stimulate choriogonadotropin synthesis also caused the cells to assume a spindle or fibroblastic morphology (Deutsch *et al.*, 1976b). Those short-chain fatty acids that did not inhibit cell growth are ineffective in mediating increased choriogonadotropin production and did not alter cellular morphology.

Choriogonadotropin production in two clonal lines of HeLa cells

HeLa₇₁ cells produce higher concentrations of choriogonadotropin constitutively (0.10m-i.u./10⁶ cells intracellularly and 12.0m-i.u./10⁶ cells secreted into media) than HeLa₆₅ cells. When grown in the

presence of 1mM-sodium butyrate for 96h, the intracellular concentration of choriogonadotropin rose to 3.4 and that in the medium to 436m-i.u./10⁶ cells. It was noteworthy that the growth of HeLa₇₁ cells was more inhibited by sodium butyrate than was that of HeLa₆₅ cells. This further supports the concept that decreased cell multiplication may be necessary for increased synthesis of this glycopeptide hormone. The validity of this concept was supported by studies with inhibitors of DNA synthesis (see below).

Induction of chorionogonadotropin synthesis in HeLa cells by inhibitors of DNA replication

Fig. 3 shows that hydroxyurea, a specific inhibitor of DNA synthesis at concentrations above 0.25mM, was a potent mediator of choriogonadotropin induction. Moreover, the concentrations of hydroxyurea

Fig. 3. Induction of choriogonadotropin by hydroxyurea and its synergism with sodium butyrate. Choriogonadotropin was measured by radioimmunoassay by using antiserum directed against the β -subunit. (a) Intracellular; (b) extracellular. ○, Cells grown with different concentrations of hydroxyurea for 96h; △, cells grown with different concentrations of hydroxyurea and 1mM-sodium butyrate for 96h.

Table 3. *Induction of human choriogonadotropin in HeLa₆₅ cells by 1-β-D-arabinofuranosylcytosine (Ara-C) and its synergism with sodium butyrate*

1-β-D-Arabinofuranosylcytosine at the final concentrations indicated were added daily to cultures for 3 successive days. It increases the activity of nucleoside deaminase, which accelerates its degradation in HeLa-cell cultures (Meyers *et al.*, 1973).

Additions to medium	Duration of growth (h)	Inhibition of growth (%)	Choriogonadotropin concentration (m.i.u./10 ⁶ cells) in HeLa ₆₅ cells			
			Intracellular		Extracellular*	
			Amounts	Proportional increase	Amounts	Proportional increase
Control (none)	120	—	0.02	—	2.3	—
Ara-C (4 μM)†	120	17	0.16	8	4.1	2
Butyrate (1 mM)†	120	63	2.20	110	55.1	24
Ara-C (4 μM) + butyrate (1 mM)†	120	79	4.50	225	92.5	40
Ara-C (8 μM)	96	36	2.92	146	20.0	9
Ara-C (20 μM)	96	71	6.04	302	80.0	35

* Secreted into media.

† Compounds were added to medium at the beginning of the experiment only.

Table 4. *Effect of hydroxyurea, 1-β-D-arabinofuranosylcytosine (Ara-C) and sodium butyrate on the incorporation of [¹⁴C]thymidine into HeLa₆₅ cells*

In experiments 1, 2 and 3, incubation with [¹⁴C]thymidine was for 5 h, and in experiment 4 it was for 2 h.

Expt. no.	Additions to culture media	Duration of incubation (h)	[¹⁴ C]Thymidine incorporated into acid-insoluble material (c.p.m./10 ⁶ cells)	Inhibition of [¹⁴ C]thymidine incorporation (%)
1	Control (none)	—	14882	—
	Hydroxyurea (1 mM)	5.5	6922	53
	Hydroxyurea (5 mM)	5.5	4197	72
	Hydroxyurea (5 mM) + butyrate (5 mM)	5.5	3552	76
2	Control (none)	—	9614	—
	Ara-C (4 μM)	5.5	4347	55
	Ara-C (40 μM)	5.5	3813	60
3	Control (none)	—	25277	—
	Butyrate (1 mM)	5.5	23413	7
	Butyrate (10 mM)	5.5	19616	22
4	Control (none)	—	10831	—
	Butyrate (1 mM)	29.0	7584	30
	Butyrate (10 mM)	29.0	2472	77

that were ineffective by themselves in promoting choriogonadotropin synthesis can potentiate the effects of sodium butyrate. Hydroxyurea is evidently a more effective inhibitor of cell growth and is also more potent than equimolar amounts of butyrate in promoting choriogonadotropin synthesis by HeLa₆₅ cells. Table 3 shows that, like hydroxyurea, another inhibitor of DNA synthesis, 1-β-D-arabinofuranosylcytosine, also produced 300-fold induction of choriogonadotropin synthesis by HeLa-cell cultures. 1-β-D-Arabinofuranosylcytosine also exhibited a

synergistic effect on sodium butyrate-mediated choriogonadotropin induction. Differences in the degree of induction with butyrate and the inhibitors of DNA synthesis depend on the concentrations used and the duration of incubation with the drugs. In general there appeared to be an approximate correlation between the concentration of choriogonadotropin produced and the degree of inhibition of cell growth. Moreover, both inhibitors of DNA synthesis used in this study caused HeLa₆₅ cells to assume a spindle or fibroblastic morphology.

Inhibition of DNA synthesis by drugs used for induction of HeLa-cell choriogonadotropin

Table 4 shows the inhibition of [^{14}C]thymidine incorporation into the acid-insoluble fraction of HeLa₆₅ cells produced by hydroxyurea, 1- β -D-arabinofuranosylcytosine and sodium butyrate used in concentrations that induce choriogonadotropin synthesis. The effects of higher concentrations of each of the drugs on DNA synthesis were also studied, since the length of incubation with these inhibitors of [^{14}C]thymidine incorporation was only 5 or 29 h compared with 48–120 h for the studies used on choriogonadotropin induction. All compounds studied inhibited DNA synthesis and cell growth, as noted during the studies on induction of choriogonadotropin synthesis.

Discussion

The use of simple chemical compounds that modulate the synthesis of proteins or polypeptides by cells provides a system for investigating the regulation of gene expression. Short-chain aliphatic acids such as butyrate alter the morphology of certain cells and induce the production of specific proteins and enzymes (Ginsburg *et al.*, 1973; Wright, 1973; Ghosh *et al.*, 1975; Deutsch *et al.*, 1976b). Erythroleukaemia cells differentiate when incubated with sodium butyrate at concentrations less than one-hundredth of that required for induction of haemoglobin synthesis by dimethyl sulphoxide (Leder & Leder, 1975; Tanaka *et al.*, 1975). In HeLa cells this simple C₄ acid mediates an increase in the activity of several membrane-associated enzymes, e.g. sialyltransferase I (Fishman *et al.*, 1974; Simmons *et al.*, 1975), alkaline phosphatase (Griffin *et al.*, 1974; Ghosh *et al.*, 1975; Deutsch *et al.*, 1976b) and 5'-nucleotidase (Deutsch *et al.*, 1976b), without affecting the activity of other enzymes putatively localized in membranes, mitochondria, lysosomes and cytosol. Butyrate also induces a dose-dependent increase in the production and secretion of the glycoprotein tropic hormone choriogonadotropin by HeLa cells (Ghosh & Cox, 1976a).

In the present study a sensitive radioimmunoassay for the β -subunit of choriogonadotropin, capable of detecting choriogonadotropin in the presence of an excess of free α -chains, was used to investigate the kinetics, structural requirements and specificity of butyrate-mediated induction of choriogonadotropin. The salt of the simple C₄ acid, butyrate, was by far the most effective inducer of choriogonadotropin synthesis. Shortening or lengthening the carbon chain by one carbon atom decreases choriogonadotropin production by 50–90%. A free terminal carboxyl group is required for optimum induction, since the introduction of a terminal amine, alcohol or ester

group abolishes the inducing activity. Other alterations of the aliphatic acids, including the replacement of hydrogen atoms by hydroxyl groups at the α , β and γ positions, the introduction of a double bond and branched-structure substitutions on other carbon atoms, also render them ineffective in increasing choriogonadotropin synthesis. A straight-chain C₄-monocarboxylate structure with a methyl group at one end and a carboxyl moiety at the other seems to be required for induction, since succinic acid, in which the terminal methyl group is replaced by a carboxyl group, is incapable of inducing choriogonadotropin in HeLa cells.

Leder & Leder (1975) have reported similar structural requirements for monocarboxylate induction of haemoglobin synthesis in mouse leukaemia cells. Simmons *et al.* (1975) have shown that the induction of sialyltransferase I in HeLa cells by short-chain fatty acids also exhibits similar structural specificities.

The monocarboxylic acids may produce their effects by binding to specific proteins through a nucleophilic attachment of the carboxyl group to an electron-poor positively charged site on the protein molecule. This chemical interaction may lead to inhibition of cell growth and de-repression of foetal genes in HeLa cells. It is noteworthy that those monocarboxylates that alter HeLa-cell morphology, inhibit their growth and induce alkaline phosphatase are also those that mediate increased choriogonadotropin synthesis. The action of butyrate seems to be on several genetic loci, since preliminary studies indicate that the synthesis of other glycoprotein tropic hormones, for example, lutropin and follitropin, also may be induced in HeLa cells by butyrate (N. K. Ghosh & R. P. Cox, unpublished work).

The mechanism by which butyrate alters gene expression in HeLa cells is not known. The findings that anti-neoplastic drugs also activate choriogonadotropin synthesis suggest that the inhibition of DNA synthesis or cell multiplication may play a role in modulation of gene expression. Little is known about the mechanism by which specialized synthetic programmes are coupled to inhibition of DNA replication. However, the acquisition of a differentiated phenotype in presumptive muscle cells is preceded by the cessation of cell proliferation (Holtzer *et al.*, 1972), and there are other similar examples (Holtzer, 1970). Butyrate and several anti-neoplastic drugs inhibit DNA synthesis and growth in HeLa-cell cultures with an associated alteration of cell shape, increase in the activity of several enzymes and an enhanced synthesis of glycopeptide tropic hormones. In HeLa cells the expression of two genes that are normally active in foetal life are altered by butyrate and by anti-neoplastic drugs, with an increase in the activity of the placental form of alkaline phosphatase (Deutsch *et al.*, 1976a) and an enhanced synthesis

of choriogonadotropin. The ectopic synthesis of trophoblastic proteins by neoplastic cells may require neither cell division nor DNA replication. These findings may have important implications for studies on neoplasia.

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References

- Cox, R. P., Elson, N. A., Tu, S. H. & Griffin, M. J. (1971) *J. Mol. Biol.* **58**, 197–215
- Dancis, J., Hutzler, J. & Cox, R. P. (1973) in *Tissue Culture: Methods and Applications* (Kruse, P. E. & Patterson, J. M. K., eds.), pp. 639–643, Academic Press, New York
- Deutsch, S. I., Ghosh, N. K. & Cox, R. P. (1976a) *Clin. Res.* **24**, 641A
- Deutsch, S. I., Silvers, D. N., Cox, R. P., Griffin, M. J. & Ghosh, N. K. (1976b) *J. Cell Sci.* **21**, 391–406
- Fishman, P. H., Simmons, J. L., Brady, R. O. & Freese, E. (1974) *Biochem. Biophys. Res. Commun.* **59**, 292–299
- Ghosh, N. K. & Cox, R. P. (1976a) *Nature (London)* **259**, 416–417
- Ghosh, N. K. & Cox, R. P. (1976b) *Abstr. Int. Congr. Biochem.* **10th**, p. 536
- Ghosh, N. K., Deutsch, S. I., Griffin, M. J. & Cox, R. P. (1975) *J. Cell. Physiol.* **86**, 663–672
- Ghosh, N. K., Rukenstein, A. & Cox, R. P. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 910
- Ginsburg, E., Solomon, D., Sreevalsan, T. & Freese, E. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2457–2461
- Griffin, M. J., Price, G. H., Bazzell, K. L., Cox, R. P. & Ghosh, N. K. (1974) *Arch. Biochem. Biophys.* **164**, 619–623
- Holtzer, H. (1970) in *Cell Differentiation* (Schjeide, O. & De Villis, J., eds.), pp. 476–493, Van Nostrand-Reinhold, Princeton, NJ
- Holtzer, H., Weintraub, H., Mayne, R. & Mochan, B. (1972) *Curr. Top. Dev. Biol.* **7**, 229–256
- Leder, A. & Leder, P. (1975) *Cell* **5**, 319–322
- Leder, A., Orkin, S. & Leder, P. (1975) *Science* **190**, 893–894
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Marcus, P. I., Cieciora, S. J. & Puck, T. T. (1956) *J. Exp. Med.* **104**, 615–624
- Meyers, R., Malathi, V. G., Cox, R. P. & Silber, R. (1973) *J. Biol. Chem.* **248**, 5909–5913
- Midgley, A. R. (1966) *Endocrinology* **79**, 10–18
- Simmons, J. L., Fishman, P. H., Freese, E. & Brady, R. O. (1975) *J. Cell Biol.* **66**, 414–424
- Tanaka, M., Levy, J., Tereda, M., Breslow, R., Rifkind, R. & Marks, P. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1003–1006
- Vaitukaitis, J. L., Braunstein, G. D. & Ross, G. T. (1972) *Am. J. Obstet. Gynecol.* **113**, 751–758
- Waymouth, C. (1959) *J. Natl. Cancer Inst.* **28**, 1003–1007
- Wright, J. A. (1973) *Exp. Cell Res.* **78**, 456–460