CHARACTERIZATION OF CYSTATHIONINE SYNTHASE AS A SELECTABLE, LIVER-SPECIFIC TRAIT IN RAT HEPATOMAS

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SUMMARY

Cell growth using homocysteine as a source of cysteine-sulphur requires two enzymes, cystathionine synthase (CS) and γ-cystathionase (CT). The second of these enzymes, CT, is apparently present in most cell lines regardless of their tissues of origin, since most cells can grow in vitro in the absence of cystine if they are provided with cystathionine, the intermediate in the pathway. Likewise, homocysteine will support the growth of many human cells. However, of a wide range of rodent cells, only well-differentiated rat hepatoma cells were found to grow using homocysteine in place of cystine. It is shown that cell growth in homocysteine-medium correlates well with the presence in the cells of detectable levels of CS. Furthermore, in cells able to grow in homocysteine-medium, it is possible to demonstrate the homocysteine-dependent trans-sulphuration of serine to cysteine. Growth in homocysteine-medium is not dependent on the release of preformed cysteine from disulphide complexes with serum proteins. In cell hybrids, and in 'dedifferentiated' variants of rat hepatomas, CS, but not CT, is subject to extinction co-ordinately with well-characterized liver-specific traits. For rodent cells, homocysteine-medium thus acts as a selective medium requiring the expression of a single liver-specific trait, CS. In addition it is shown that, in certain hepatoma variants, CS is regulated co-ordinately with a urea-cycle enzyme (carbamoyl phosphate synthetase I) by glucocorticoids and cyclic-AMP.

Cell death through cysteine starvation is briefly considered. The immediate cause of death is apparently an insufficient supply of reduced glutathione. Selenium and vitamin E assist cell growth when the supply of cysteine is limiting.

INTRODUCTION

Well-differentiated hepatomas can synthesize certain nutrients that are normally regarded as essential components of tissue-culture medium. This has provided a number of useful systems for the selective culture of cells expressing liver-specific traits. Hepatomas have been grown in the absence of glucose (Bertolotti, 1977), and in the absence of tyrosine (Choo et al. 1976; Haggerty et al. 1975), and in medium containing ornithine in place of arginine (Niwa et al. 1979; Widman et al. 1979). This paper describes an investigation of the ability of hepatomas and other cells to grow using homocysteine in place of cystine. Cysteine can be produced from serine, the sulphur being derived from homocysteine. This trans-sulphuration proceeds via cystathionine and is catalysed by two enzymes, cystathionine synthase (CS) and γ-cystathionase (CT) (Fig. 1). In the rat, these two enzymes are found predominantly in the liver, pancreas and kidney (Mudd et al. 1965). Naylor et al. (1976) showed that a range of mouse and rat cells could grow using cystathionine in place of cystine,

Key words: hepatoma, differentiation, selective growth, homocysteine, cystathionine synthase.
Fig. 1. The production of cysteine by the trans-sulphuration of serine. Enzymes: CS, cystathionine synthase (EC 4.2.1.21); CT, γ-cystathionase (EC 4.4.1.1).}

which suggests the tissue-specific restriction of CT is not maintained in vitro. However, none of these cells (including HTC, a rat hepatoma with limited expression of liver-specific traits) could grow using homocystine. It would seem that a study of sulphur-amino acid metabolism in well-differentiated hepatomas might cast light on the regulation of CS, and possibly provide a new selective medium for a liver-specific trait. To characterize its expression further, CS has also been measured in 'dedifferentiated' hepatomas and in hybrids made by fusing a rat hepatoma to a mouse fibroblast cell line. A better understanding of the regulation of CS is desirable in view of the effects of CS deficiency seen in homocystinuria (Gerritsen et al. 1962; Carson et al. 1963), and in view of our incomplete understanding of the mechanisms responsible for tissue-specific gene expression.

MATERIALS AND METHODS

Cells

The species and tissues of origin of the cells are listed in Table 1. Most of the cell lines are described fully elsewhere: φ5, Fu5-TG1, Arg-6D (Goss, 1984a); 7777 (Morris & Wagner, 1968); Ad-1, 14, H15 (Goss, 1984b); HTC (Thompson et al. 1966); Y3.Ag 1.2.3 (Galfré et al. 1979); NRK TG3/B7 Tc4 (Marshall, 1980); L6-CAP, a chloramphenicol-resistant derivative made in this laboratory from L6 (Yaffe, 1968); Hunt's tumour, a fibrosarcoma isolated in this laboratory from a γ-irradiated hooded rat, by Dr S. V. Hunt; Pu-19 (Jonasson et al. 1977); L-TK~ (Kitt et al. 1966); D98/AH2 (Szybalski et al. 1962; Gartler, 1967); RT112 (Marshall et al. 1977); Raji (Epstein et al. 1967); HT1080 (Rasheed et al. 1974); MRC-5 (Jacobs et al. 1970). C57-black fibroblast cultures were prepared from 11-13 day embryos and were used at passage 4-8. Lφ is a hybrid clone isolated in HAT medium (Littlefield, 1964) from a Sendai-virus-induced fusion of L-TK~ and φ5 cells. Lφ expresses both rat and mouse lactate dehydrogenase A (S. J. Goss unpublished data).

Culture media

An enriched form of Eagle's Minimal Essential Medium, supplemented with 5% or 10% (w/v) foetal calf serum, was used for the routine maintenance of all cell cultures. This medium was prepared as described elsewhere ('arginine-medium' of Goss, 1984a), except that N-acetylglutamate was omitted. Selective media were made with DL-homocystine (0.8 mM), or l-homocystine (0.8 mM), or (+)cystathionine (1.6 mM) in place of cystine, and the foetal calf serum was then extensively dialysed before use (Goss, 1984a). With the exception of certain preliminary experiments, in all cases where dialysed serum was used, sodium selenite (30 nM) was added to the medium. Bovine insulin (10 nM) and dexamethasone acetate (50 nM) were added to media used for φ cells and their hybrids, and the dexamethasone supplement alone was used for 7777 cells. To obtain good growth of Raji cells in media made with dialysed serum, it was necessary to supply additional non-essential amino acids as in Iscove's medium: 'Inducing medium' is routine growth medium supplemented with dexamethasone acetate (5.56 nM), cholera toxin (5×10^-10 M),
### Table 1. Cell growth using alternative sources of cysteine-sulphur

<table>
<thead>
<tr>
<th>Cell and origin</th>
<th>Growth in cystine-free medium containing: Homocysteine</th>
<th>Cystathionine synthase activity (nmol h⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>φ₁</td>
<td>Hepatoma</td>
<td>+</td>
</tr>
<tr>
<td>Fu5-TG1</td>
<td>Hepatoma</td>
<td>+</td>
</tr>
<tr>
<td>7777</td>
<td>Hepatoma</td>
<td>+</td>
</tr>
<tr>
<td>HTC</td>
<td>Hepatoma</td>
<td>−</td>
</tr>
<tr>
<td>Y3.Ag 1.2.3</td>
<td>Myeloma</td>
<td>−</td>
</tr>
<tr>
<td>NRK TG3/3B77Sc4</td>
<td>Kidney fibroblast</td>
<td>−</td>
</tr>
<tr>
<td>L6-CAP</td>
<td>Myoblast</td>
<td>−</td>
</tr>
<tr>
<td>Hunt's tumour</td>
<td>Fibrosarcoma</td>
<td>−</td>
</tr>
<tr>
<td>Mouse cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pg-19</td>
<td>Melanoma</td>
<td>−</td>
</tr>
<tr>
<td>L-TK⁻</td>
<td>Fibroblast line</td>
<td>−</td>
</tr>
<tr>
<td>C57-black</td>
<td>Diploid fibroblast</td>
<td>−</td>
</tr>
<tr>
<td>707 B1011C3</td>
<td>Erythroleukaemia</td>
<td>−</td>
</tr>
<tr>
<td>Human cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLC/PRF/S</td>
<td>Hepatoma</td>
<td>+</td>
</tr>
<tr>
<td>Bu-25</td>
<td>Cervical carcinoma (HeLa)</td>
<td>+</td>
</tr>
<tr>
<td>D98/AH2</td>
<td>Cervical carcinoma (HeLa)</td>
<td>+</td>
</tr>
<tr>
<td>RT112</td>
<td>Urinary bladder carcinoma</td>
<td>+</td>
</tr>
<tr>
<td>Raji</td>
<td>Lymphoma</td>
<td>+</td>
</tr>
<tr>
<td>HT1080</td>
<td>Fibrosarcoma</td>
<td>−</td>
</tr>
<tr>
<td>MRC5</td>
<td>Diploid fibroblast</td>
<td>−</td>
</tr>
</tbody>
</table>

Cell growth: +, growth comparable with that in the presence of cystine; −, no growth, cells degenerate. Assays: nil, <1 nmol h⁻¹ mg⁻¹, other results rounded to the nearest unit; n.d., not done.

*This surprising result is considered in the text.*

### Enzyme assays

Cells to be assayed were grown in complete growth medium (i.e. with cystine). The methods for assaying ornithine transcarbamoylase (EC 2.1.3.3) and carbamoyl phosphate synthetase I (EC 2.7.2.5), and for inducing and assaying tyrosine aminotransferase (EC 2.6.1.5) are given elsewhere (Goss, 1984). Cystathionine synthase (EC 4.2.1.21) was assayed by a method adapted from Mudd et al. (1965). Cells were suspended and sonicated in lysis buffer to give a protein concentration of around 10 mg ml⁻¹ (lysis buffer: 300 mM-Tris·HCl, 5 mM-EDTA, 0·3 mM-(-)-cystathionine, pH 8·3). Protein was estimated according to the method of Lowry et al. (1951). The sonicates could be stored at −20°C for up to 3 weeks without loss of activity. Before the assay, 75 μl sonicate were pre-incubated for 30 min at 37°C with 25 μl homocysteine solution (150 mM-DL-homocysteine, 150 mM-β-mercaptoethanol) in lysis buffer, stored in small samples at −70°C and 25 μl pyridoxal phosphate solution (18 mM-pyridoxal phosphate in 1/10 strength lysis buffer, pH 8·3, stored at −20°C). The assay was started by the addition of 25 μl [U-¹⁴C]serine solution (6 mM serine, 8 μCi ml⁻¹: Amersham, UK). The incubation was continued for up to 4 h, 30-μl samples being withdrawn at intervals and spotted onto Whatman 3MM chromatography paper. A descending chromatogram...
SJ. Goss was run for 14 h with ethanol/water/\(0.88\) ammonia (18:1:1, by vol.) as eluant (Smith, 1969). Each track was cut into 12-mm slices, and the radioactivity of each slice was measured by scintillation counting. The immediate reaction product, cystathionine, and its possible further metabolites, cysteine and cystine, form one peak in this system, in slices 1–3. Unreacted serine migrates to slices 7–10. Cystathionine synthase activity was estimated from a regression against time of the fractional conversion of serine to material in the cystathionine peak. To maximize the sensitivity of the assay, the reaction mixture is formulated with a sub-saturating concentration of serine (i.e. \(K_m \times 60\%\)). As a result, during the assay, the rate of reaction declines exponentially. However, provided less than 10% of the serine had reacted, it was considered to be an unnecessary sophistication to use a logarithmic regression. The amount of enzyme detected was proportional to protein assayed for the range 225–810 ng. Under typical conditions, the limit of sensitivity was 1 nmol h\(^{-1}\) mg\(^{-1}\), and duplicate assays generally agreed to within 10%. The assay results given below are derived from single experiments: the data are used simply to demonstrate the detectability of CS.

**Demonstration of trans-sulphuration of \(^{14}\)C-serine to \(^{14}\)C-cysteine**

The cells were labelled as they reached confluency in 25 cm\(^2\) tissue-culture flasks. The cultures were first starved of serine and cystine for 6 h by exposure to routine growth medium made without non-essential amino acids and cystine, and with 1% dialysed foetal calf serum. This medium was then supplemented with alanine and glycine (both 0.5 mM) and either with \([U-^{14}\text{C}]\text{cysteine (0.5 \(\mu\)Ci m\(^{-1}\); Amersham, UK) or with \([U-^{14}\text{C}]\text{serine (1 \(\mu\)Ci m\(^{-1}\); Amersham, UK}.\) Labelling with serine was done in the presence or absence of DL-homocysteine (0–8 mM). The medium contained large amounts of 'cold' alanine and glycine in order to quench the labelling of cellular protein with these amino acids, both of which can readily be synthesized from serine. The cells were labelled at 37°C for 5 h, and then harvested and lysed with 2 ml water. The proteins were precipitated by the addition of 2 ml ice-cold 10% trichloroacetic acid, and the precipitate was extracted twice for 5 min at 4°C with 8 ml 5% trichloroacetic acid. The precipitate was then washed with ethanol and diethylether and dried, it was taken up in 1 ml 5 M HCl, and the solution was degassed and refluxed in vacuo at 115°C for 18 h. The hydrolysate was dried over KOH and P\(_2\)O\(_5\), dissolved in water and freeze-dried. The residue was taken up in 50 µl water, and 5–10 µl (approx. 10,000 cts min\(^{-1}\)) were spotted onto Whatman 3MM chromatography paper. A descending chromatogram was run for 18.5 h with \(n\)-butanol/glacial acetic acid/water (12:3:5, by vol.) as eluant (Smith, 1969). The tracks were cut into 12 mm slices (solvent front at slice 35), and the radioactivity in each slice was measured by scintillation counting. The cystine and serine peaks were identified by 'spiking' the samples with \(^{14}\)C-labelled standards.

**RESULTS**

**Cell growth using alternative sources of cysteine-sulphur**

A wide variety of rat, mouse and human cells have been tested for their ability to grow in cystine-free medium supplemented with 5% or 10% foetal calf serum. Adherent cells were seeded at approximately 1/5 confluency, mouse erythro-leukaemia cells at 10\(^4\) ml\(^{-1}\), and human lymphoma cells at 10\(^6\) ml\(^{-1}\). In every case, if no alternative source of cysteine-sulphur was added to the medium, the cells died within 2 days. If cystathionine was added to the medium, most cells were able to grow (Table 1), in agreement with the findings of Naylor et al. (1976). In contrast, cystine-free medium supplemented with homocysteine ('homocysteine-medium') permitted the growth of relatively few types of cell. Of the rodent cells, only the hepatomas \(\phi_1\) and Fu5-TG1 grew well with no cell death. The hepatoma 7777 grew poorly at first and some cells died, but the culture resumed normal growth after 1 week. HTC, another hepatoma, did not grow, and slowly degenerated, a finding
Hepatoma growth in homocysteine-medium is similar to that of Naylor et al. (1976). All other rodent cells tested died rapidly, usually on the second day of the test. The ability of rat and mouse cells to grow in homocysteine-medium correlates absolutely with the presence of detectable CS, which is apparently behaving as a tissue-specific trait in these cells (Table 1). Human cells differ from rodent cells in that, in vitro, human cells from many tissues have CS and can grow using homocysteine as a source of cysteine-sulphur. The results shown in Table 1 confirm and extend the results of others in this respect (Eagle et al. 1961, 1966; Naylor et al. 1976). Fibroblasts were the only human cell type to die in homocysteine-medium: in the case of diploid fibroblasts it is believed to be the activity of CT that is limiting (Hankinson & Jacoby, 1975).

Cell growth in homocysteine-medium is dependent on de novo cysteine synthesis

Whilst the correlation of CS activity with the ability of cells to grow in homocysteine-medium strongly suggests the dependence of such growth on de novo cysteine synthesis, it must be remembered that a sulphhydryl compound such as homocysteine can release significant amounts of pre-formed cysteine from its covalent association (via disulphide bridges) with serum proteins (Eagle et al. 1960). Three lines of evidence argue that such preformed cysteine does not contribute significantly to cell growth in homocysteine-medium in the present experiments.

1. A batch of dialysed serum was prepared with 0.8 mM-DL-homocysteine in the dialysis fluid. Any serum-bound cysteine that could be released by homocysteine should thus have been removed. Homocysteine-medium made with this serum supported normal growth of the hepatomas φ1 and Fu5-TG1, and of the human cells Raji and D98/AH2. The same medium was exceptionally rapidly lethal to HTC and MRC-5 cells. The slower degeneration of these two cell lines in homocysteine-medium made with standard dialysed serum suggests that such medium is indeed contaminated with trace amounts of cysteine released from serum proteins, but clearly insufficient to support cell growth.

2. Essentially the same observations were made with homocysteine-medium containing serum treated before dialysis with 10 mM-sodium dithionite (an alternative method for removing serum-bound cysteine; Eagle et al. 1960). Additionally, three 7777 derivatives were tested and grew well in homocysteine-medium prepared with this serum.

3. A number of cells able to grow in homocysteine-medium were also tested in medium made with homocystine in place of cysteine. Homocystine cannot release serum-bound cysteine (Eagle et al. 1960). D98/AH2 and Fu5-TG1 grew well in homocystine-medium, however, it was not possible to grow either φ1 or 7777 cells in this medium. (An explanation of this unexpected finding is considered in the final section of Results.)

Cells able to grow in homocysteine-medium synthesize cysteine from serine

H15, which is considered in detail below, is a partially 'dedifferentiated' variant of 7777. H15 cells normally lack CS, and they cannot grow in homocysteine-medium unless CS expression is first induced in them by hormone treatment. These cells,
Fig. 2

(A) Chromatogram slice no. 8, Cys and Ser, Conversion 0.04%.

(B) Chromatogram slice no. 9, Conversion 7.3%.

(C) Chromatogram slice no. 10, Conversion 3.0%.
both induced and non-induced, were transferred to medium containing \([14C]\)serine. After a period of labelling, they were harvested and their proteins hydrolysed. The hydrolysates were chromatographed to reveal any \([14C]\)cysteine that had been synthesized from the \([14C]\)serine (Fig. 2). It is clear that the cells convert serine to cysteine only when they are grown in medium that induces CS expression. Furthermore, the extent of this conversion is reduced if homocysteine is omitted from the medium, in which case the \textit{trans}-sulphuration presumably depends on the endogenous supply of homocysteine from \textit{S}-adenosylmethionine. The ability of these cells to grow in homocysteine-medium is correlated both with CS activity and with detectable \textit{de novo} cysteine synthesis. A similar homocysteine-dependent synthesis of cysteine was also demonstrated in Fu5-TG1 cells (data not shown).

\textit{CS expression in hybrid cells}

\(L\phi\) is a hybrid clone derived from a fusion of L-TK\textsuperscript{−} mouse fibroblasts and \(\phi_1\) rat hepatoma cells. The expression of liver-specific traits is very stably suppressed in \(L\phi\). Nevertheless, it was possible, by plating the cells in tyrosine-free medium and so selecting for phenylalanine hydroxylase expression, to isolate a revertant, \(L\phi\text{rev}\), that had regained its liver-specific properties (work done by Dr R. J. Gibbons in this laboratory). Table 2 summarizes the properties of the parental cells and the hybrids. It will be seen that CS and the ability to grow in homocysteine-medium are both expressed co-ordinately with well-known liver-specific traits.

\textit{CS expression in dedifferentiated hepatoma variants}

In experiments designed to investigate the factors controlling tissue-specific gene expression, variant hepatomas have been isolated that have lost the ability to grow using ornithine in place of arginine (Goss, 1984a). These variants are unable to convert ornithine to citrulline, which requires the liver-specific urea-cycle enzymes ornithine transcarbamoylase and carbamoyl phosphate synthetase I (CPS-I). Arg-6D is one such derivative of \(\phi_1\). Arg-6D is generally dedifferentiated in that it has also lost the ability to grow without glucose and without tyrosine, and does not express dexamethasone-inducible tyrosine aminotransferase (Goss, 1984a). Further tests have now shown that Arg-6D also lacks detectable CS and is unable to

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Fig. 2. Cysteine synthesis in hepatoma cells induced to express CS. CS\textsuperscript{−} cultures of H15 cells were prepared in routine growth medium, and CS\textsuperscript{+} cultures in inducing medium (formulation: see Materials and Methods). The graphs show radioactivity in chromatograms of protein hydrolysates prepared from the cells after they had been labelled with \([14C]\)serine or \([14C]\)cysteine. A. Non-induced cells labelled with \([14C]\)serine in the presence of homocysteine; (----) induced cells labelled with \([14C]\)cysteine. B. Induced cells labelled with \([14C]\)serine in the presence of homocysteine. C. Induced cells labelled with \([14C]\)serine in the absence of homocysteine. All three graphs for cells labelled with \([14C]\)serine are plotted in two parts, with the left-hand side on a \(2\times\) expanded scale to show the newly synthesized cystine more clearly. In each case the radioactivity in newly synthesized cystine is given as a percentage of the radioactivity in the serine peak. It was not possible to identify the invariant metabolite of serine in the unlabelled peak. The results in A for cells labelled with cysteine are plotted without expansion, according to the right-hand scale.
Table 2. Co-ordinate regulation of liver-specific traits, including CS, in a hepatoma x fibroblast hybrid cell

<table>
<thead>
<tr>
<th>Cell</th>
<th>Growth in cystathionine-medium</th>
<th>Growth in homocystine-medium</th>
<th>CS (nmol h⁻¹ mg⁻¹)</th>
<th>CPS-I (nmol min⁻¹ mg⁻¹)</th>
<th>OTC (nmol min⁻¹ mg⁻¹)</th>
<th>TAT (nmol min⁻¹ mg⁻¹)</th>
<th>Basal</th>
<th>Induced</th>
<th>Growth without tyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-TK⁻</td>
<td>+</td>
<td>+</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>18</td>
<td>98</td>
<td>+</td>
</tr>
<tr>
<td>L-φ</td>
<td>-</td>
<td>-</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>L-φrev</td>
<td>n.d.</td>
<td>+</td>
<td>nil</td>
<td>nil</td>
<td>0.5</td>
<td>nil</td>
<td>2</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>

Assays: CS, cystathionine synthase, nil < 1 nmol h⁻¹ mg⁻¹; CPS-I and OTC, carbamoyl phosphate synthetase I and ornithine transcarbamoylase; nil, <0.1 nmol min⁻¹ mg⁻¹; TAT, tyrosine aminotransferase; induction by exposure for 36 h to medium with 1 μM dexamethasone acetate.
Hepatoma growth in homocysteine-medium

Table 3. The co-ordinate induction of CS and CPS-I in variant hepatomas by dexamethasone and cholera toxin

<table>
<thead>
<tr>
<th>Cell</th>
<th>Medium</th>
<th>CPS-I activity (nmol min⁻¹ mg⁻¹)</th>
<th>CS activity (nmol h⁻¹ mg⁻¹)</th>
<th>Growth in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Homocysteine Cystathionine</td>
</tr>
<tr>
<td>I4</td>
<td>Without hormones</td>
<td>nil</td>
<td>nil</td>
<td>-</td>
</tr>
<tr>
<td>I4</td>
<td>Full ‘inducing’</td>
<td>2</td>
<td>14</td>
<td>+ n.d.</td>
</tr>
<tr>
<td>H15</td>
<td>Without hormones</td>
<td>0.1</td>
<td>nil</td>
<td>-</td>
</tr>
<tr>
<td>H15</td>
<td>Inducing – dex.</td>
<td>0.2</td>
<td>nil</td>
<td>n.d.</td>
</tr>
<tr>
<td>H15</td>
<td>Inducing – ch. tox. – IBMX</td>
<td>0.1</td>
<td>nil</td>
<td>- n.d.</td>
</tr>
<tr>
<td>H15</td>
<td>Full inducing</td>
<td>2</td>
<td>16</td>
<td>+ n.d.</td>
</tr>
<tr>
<td>Ad-1</td>
<td>Without hormones</td>
<td>nil</td>
<td>nil</td>
<td>-</td>
</tr>
<tr>
<td>Ad-1</td>
<td>Full inducing</td>
<td>1</td>
<td>35</td>
<td>n.d. n.d.</td>
</tr>
</tbody>
</table>

Induction was observed in hepatomas growing in the presence of cystine. Full ‘inducing’ medium contains dexamethasone (dex.), and cholera toxin (ch. tox.) and isobutylmethylxanthine (IBMX) to elevate intracellular cyclic AMP (see Materials and Methods). Assays were done at the third passage, at which stage the cells were subcultured at 1/5 into equivalent media with alternative sources of cysteine for the growth tests. CS: nil, <1 nmol h⁻¹ mg⁻¹. CPS-I: nil, <0.1 nmol min⁻¹ mg⁻¹. n.d., not done.

grow in homocysteine-medium: CS is once again behaving as a liver-specific trait. Arg-6D cells retain the ability to grow using cystathionine as a source of cysteine.

Further light has been cast on the regulation of CS by studies using certain other variant hepatomas also originally selected for arginine auxotrophy. Ad-1 (derived from ø1) and I4 and H15 (derived from 7777) are variants that express CPS-I only if they are stimulated both by a glucocorticoid and by elevated intracellular cyclic AMP (Goss, 1984). Table 3 shows that this double stimulation is also necessary if these cells are to express CS and become capable of growing in homocysteine-medium.

Two liver-specific traits, CPS-I and CS, despite their being metabolically unrelated, are thus seen to share common regulatory factors. It is, of course, highly likely that there are additional factors capable of affecting either enzyme individually. The tissue distributions of CS and CPS-I are not identical (compare Jones et al. (1961) with Mudd et al. (1965)).

Selenium and vitamin E assist cell growth when the supply of cysteine is limiting

Some observations reported above are unexpected and deserve further consideration. For example, two hepatomas that can grow in homocysteine-medium die if the homocysteine is replaced with homocystine. Possibly related, is the paradoxical finding that Raji cells can grow in homocysteine-medium, but not in cystathionine-medium (Table 1). In both these instances, it has been shown that growth in homocysteine-medium is not simply an artifact due to preformed cysteine released by reduction from the serum proteins. One possible explanation is that homocystine and cystathionine cannot readily enter these cells. Another explanation arises from a consideration of the roles of cysteine in cells. Cysteine is used not only for protein synthesis, but also for the production of glutathione, a major intracellular
reducing agent. Glutathione is used by the selenium-containing enzyme, glutathione peroxidase, to inactivate toxic peroxides. Homocysteine-medium provides a strongly reducing environment, which should assist cell survival, either by limiting oxidative damage directly or by helping maintain the cellular glutathione pool in a reduced state. In contrast, media made with homocystine or cystathionine could afford no such protection to the cells. It might then be cellular susceptibility to oxidative damage that explains why some cells die unexpectedly in medium made with homocystine or cystathionine. Our preliminary experiments on Fu5-TG1 cells can be interpreted in this light: it was found that these cells died after 3 days in cystathionine-medium, unless the medium was supplemented either with selenium (30 nm-sodium selenite) or with vitamin E (α-tocopheryl acetate, 5 μM). Selenium presumably increases the efficiency with which low levels of reduced glutathione can be used, whilst vitamin E, an anti-oxidant, should reduce the need for the glutathione peroxidase system. These supplements also improve the cloning of Fu5-TG1 in homocysteine-medium. When 300 cells are plated in a 25 cm² tissue-culture flask in homocysteine-medium, the plating efficiency is less than 1% without the supplements, but is 8–10% if both selenium and vitamin E are added. Neither supplement had any detectable effect on growth at high cell densities in homocysteine-medium.

**DISCUSSION**

The experiments described in this paper have shown that for cells to grow in homocysteine-medium they must express detectable levels of CS. Such cell growth is associated with the synthesis of cysteine by the trans-sulphuration of serine. In human cells *in vitro*, CS has widespread expression, regardless of the histogenetic origin of the cells. In contrast, in rodent cells, CS is detectable only in certain well-differentiated rat hepatomas. Since most rodent cells can grow using cystathionine as a source of cysteine, it appears that, of the two enzymes (CS and CT) needed for trans-sulphuration, it is only the first that behaves as a tissue-specific trait in vitro. This conclusion is supported by the pattern of CS expression in hepatoma hybrids and variants. In both these situations, CS is regulated co-ordinately with other liver-specific functions, whilst CT is apparently expressed constitutively. Homocysteine-medium thus selects for a single tissue-specific enzyme.

It is of interest that, in some hepatoma variants, CS shows the same hormonal modulation as CPS-I. The difference between these cells and others that express both enzymes without hormonal stimulation (e.g. Fu5-TG1; this paper and Goss, 1984a) presumably lies in the general mechanisms by which the cells respond to hormones, rather than in distinct, but equivalent, lesions affecting the production of each enzyme independently. In future experiments, it may be worthwhile attempting to dissociate CPS-I and CS expression by selecting against CPS-I whilst maintaining the cells in homocysteine-medium. This approach should yield new classes of variants and so further elucidate the factors controlling the expression of liver-specific traits.
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