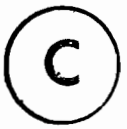
FOLATE ACCUMULATION AND TURNOVER IN CULTURED HUMAN FIBROBLASTS

by

Jack Hilton



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ABSTRACT

Cultured human fibroblasts formed polyglutamate folate from ^{3}H PteGlu in the culture medium. Intracellular polyglutamate increased with duration of incubation, reaching a maximum after about 10 days.

Intracellular folate was proportional to the PteGlu in which the cells were incubated. Cells in logarithmic growth accumulated intracellular folate in higher concentrations than did confluent cells. In confluent cells grown in PteGlu concentrations in excess of those required to sustain a maximum growth rate, a larger proportion of folate was polyglutamate than at lower PteGlu concentrations.

Fibroblasts deprived of folate lost polyglutamate folate in an exponential manner with a $T_{1/2}$ of 3.5 days; a small fraction of which entered the pool of intracellular monoglutamate to prevent its decrease over the first 2-3 days of deprivation. Both polyglutamate and monoglutamate folates effluxed from the cells and accumulated in the culture medium during folate deprivation, a small proportion of these were freely exchangeable with fresh medium.

RESUME

Les fibroblastes humains en culture forment du polyglutamate de folate à partir du ^{3}H GlutPté se trouvant dans le bouillon de culture. La concentration de polyglutamate intracellulaire augmente avec la durée de l'incubation, atteignant un niveau maximum après environ dix jours.

La concentration de folate intracellulaire est proportionelle à la concentration du GluPté dans lequel les cellules ont été incubées. Les cellules en phase de croissance logarithmique accumulent le folate intracellulaire en de plus hautes concentration que les cellules confluentes. Dans les cellules confluentes élevées en présence d'une concentration de GluPté excédant celle requise pour maintenir un taux de croissance maximum, la proportion de folate se retrouvant sous la forme de polyglutamate est plus large pour les concentrations de GluPté plus élevées.

Les fibroblastes humains privés de folate perdent le polyglutamate de folate d'une façon exponentielle avec un $T_{1/2}$ de 3.5 jours; une petite fraction de ce composé entre dans le mélange intracellulaire de monoglutamate prévenant sa diminution durant les 2 ou 3 premiers jours de privation. Le polyglutamate et le monoglutamate de folate s'accumulent dans le bouillon de culture durant le manque de folate; une petite proportion de ces composés s'échange librement avec le bouillon frais de culture.

CLAIMS TO ORIGINALITY

1. Progressive accumulation of intracellular polyglutamate folate was observed by human fibroblasts cultured in ^{3}H PteGlu *in vitro*.
2. The rate of intracellular folate accumulation was proportional to extracellular folate in the medium over 4 1/2 logs. Intracellular folate concentration did not exceed extracellular folate concentration even after prolonged incubation.
3. In confluent human fibroblasts grown in PteGlu concentrations in excess of those required to sustain a maximum growth rate, a larger proportion of folate was polyglutamate than at lower PteGlu concentrations.
4. A small fraction (3%) of the intracellular folate was freely exchangeable with fresh folate-free medium.
5. The remaining amount of intracellular folate left the cell during incubation in folate-free medium. The majority of the folate lost was polyglutamate folate, with very little conversion of the polyglutamate to monoglutamate folate.
6. A minority of intracellular polyglutamate appeared to be in equilibrium with intracellular monoglutamate.

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To Dr. David Rosenblatt I would like to extend my sincere thanks for so many helpful suggestions throughout my research, as well as supplying and supervising the cell cultures used in these studies. Dr. Rosenblatt has not only been a very effective supervisor but a very good friend.

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TABLE OF CONTENTS

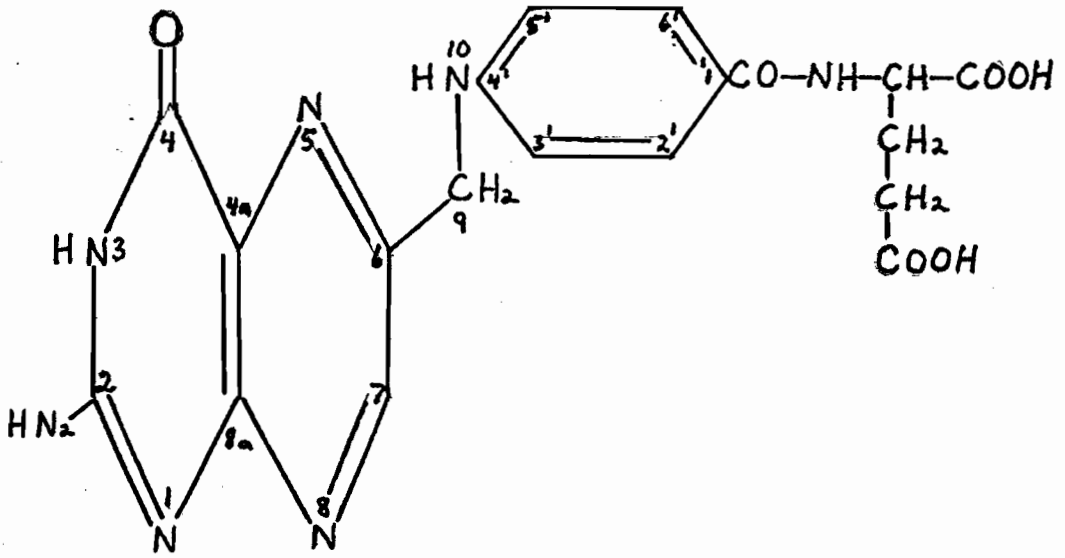
HISTORICAL REVIEW

Working in India in 1931, Wills, a nutritional scientist, reported anemias found in vegetarian and meat-eating pregnant women, characterized by increased red blood cell size and presence of megaloblasts in the blood[1]. In subsequent years, Wills was able to cure the anemia by administration of crude liver and yeast extracts, although preparations of purified liver extracts which were effective in American patients with pernicious anemia failed to correct the anemic condition [2,3]. In 1938, When a similar nutritional deficiency was observed in monkeys, the unknown factor which corrected the nutritional deficiency was termed Compound M[4]. Mitchell et al in 1941, extracted from four tons of spinach a pure substance described as an acid nutrilite with molecular weight of approximately 500[5]. The new compound was found to be an active growth factor for Streptococcus lactis[5] and rats[5]. It was eventually named folic acid, from the Latin, "folium", meaning leaf. The name appeared to be appropriate, as the leaves of all plants examined, contained the factor[5]. In 1943, Stokstad isolated a growth factor from yeast and liver with the same absorption spectrum and growth potency for Lactobacillus casei, as the factor isolated from spinach by Mitchell and co-workers two years previously[6]. It was later found that both growth factors were derived from the same compound, now termed folic acid.

Folic acid is a water soluble vitamin composed of a pteridine ring attached to a para-aminobenzoylglutamate portion, with the glutamate

portion containing one or more glutamate residues as shown in Fig. 1[7].

FIGURE 1



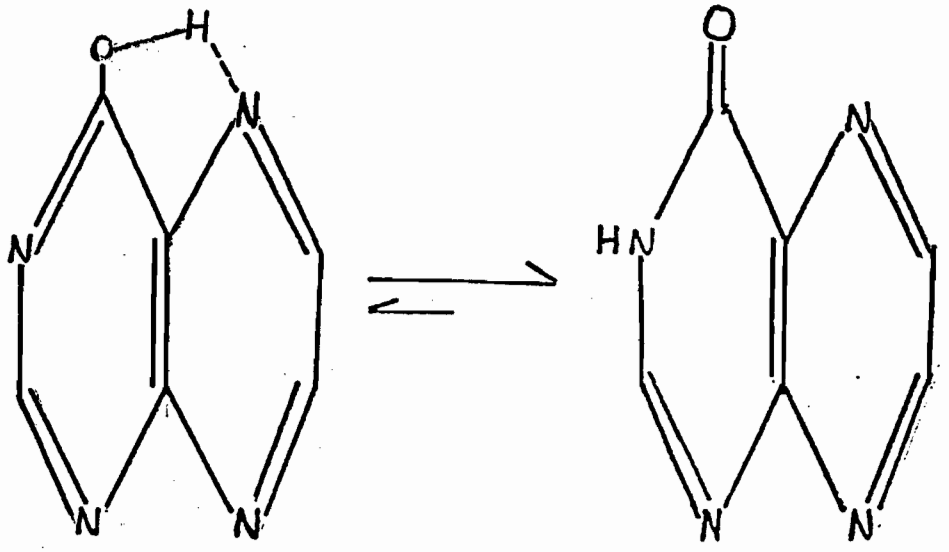
FOLIC ACID

Therefore the molecule possessing the structure N-[4- (2-amino-4-hydroxy-6-pteridinyl) methyl] amino benzoyl] glutamic acid, has been referred to as both pteroylglutamic acid (PteGlu)[8] and folic acid. Folic acid is now defined as the non-reduced unsubstituted monoglutamic acid conjugate of

pteroic acid, whereas folate generally refers to the whole range of related pteroylglutamate compounds which include monoglutamates, polyglutamates and their reduced or substituted analogues[9]. The fully reduced compound is referred to as either 5, 6, 7, 8-tetrahydrofolate (THF) 5, 6, 7, 8-tetrahydropteroylglutamic acid (H_4PteGlu).

The term pterin actually originated as an abbreviation of the longer form pteridine but was eventually proposed as the proper designation for all derivatives of 2-amino-4-hydroxy-pteridine[10]. Characteristically these pterins have relatively low solubility in water but folic acid has a higher solubility in water because of the polar effect of the glutamic acid moiety[11]. Although the pterin portion of folate is commonly described as the 2-amino-4-hydroxy-pteridine tautomer, studies indicate that the equilibrium favors the 4-oxo (cyclic amide) rather than the 4-hydroxy enolic form[12-17].

FIGURE 2

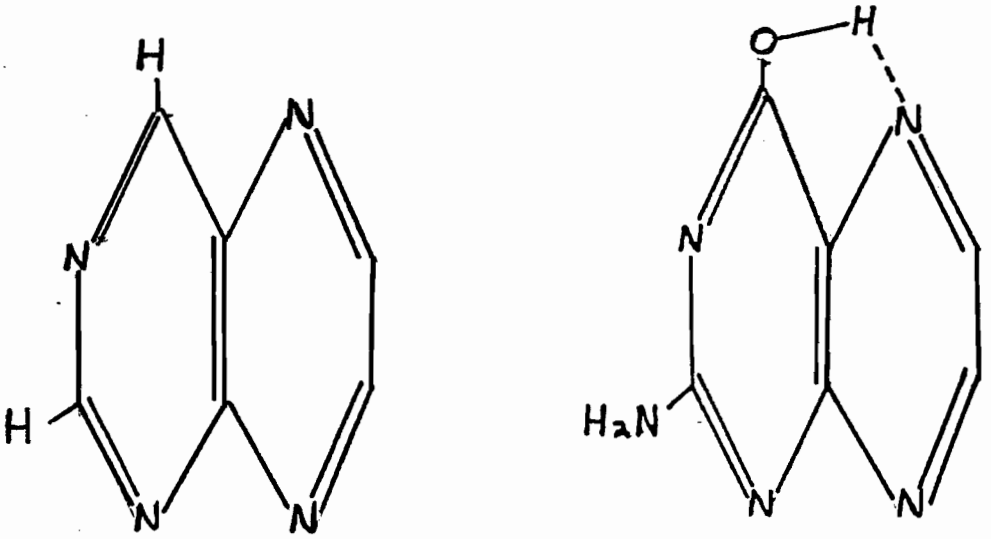


4-HYDROXY ENOLIC

4-OXO CYCLIC AMIDE

The pterin ring of folic acid is stabilized by the 2-amino, 4-hydroxy groups, which act as electron-liberating substituents tending to compensate for the Π electrons withdrawn by the ring nitrogens in pteridines[12].

FIGURE 3



PTERIDINE PTERIN

By compensating for the electron loss the pterins do not have the destabilization observed in pteridines without the 2-amino, 4-hydroxy substitutions. Since pterins are relatively resistant to oxidation[12],

investigations utilizing mild oxidation to determine the nature of alkyl sidechains have been possible[19,20]. These studies revealed that oxidation of alkyl sidechains in the 6 or 7 positions by alkaline permanganate or acidic hydrogen peroxide yields pterin -6 or 7-carboxylic acid depending on whether the alkyl side chain is on the 6 or 7 position[20-22]. Although electrophilic substitutions on the pterin ring carbons have not been reported, alkylation may occur on any or several of the ring nitrogens or on the oxygens of hydroxyl groups, depending on the reaction conditions and steric factors[12,23]. When pteridines become hydrogenated, unlike the parent compound, the number of electron liberating substituents which are present (such as the 2-amino and 4-hydroxy groups of pterins) decrease the stability of the compound[24-26].

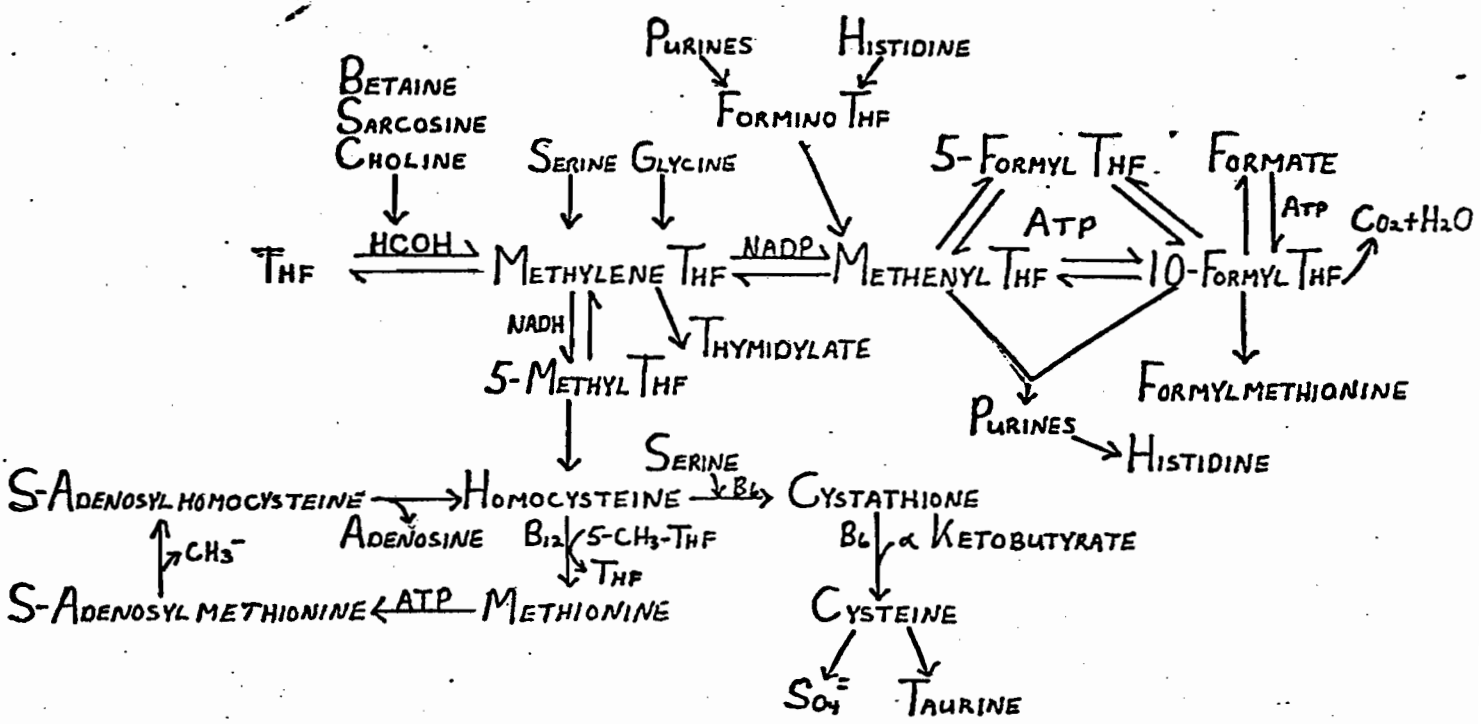
Folic acid will, in the presence of nitrous acid and low temperature, produce a 10-nitroso derivative[27]. Folic acid and its derivatives are also reported to be readily halogenated[28-30], or nitrated in the benzene ring to produce dichloro or 3',5'.-dinitro derivatives[29]. In the presence of sunlight[31], irradiation[32], hydrogen peroxide and other alkaline and acidic hydrolysis[33], folic acid is decomposed to a number of pterin derivatives and p-aminobenzoylglutamic acid.

Most folates exist in nature with more than one glutamate residue attached to the para-aminobenzyl portion of the folate molecule. These glutamate residues were found to be attached to the folate molecule by γ-glutamyl linkages[7].

The function of folic acid is as a coenzyme, which is used in the transfer of one carbon units. In order to participate in this role, folic

acid or dihydrofolic acid is reduced by the enzyme 5, 6, 7, 8-tetrahydrofolate: NAD(P) oxidoreductase (1.5.1.3), commonly termed dihydrofolate reductase. Once in the 5, 6, 7, 8-tetrahydrofolate form, different coenzymes are formed by enzymatic addition of different one carbon fragments, to form the different folate coenzymes required in cell metabolism. The known metabolic functions of the various folate derivatives and their interrelationships are listed in Fig. 4.

Fig 4.



34-36

FOLATE METABOLISM

FIG 4

The mechanism of these various folate dependent reactions has been extensively studied, and in the following section a detailed description will be presented on the present status of the mechanisms of the best studied ones. In the review, the mechanisms discussed will demonstrate which critical reactive groups on the folate molecule are responsible for the reactions and whether the polyglutamate form of folate is a more effective substrate for these reactions than the monoglutamate form. The purpose of the study described in this thesis was to study folate accumulation in normal human fibroblasts and to relate this accumulation and distribution of folate forms to the growth of the cells.

The distribution of folate derivatives found inside the cell may have an effect on enzyme function[37]. To provide an understanding of why some enzymic reactions preferentially utilize a particular folate derivative, a brief description of two folate dependent enzymes and their interaction with folate will follow.

Dihydrofolate Reductase (DHFR)

This enzyme participates in the reaction described by the equation below. The rate of reaction of equation (1) is greater than

(1) $H_2PteGlu + NADPH + H^+ \rightleftharpoons H_4PteGlu + NAPD^+$

(2) $PteGlu + 2\ NADPH + 2H^+ \rightleftharpoons H_4PteGlu + 2\ NADP^+$

that of equation (2) at physiologic pH.

Bacterial Dihydrofolate Reductase

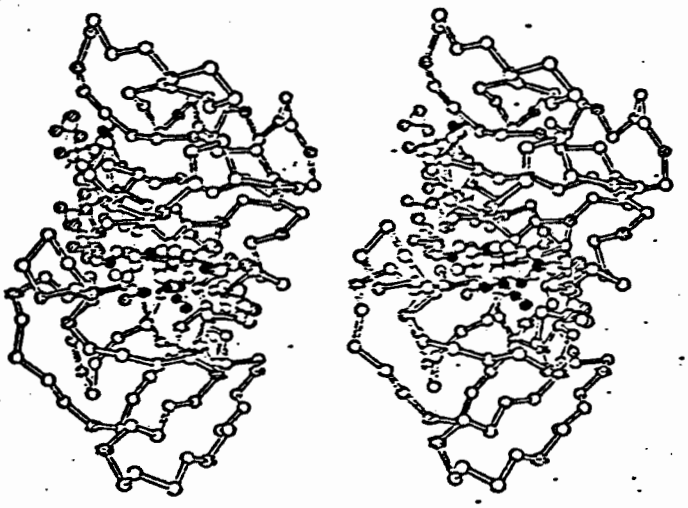
Recently it was found using x-ray diffraction of dihydrofolate reductase isolated from E.coli, that the p-aminobenzoyl portion of methotrexate

was bound in a hydrophobic pocket of the enzyme by side chains of Leu-28, Ile-50, and Leu-54. The glutamate portion was bound at the enzyme surface with the side chains of Arg-57 hydrogen bonded to the α-carboxyl group of the folate[38]. The α-carboxyl groups of the polyglutamates were found to react with side chains of the dihydrofolate reductase, which were found to be different with the two types of reductase examined in L.casei and E.coli[38]. The presence of different forms of dihydrofolate reductase has similarly been observed in different strains of E.coli[39]. The presence or absence of the α-carboxyl binding sites on the enzyme may be responsible for the greater affinity of the folate polyglutamate or monoglutamate substrate to the enzyme and may explain why in some cells there is a preference of the reductase for polyglutamates[40], whereas in others no such preference has been reported[41].

X-ray crystallographic studies conducted on dihydrofolate reductase isolated from L.casei and E.coli indicate that the pyrimidine portion of methotrexate, a 10-methyl-4 amino-4 deoxy analogue of PteGlu, was buried in a primary hydrophobic pocket, while the p-aminobenzoyl portion of the methotrexate was located in a second hydrophobic pocket[38]. The nicotinamide ring of NADPH has been found to be in close proximity to the pyrazine ring of MTX with both sharing one hydrophobic cleft of the enzyme[38]. The adenine portion of NADPH was found in a second hydrophobic cleft separate from the one to which the nicotinamide ring of NADPH was bound[38]. In the same study, in the primary hydrophobic pocket, the C(4) carbon of the nicotinamide ring was in close proximity to the N(5) - C (6) bond of the pyrazine ring of MTX. The ribose phosphate portion of the NADPH appear to be involved in numerous hydrogen bond

interactions with the dihydrofolate reductase backbone chains[38].

Fig 5



38

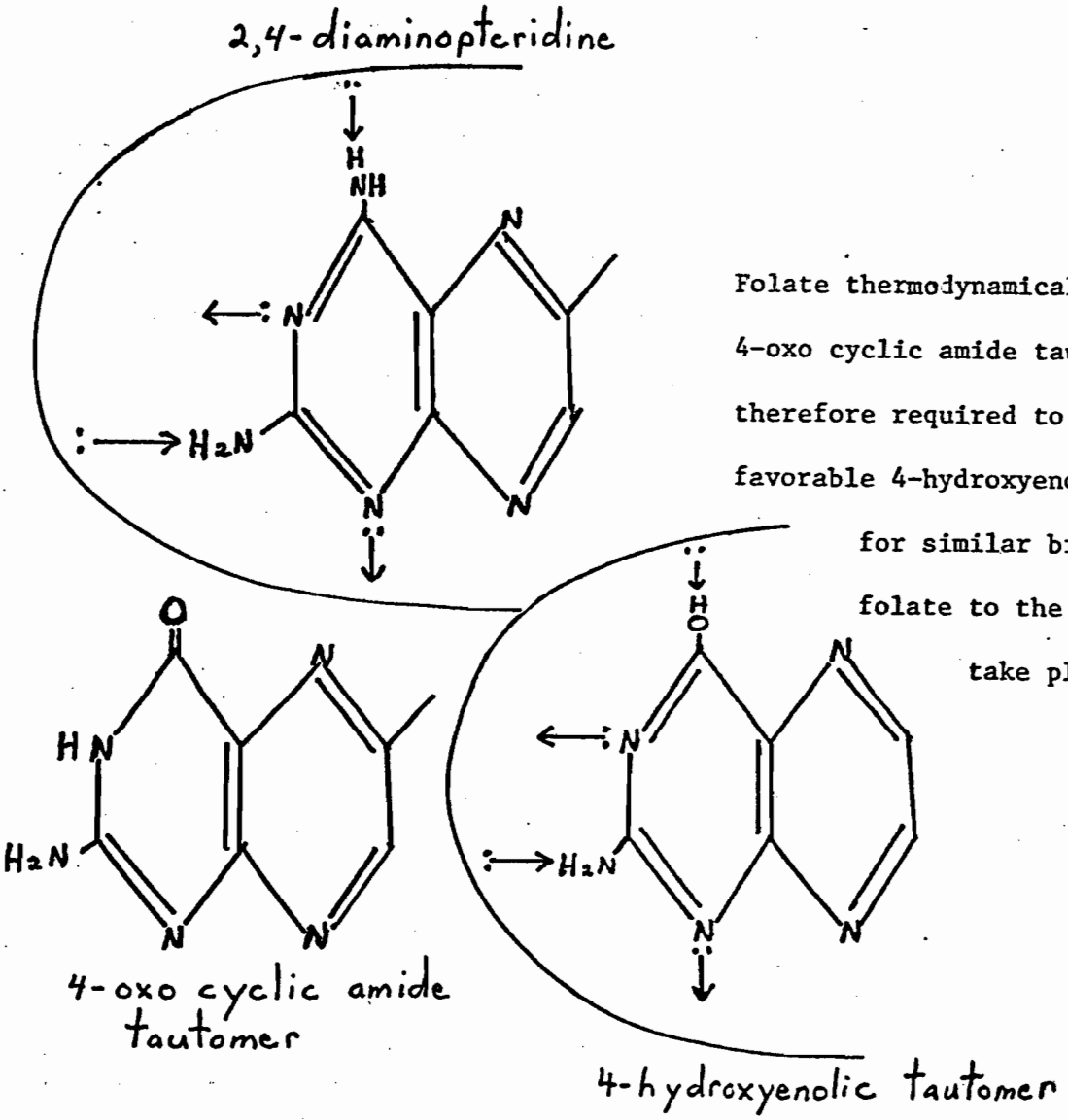
Fig. 5 Stereo drawing of the 162 α-carbon atoms in *L. casei* DHFR. Also shown are bound molecules of NADPH and MTX. Nitrogen and oxygen atoms are indicated by blackening and shading respectively.

Although direct evidence has not been presented in the bacterial system, it would appear based on kinetic results obtained from a methotrexate-resistant strain of Streptococcus faecalis, that NADPH first binds to dihydrofolat reductase, then followed by folate in an ordered sequential mechanism[42].

Mammalian Dihydrofolate Reductase

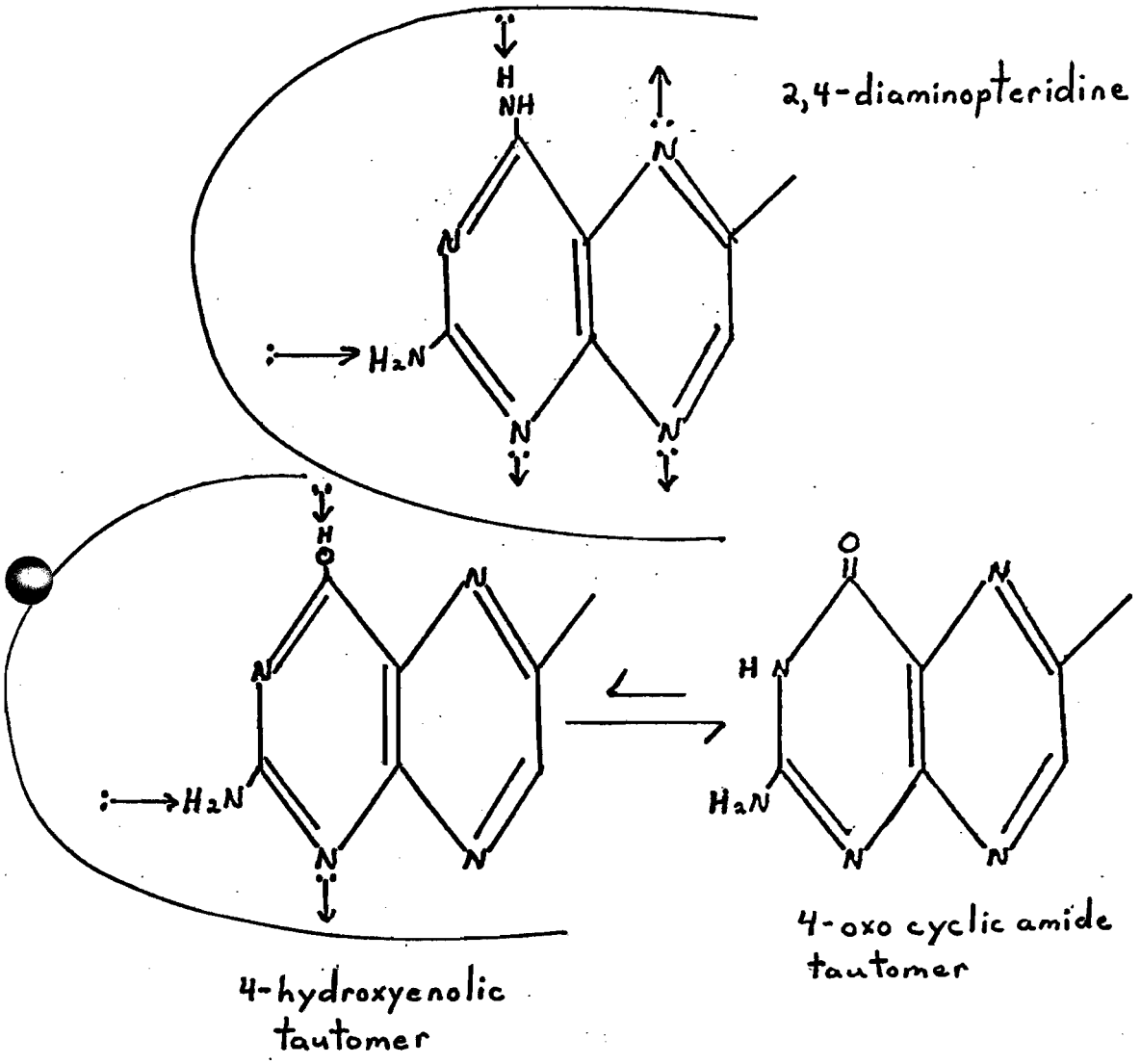
Dihydrofolate reductase examined from Ehrlich ascites carcinoma cells was found to bind pteroate only 1/80 as well as folate [43,44]. This observation suggests that the glutamate region of the folate has an important function in the binding of folate to the reductase. Studies conducted with the human enzyme, obtained from acute myelogenous leukemic and acute lymphocytic leukemic cells showed that the polyglutamate folate has a ten fold lower Km[40]. Such preference of the reductase for polyglutamates was not observed in methotrexate resistant human KB cells, with the Km s of folate polyglutamates and monoglutamates both being approximately 0.58 uM[41]. The difference in the affinity of the enzyme for folates of different glutamate length may be explained by the presence of different forms of dihydrofolate reductase, as described in bacterial systems [38,39]. Based on this evidence, it would appear that the glutamate is important for the binding of dihydrofolate reductase to folate, however the actual number of the glutamates may not be important, depending on the dihydrofolate reductase present. Dihydrofolate reductase obtained from chick liver bound 6-methylpterin only 1/100,000 times as well as folate[45], and pigeon liver reductase was found to bind p-aminobenzoylglutamate quite significantly [46]. This evidence in conjunction with the x-ray crystallography studies conducted in the bacterial systems would indicate that the p-aminobenzoylglutamate portion of folate has a binding site on the enzymes.

It has been observed that when folate was converted to its 4-amino analogue (aminopterin) affinity for the enzyme was increased more than 10,000 fold[47]. Working with enzyme purified from pigeon liver, Baker suggested that the increased affinity of the diamino-compound might be a result of the generation of a protonated species which combines with an anionic site on the enzyme[48]. Zakrzewski suggested that 2,4-diamino-pyrimidine forms hydrogen bonds with the reductase[49,50] as described below.



Folate thermodynamically favors the 4-oxo cyclic amide tautomer and is therefore required to form the less favorable 4-hydroxyenolic tautomer for similar binding of folate to the enzyme to take place.

Baker et al,[51] has modified this model to include the generation of a hydrogen bond which involves N(5) and N(8) of the pteridine ring of aminopterin and methotroxate, but not of folate[51] as shown below.



Based on the evidence in support of hydrogen bond formation[44,52] and cationic charge of the pyrimidine combining with an anionic charge on the enzyme[38], both appear involved in explaining the greater binding of

2,4-diaminopyrimidines than of 2-amino, 4-hydroxypyrimidines to dihydrofolate reductase.

It is very difficult to determine whether the mechanism for binding folate and NADPH to dihydrofolate reductase by the mammalian enzyme is an ordered or random sequential mechanism. Evidence obtained from reductase obtained from mouse L1210 lymphoma cells indicates that the dissociation constant for NADPH (5×10^{-8}M) is only 4 times less than that of PteGlu (3×10^{-7}M). It has been observed in the same cellular system that triamterene (2, 4, 7-triamino-6-phenylpteridine) dissociates 60 times faster from the enzyme-triamterene complex than from the enzyme-NADPH-triamterene complex. If H_2PteGlu follows this pattern, and has a greater affinity for the enzyme-NADPH-complex than for the enzyme alone, and if the 4 fold greater affinity of the reductase for NADPH over folate is real, the reaction may in fact be ordered and sequential. This conclusion may not be correct however because in enzyme purified from L1210 lymphoma cells resistant to methotrexate, there was no binding preference observed in either of the reactions listed below[42].

This would certainly suggest a random sequential reaction.

(1) enzyme-NADPH-complexed with triamterene[42]

(2) enzyme-triamterene-complexed with NADPH

Comparison of Bacterial and Mammalian Enzyme

It has been noted that the mammalian dihydrofolate reductase isolated from porcine liver differs from that of the bacterial reductases purified from L.casei and E. coli. The observation that the dissociation

constants for NADPH and folate are 1.4×10^{-7} and 3.6×10^{-7}M respectively[53], would support the suggestion that mammalian enzyme could bind both ligands simultaneously and that there was only one high affinity site in the enzyme, unlike the two observed in the bacterial system. The Michaelis constants[53], for both NADPH and H_2PteGlu are lower for the mammalian reductase than for the bacterial[53]. The bacterial enzyme had a larger turnover number[53].

Comparison of Michaelis constants for bacterial and mammalian DHFR

	E.coli	Porcine
	Km in μM	
NADPH	6.45 ± .9	.55 ± .28
H_2PteGlu	.44 ± .05	.05 ± .016

Although there is little direct evidence available in either the bacterial or mammalian enzymes in relation to its action on the folate substrate, it has been suggested that a histidine in the active site of the enzyme is responsible for donating a proton to N(8) and N(5) of the pyrazine ring of folate[51]. Polarographic investigation of PteGlu showed

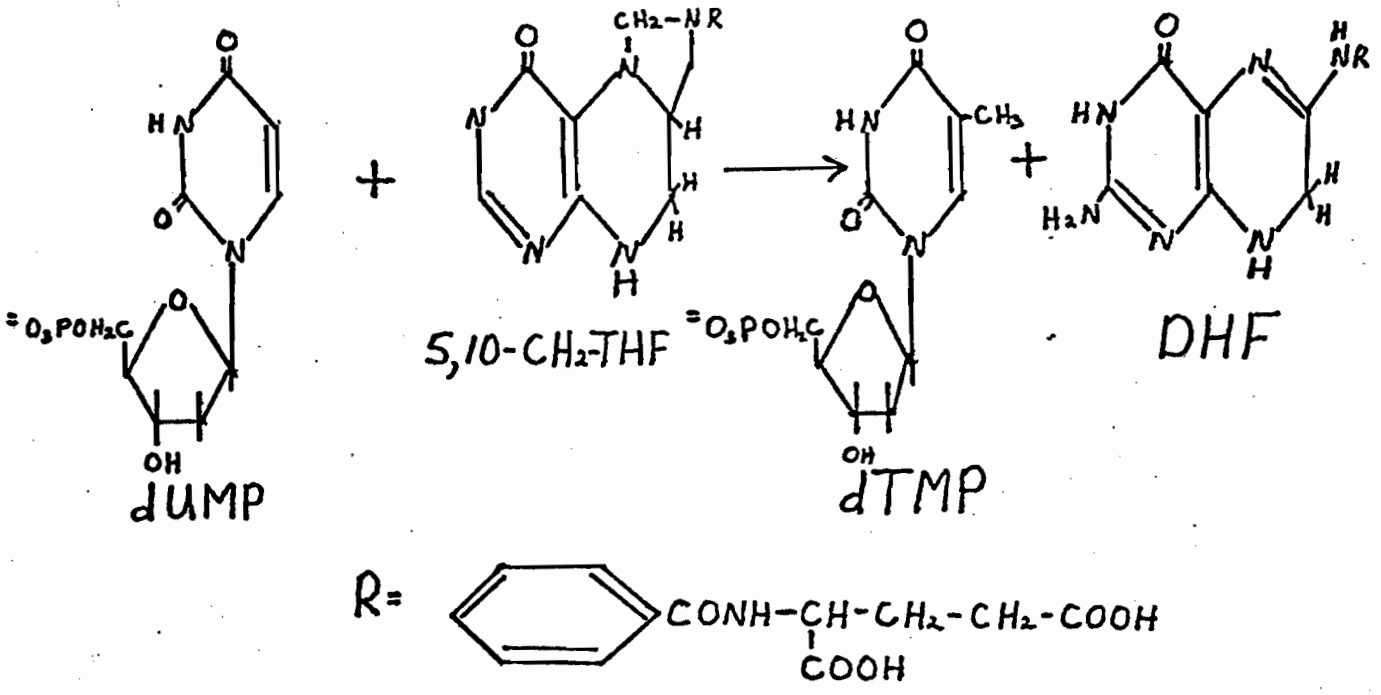
that PteGlu was reduced in the first step to 5, 8-H_2PteGlu and then to 7, 8-H_2PteGlu[54,55]. Further reduction of 7, 8-H_2PteGlu occurs across the N(5) - C(6) bond of the dihydropteridine ring[38]. X-ray crystallographic analysis of dihydrofolate reductase isolated from bacteria[38], showed the C(4) carbon of the nicotinamide ring of NADPH "A side" close to the N(5) - C(6) bond of the pyrazine ring of MTX[38]. The study further illustrates that the transferable hydride ion probably comes from the A side of the nicotinamide ring, since the B side points down and away from the pyrazine ring.[38]

Thymidylate Synthetase

Thymidylate Synthetase (Bacterial)

It was found in 1944 that one could replace the requirement of folate in the bacterial system, S. faecalis by supplementing the medium with thymine or thymidine at 5000 times the optimum concentration of folate required by the cells[56]. It was therefore concluded that there was a folate derivative which was required as a coenzyme in the synthesis of thymidine derivatives. This was found to be 5, 10-methylene-H_4PteGlu and is now known to be formed from H_4PteGlu by non-enzymic reaction with formaldehyde and by enzymic reaction with serine as well as enzymic reduction of 5, 10-methenyl-H_4PteGlu[57-64]. Deoxyuridine monophosphate (dUMP) was proved to be the substrate, and by the action of thymidylate synthetase (EC 2.1.1.45), dUMP was found to be methylated to dTMP[62-64] as shown in Fig. 6.

FIGURE 6



In the binding of dUMP and 5, 10-methylene-H_4PteGlu to the synthetase, it has been found that the reaction is initiated by nucleophilic binding of a cysteinyl residue located on the active site of the enzyme, to the 6-position of the pyrimidine ring of dUMP, resulting in saturation of the 5, 6 position of dUMP [66]. Amino acid analysis of thymidylate synthetase isolated from L.casei revealed a cysteine residue at position 198. This was presumed to be the nucleophile responsible for ternary complex formation[66].

Mammalian Thymidylate Synthetase

Although NADPH also stimulates the production of thymidylate, as described in extracts of calf thymus[65], it probably does so in conjunction with the action of dihydrofolate reductase, where both produce more H_4PteGlu and consequently 5, 10-CH_2 - H_4 PteGlu, the required substrate for TMP biosynthesis[65]. This theory is represented by the equations given below.

$$\text{dUMP} + \text{5, 10-methylene} - H_4\ \text{PteGlu} \rightleftharpoons \text{dTMP} + H_2\ \text{PteGlu}$$

$$H_2\text{PteGlu} + \text{NADPH} + H^+ \rightleftharpoons H_4\ \text{PteGlu} + \text{NADP}^+$$

$$H_4\ \text{PteGlu} + \text{HCOH} \rightleftharpoons \text{5, 10- methylene} - H_4\ \text{PteGlu}$$

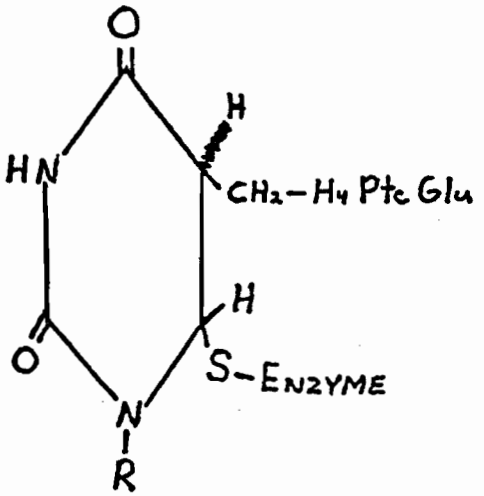
Thymidylate synthetase isolated from chick embryo was found to have a molecular weight of 58,000 and a pH optimum of 6.5 at 37°C[67]. Its concentration in cells ranges from trace amounts found in relatively non-proliferating cells, to greatly elevated levels found in rapidly proliferating cells [68-70]. In rat liver hepatoma the activity of the enzyme was increased from 1.3 nmol/h/mg found in normal liver to 24.5 nmol/h/mg found in Novikoff hepatoma[71].

Kinetic studies conducted on thymidylate synthetase isolated from chick embryo[67] and human leukemic blast cells[72] indicate that the synthetase first binds dUMP, then binds 5, 10-CH_2-H_4 PteGlu and hence is an ordered reaction.

Mechanism of Thymidylate Synthetase

Using fluorodeoxyuridine which binds covalently to the enzyme, disruption of the enzyme-dUMP complex with cyanogen bromide has revealed that the active site of the enzyme, which bound the ternary complex formed, is invisioned to appear as shown below. It is formed by the attack on 5, 10-CH_2-H_4 PteGlu or its cationic imine, by the carbanion generated by addition of the active site nucleophile (cys. 198) to carbon 6 of the pyrimidine ring[73].

FIGURE 7



R = 5'-PHOSPHO-2'-DEOXYRIBOSE [73]

There appear to be two separate binding areas on the thymidylate synthetase for both dUMP and 5, 10-CH_2-H_4PteGlu. Evidence for this includes the fact that sulfhydryl inhibitors prevent binding of dUMP to the synthetase and γ-glutamyl conjugase prevents the binding of 5, 10-CH_2-H_4PteGlu[74]. Although 5, 10-CH_2-H_4PteGlu does not bind directly to the thymidylate synthetase in the absence of dUMP 5, 10-CH_2-H_4 $PteGlu_{4-7}$ will [74,75]. With the observation that PteGlu (Ki: 1.5×10^{-4}M) does not inhibit thymidylate synthetase as well as $PteGlu_6$(Ki: 6×10^{-7}M) and that inhibition of thymidylate synthetase is increased by the addition of glutamates to the PteGlu molecule [66,76,77], it is possible that the glutamate portion of PteGlu has a definite binding site on thymidylate synthetase.

Folate Accumulation

It has been observed that some bacteria[78], erythroid[79] and granulocyte [80] precursors, as well as some other cells [81, 82], accumulate folate to a concentration which is in excess of the folate level in the exogenous medium. This was also observed in L.casei[83].

Most naturally occuring folate is found in the polyglutamate form consisting of from one to seven glutamates, with the predominant form found in mammalian cells being probably pentaglutamate[84].

The reliability of procedures which have been used in the recent past to determine exact polyglutamate chain length[85,86] has been recently challenged by the observation that some H_4PteGlu derivatives were not cleaved, by zinc acid reduction or potassium permanganate (K M_nO_4) oxidation[87].

Studies have indicated that polyglutamates constitute between 70-90% of intracellular folate. Measurement of intracellular polyglutamate folate has been hampered by the action of γ-glutamyl carboxypeptidase (conjugase) an enzyme specific for the γ-glutamyl bond of polyglutamates which hydrolyses folate polyglutamates to monoglutamates[88].

Monoglutamate folate levels in the rat uterine tissue and Physarium polycephalum have been reported to be increased during rapid cellular proliferation[89], however other studies with leukemic blast cells, and mature leukocytes showed no such increase[90].

The importance of polyglutamate folate in the cell has been demonstrated in a Chinese hamster cell line auxotrophic for glycine, adenosine and thymidine as well as having a decreased level of intracellular folate[91]. The defect in these cells was identified later as low polyglutamate synthetase (ligase) activity, resulting in abnormally low folate polyglutamate levels found inside the cell[92]. In other studies polyglutamates have been shown to have lower Km values for enzymes than have monoglutamates. (Table 1)

TABLE 1

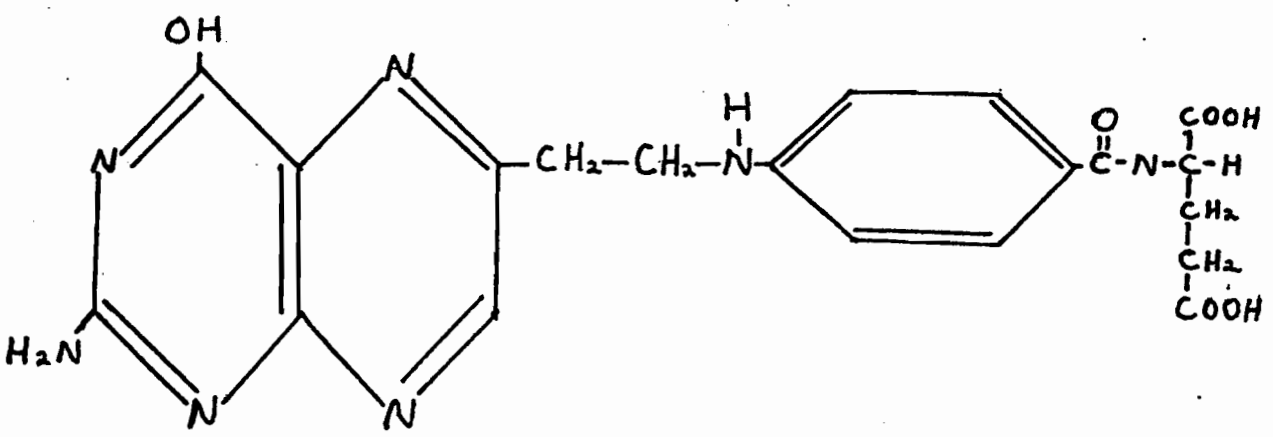
	Enzyme	Monoglutamate	Polyglutamate	Source
Ki	Thymidylate Synthetase	1.5×10^{-4} M	6×10^{-7} M	L.casei[93]
Km	Methionine Synthetase	7.3×10^{-5} M	2.2×10^{-5} M	Bovine Brain[94]
Km	AICAR Transformylase	6.7×10^{-4} M	1.83×10^{-6} M	Chicken Liver[95]
Km	Forminotransferase cyclodeaminase	4.8×10^{-5} M	3.5×10^{-6} M	Porcine Liver[96]

Studies of folate polyglutamate synthesis have now been carried out in guinea pig, rat, hamster, sheep, monkey and human tissues [97-103]. Most of these studies have been conducted in intact animals using radiolabelled folates. These studies have demonstrated that folate polyglutamates may be identified in cells as early as four hours after injection of radiolabelled PteGlu[104]. Other studies utilizing radiolabelled PteGlu demonstrated that the PteGlu reached equilibrium with endogenous

liver folate during a 3 day period in guinea pig or rat liver[99,102], and in monkey kidney even after three days, full equilibrium had not taken place[98]. Evidence has been presented in both L.casei[105] and rat liver[99] which would indicate that triglutamate formation might be a rate limiting step in polyglutamate synthesis, but with the purified enzyme derived from Chinese Hamster ovary cells, the rate limiting step appeared to be the addition of the first glutamate, with additional glutamates being added sequentially[107]. In rat liver and bovine brain it would appear that polyglutamate synthesis occured by sequential addition of single glutamate residues on the folate monoglutamate[108-109]. Data further indicates that liver exposed to PteGlu for extended periods of time synthesizes polyglutamates which increase in length with time and eventually form heptaglutamates[110]. One must re-emphasize however that methods used to determine polyglutamate chain length in these studies have been challenged.

Polyglutamate synthesis involves one or more ligase, (synthetase, polyglutamate synthetase) enzymes, which add glutamate residues

FIGURE 8



HOMOFOLATE

sequentially to the various folate derivatives [107]. Studies with semi-purified preparations had indicated that in the mold Neurospora crassa there might be two different synthetase enzymes: one enzyme which could convert $H_4PteGlu_2$ and another which was responsible for higher conjugates[111]. In a preparation of partially purified enzyme from the bacterium E.coli, a synthetase was identified which had a folate specificity for 10-formyl-H_4PteGlu, 10-formyl-H_4 homofolate (Fig. 8), 5, 10-methylene-H_4PteGlu H_4PteGlu[112]. H_2PteGlu, 5-methyl-H_4PteGlu, 5-formyl-H_4PteGlu and PteGlu were found to be unsuitable for polyglutamate synthesis by this preparation. In extracts of sheep liver it was found that synthetase had a specificity pattern of H_4PteGlu (100%), 5-methyl-H_4PteGlu (50%), 5-formyl-H_4PteGlu (40%)[106]. Studies with purified synthetase isolated by Taylor and co-workers used Chinese hamster ovary and V-79 lung cells [92]. The cellular sonicates from both of these cell lines have revealed that the folate specificity of synthetase for diglutamate synthesis was H_4PteGlu, H_4 homofolate, 5-formyl-H_4PteGlu, 5-methyl-H_4PteGlu, with the reaction catalysis optimal between pH 8.5 and 10.2 and dependence on magnesium ions and a purine nucleotide triphosphate[92].

Transport of Folate into Cells

Polyglutamate Transport

The study of folate polyglutamate transport into cells has been limited by the problem of possible hydrolysis of these prior to entry. These studies have utilized bacteria [113], human bone marrow cells, and cultured stimulated lymphocytes[114]. Polyglutamate folates enter L.casei with the affinity constant and maximum velocity of folate uptake decreasing with increasing glutamate length[113]. In human bone marrow cells and lymphocytes in culture, triglutamate folates were only transported 1-5% as rapidly as were mono and diglutamates[114]. These studies suggest that some polyglutamates are transported into some cells, (more so probably in the bacterium than in the human cell) but do so only slowly.

Monoglutamate Transport

Contrary to polyglutamate transport into the cell, monoglutamate transport has been studied in depth, and characterized in bacteria[113,115] erythrocytes[116,117], L1210 mouse leukemic cells[118], hepatocytes[119], and bone marrow cells in culture[114]. These transport systems have been characterized as carrier mediated, energy dependent, and partially sensitive to high anion concentration[120].

In isolated hepatocytes grown in different concentrations of 5-methyl-H_4PteGlu, transport included two components: one saturatable at low 5-methyl-H_4PteGlu levels and another non-saturatable at concentrations of 5-methyl-H_4PteGlu as high as 20 μM[119]. Both of these systems appeared to be inhibited by metabolic inhibitors (2, 4-dinitrophenol, NaCN, and

sodium arsenate). The saturatable component of these cells was shown to have a concentration for 1/2 maximum velocity of transport of about 0.9 uM and was inhibited slightly by PteGlu[119].

Efflux of 5-methyl-H_4PteGlu from hepatocytes appeared to proceed by a mechanism separate from influx[119]. When hepatocytes were grown in medium deficient in vitamin B_{12} the efflux mechanism was twice as rapid as in normal cells, whereas the influx was unaffected[119].

A human family studied by Branda and co-workers developed folate-induced remission of aplastic anemia[121]. They appeared to have a familial defect in uptake of 5-methyl-H_4PteGlu in cultured lymphocytes and bone marrow cells, but PteGlu transport appeared normal[121].

The purpose of the study described in this thesis was to study folate accumulation in normal human fibroblasts and to relate this accumulation and distribution of folate forms to the growth of the cells.

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CHAPTER 1

DETERMINATION OF INTRACELLULAR FOLATES IN HUMAN FIBROBLASTS

INTRODUCTION

The purpose of the study was to utilize human fibroblasts cultured in vitro, to determine folate accumulation and turnover, when the cells were grown in medium containing PteGlu. Although PteGlu is not a naturally occurring compound, it was used because of stability and as a marker for metabolic alteration of intracellular folate.

In the past, attempts were made to determine chain length of polyglutamate folates in cells by cleaving the C-9, N-10 bond of the various folates by either zinc acid reduction[1] or potassium permanganate oxidation[2]. Cleaving of the C-9, N-10 bond was done to convert the mixture of folates present into a pteridine and a mixture of p-aminobenzoylglutamate$_n$ differing only in glutamate chain length. Two recent reports have indicated that these techniques do not reproducibly break this bond in all folates, and that PAB-glutamates may not be stable under these conditions[3,4].

Other methods used in the determination of polyglutamate chain length include gel filtration[5,6] ion exchange chromatography[7], thin layer chromatography, high voltage electrophresis[8], and high pressure liquid chromatography (HPLC)[9].

The method used to separate polyglutamates and monoglutamates in our studies was gel filtration, utilizing Sephadex G-25.

Sephadex chromatography separates the various folates both by molecular size and adsorption to the gel. Because of adsorption of the various folates to the gel, actual glutamate length is hard to discern, but the procedure reliably separates long polyglutamates from monoglutamates[5,6].

Materials and Methods

PteGlu labelled with ^{3}H (3' 5', 7, 9: specific activity 0.5, 5 and 45 Ci/mmole) and with ^{14}C (2-^{14}C; specific activity 50 mCi/mmole) was purchased from Amersham-Searle Corp., D, L-5-formyl-H_4 PteGlu from Lederle Products Department, American Cyanamid of Canada Ltd., Aquasol scintillation cocktail from New England Nuclear Corp., and PteGlu and 5-methyl-H_4PteGlu without radioactive label (reagent grade) were purchased commercially.

[^{3}H] PteGlu used in some studies was purified by stepwise elution with ammonium bicarbonate from DEAE cellulose[10]. [^{3}H] PteGlu used in other studies was not purified before use. It contained 6% of impurities when examined by DEAE cellulose chromatography. The impurities when filtered on Sephadex G-25 were found to elute between polyglutamate and monoglutamate folates but were not observed in the cell extracts. The small proportion of contaminants found in the medium were therefore not included in the calculations pertaining to the intracellular folate levels.

Cell Culturing

Normal human fibroblasts, strains MCH23 and MCH38 were obtained from the Repository for Human Mutant Cell Strains, Montreal Children's Hospital.

The cells were cultured at the Montreal Children's Hospital under the supervision of Dr. D. Rosenblatt. The cells were kept frozen in liquid nitrogen in 0.5 ml glycerol media (10% glycerol in MEM) at a concentration of 1.0 - 2.0 x 10^6 cells 1.2 ml cryule glass vial. The cells were then thawed and grown in petri dishes (Falcon) of 100 cm^2 surface area containing 10 ml of medium in an atmosphere of 5% CO_2 and 95% air[11]. The fibroblasts used in these experiments had gone through 20-23 generations, with a total life span of 48 generations. For some experiments fibroblasts were grown in roller bottles of 690 cm surface area, containing 50-100 ml of medium and the cells harvested with 0.25% trypsin as previously described[11]. All cells were determined to be free of Mycoplasma contamination by incorporation of ^3H uridine and ^3H uracil into RNA as described by Snider et al[12].

The standard medium consisted of Eagles minimal essential medium plus nonessential amino acids (Grand Island Biological Co.) but lacking PteGlu. To this standard medium were added as indicated PteGlu and 10% fetal calf serum (Gray Indust. Inc.) dialysed for 20h. against a 10 fold volume of 0.9% sodium chloride with two changes at 4 degrees C. The cells were rapidly rinsed four times in Dublecco s phosphate buffered saline solution (pH 7.4) and then incubated for sixty min at 37 C in phosphate buffered saline. They were then released from the surface of the dishes with a rubber policeman in 0.05M potassium phosphate buffer (pH 9.2) supplemented with 10 mM mercaptoethanol.

Cellular Extraction

Harvested cells were sonicated four times at maximum power in a Bronson Sonifier for 30 sec, heated to 100 C forl0 min, centrifuged at 700g for 10 min and the supernatant collected. This supernatant

was assayed or chromatographed immediately following sample extraction without storage.

Sephadex G-25 Chromatography

Two ml aliquots of cell extracts were applied to columns (1.6 x 36 cm) of Sephadex G-25, fine grade. They were filtered with 0.1M ammonium bicarbonate (pH 8.2) at room temperature at a flow rate of 60 ml/h. Forty, 5 ml samples were collected.

Usual filtration time was 200 min. Columns were standardized with blue dextran, (Blue Dextran 2000 Pharmacia Co.), $^{3}H\ H_2O$, 5-methyl-H_4 PteGlu, 5-formyl-H_4 PteGlu, PteGlu and with two folate polyglutamate preparations: an extract of Lactobacillus casei grown for 18 h in ^{3}H PteGlu, and authentic $PteGlu_5$ (kindly supplied by C.M. Baugh, Dept. of Biochemistry, Univ. of Southern Alabama, Mobile). The collected fractions of fibroblast extracts filtered through Sephadex G-25 were assayed microbiologically before and after conjugase treatment. The microbiological assay of intracellular folates used in the study was conducted by Ms. Sally Lue-Shing[13]. The assay organism used was Lactobacillus casei, ATC 7469 and Pediococcus cerevisiae, ATCC 8081. These organisms were maintained in maintenance medium (Difco Lab) and subcultured weekly.

Polyglutamates were deconjugated to monoglutamates by treating all the fractions collected with 10% (vol/vol)[1] folate depleted human serum for 90 min. at 37°C and pH 4.5[13,14]. The pH was then adjusted to 6.9 and .8 ml of each fraction was mixed with concentrated assay medium containing the assay organism, and incubated without precipitation of protein[13]. Standards and controls were assayed in a similar fashion. For the microbiological assays, care was taken to ensure that the samples

were diluted into the range where growth of the microorganism was directly proportional to the folate concentration.

Breakdown of polyglutamate folates during the extraction procedure was tested by splitting an extract containing a known amount of radio-labelled polyglutamates into two samples; one placed in a dish containing cells and subjected to the entire extraction procedure while the other remained untreated. Both extracts were then filtered through Sephadex G-25 with folate polyglutamates and monoglutamates as well as PteGlu levels being analyzed by radioactive counts.

Results

Extracts of fibroblasts were fitered through Sephadex G-25, and the fractions were analyzed both by radioactive counting and micro-biological assay before and after conjugase treatment to determine the folates present. Folate extracted from fibroblasts and from the gel refiltered as polyglutamate folate, when tested without exposure to conjugase and as monoglutamate when treated with conjugase. (Fig. 1). These standardization procedures allowed identification of folates filtering in fractions seven to eleven (Kd= 0)[15] as those probably with more than three glutamate residues per molecule (based on failure to support growth of L.casei without conjugase treatment) those filtering in fraction thirteen to seventeen (Kd = .8) as reduced monoglutamates, (with possible contamination by short chain polyglutamates) and those fractions 21-25 as PteGlu (Kd = 2.2) (Fig. 2).

As can be observed from Table 1, when a known amount of polyglutamates were exposed to our extraction procedure there was approximately 3%

(SD ± .5) breakdown of polyglutamates to monoglutamates.

Discussion

Although the presence of polyglutamate folates in cells has been reported for more than thirty years, studies to examine the function of these materials in cells have been delayed by the absence of a reliable, generally accepted technique for preservation of polyglutamates during extraction of cells, measurement of polyglutamates, and determination of chain length. The objective of this study was to test various procedures for the determination of polyglutamate folates in human cells.

The technique of gel filtration (using Sephadex G-25) separates folate both by molecular size, and by interaction of different pteridine structures with the gel [5,6]. In our studies, we have used this method to separate polyglutamates from folates with short chains. The similarity in mobility through the gel of monoglutamate standards and what we term intracellular monoglutamates suggests that contamination of the latter by short chained polyglutamates is not probable. All polyglutamate folates filtered in the excluded volume: $PteGlu_5$ filtered in this position although the oxidized unsubstituted pteridine appears to have a high affinity for the gel, and PteGlu filtered in more than twice the volume of the gel. During the extraction procedure approximately 3% of the intracellular polyglutamates were found to breakdown into monoglutamates, indicating that the 25-40% monoglutamates found inside the cell were not totally derived by polyglutamate hydrolysis during extraction.

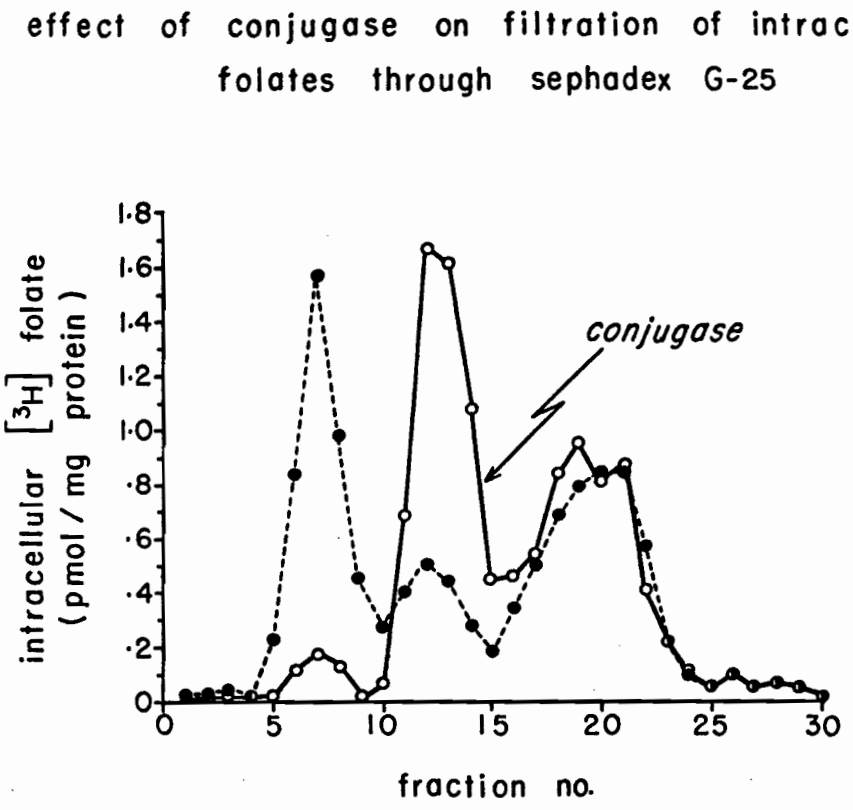

Figure 1. Effect of conjugase on filtration of intracellular folates through Sephadex G-25. Confluent fibroblasts were grown in 2.3 uM (^{3}H) PteGlu for 3 days. After extraction, half the extract was treated with serum conjugase for 90 min. (—o—) and the other half not (- - • - -). Extracts were filtered through Sephadex G-25 (1.6 x 36 cm) with 0.1 M ammonium bicarbonate (pH 8.2).

folate in fibroblasts after incubation in folic acid

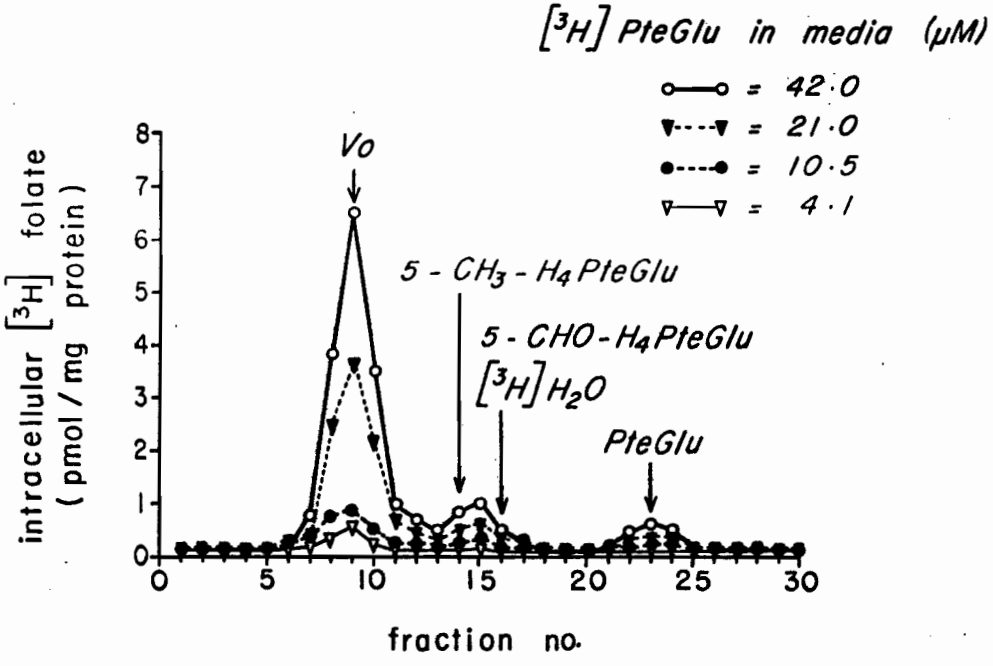

Figure 2. Folate in fibroblasts after incubation in PteGlu. Confluent cells were grown in (^{3}H) PteGlu for 24 h and extracts filtered through Sephadex G-25. Filtration position of Blue Dextran 2000, D,L-5-CHO-H_4 PteGlu, D,L-5-CH_3 H_4 PteGlu, PteGlu and (^{3}H) H_2O were also indicated.

Effect of extraction procedure on the distribution of polyglutamate and monoglutamate folate.

TABLE 1

	% Polyglu	% Monoglu
Without Extraction Procedure	71	29
With Extraction Procedure	68	32
% Breakdown of Polys to Monos by Extraction Procedure	3	

Figures represent the mean of 2 experiments each done in triplicate.

Mean coefficient of variation (C.V.) = 4.7%.

FOLATE REQUIREMENT OF HUMAN FIBROBLASTS

INTRODUCTION

To study folate metabolism in cells grown in inadequate and excess concentrations of PteGlu, the folate requirement of human fibroblasts was determined. It has been observed that normal folate levels in the serum are between eight and twelve ng/ml, with serum values of four ng/ml and below resulting sometimes in manifestations of folate deficiency[16,17]. In mouse L_{1210} cells it was observed that the nutritional requirement for optimal growth was forty-four ng/ml[18]. The purpose of this study was to determine the folate requirement for human fibroblasts so that comparative studies with other cellular systems could be made, as well as using the data to determine folate sufficiency in the fibroblast when the cell was exposed to various experimental conditions.

Methods

Human fibroblasts were maintained until used in medium containing 2.2×10^{-6} M unlabelled PteGlu. They were then replated and grown at low cell density in folate-free medium for six days. The fibroblasts were then equally distributed (10^5 cells/roller) into replicate roller bottles and subcultured in medium containing different concentrations of unlabelled PteGlu (10^{-9}-10^{-6}M). After the cells had grown to confluence at day 11, 10^5 cells were again subcultured into the same PteGlu concentrations. The cells were again allowed to grow to confluence and the same subculturing procedure as previously mentioned were carried out

at days twenty and thirty-two. On day fifty after the initial subculture had been performed, cells were harvested with trysin and counted in a Coulter electronic cell counter.

Results

The cell growth of fibroblasts appeared to be maximal when grown in PteGlu concentrations at 5×10^{-8} M or higher. Very little growth was observed with fibroblasts grown in 10^{-8}M, or 10^{-9}M PteGlu. The folate requirement of human fibroblasts cultured in vitro was higher than 10^{-8} M PteGlu and very probably close to 5×10^{-8} M PteGlu (Table 2).

Discussion

The minimum folate requirement of human fibroblasts in logarithmic growth was very close to 1×10^{-8}M PteGlu, which represents 4.4 ng/ml. With the folate concentration required for normal metabolism in human serum being above 4 ng/ml[16], it would appear that the folate requirement of growing human fibroblasts studied in vitro was very similar to the in vivo folate requirement found in human serum. In order to detect folate deficiency in the cells, it required 6 days in folate-free medium and subculturing into low concentrations of PteGlu (10^{-9}-10^{-8} M) for 4 passages. We have no data at the present time on folate requirements for confluent cells. The similarity between the human serum folate requirement and our cellular model system suggests that the human fibroblast in vitro might be a good model for in vivo folate metabolism, and comparison of folate metabolism in cells grown in excess and inadequate folate.

TABLE 2

Fibroblasts per dish after log growth in different concentrations of folate

(PteGlu) in medium (M)	10^{-6}	10^{-7}	5×10^{-8}	10^{-8}	10^{-9}
Cells x 10^{-5}	7.2	7	7	1.8	1.2

Cells were subcultured at low cell density in these concentrations on days 0, 11, 20, and 32. The number of cells per dish was determined on day 50.

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CHAPTER 2

DETERMINATION OF INTRACELLULAR CONCENTRATION OF ^{3}H FOLATE AND MICROBIOLOGICALLY-ACTIVE FOLATE

INTRODUCTION

In most *in vitro* cultured cell systems, as well as in intact whole animals, the folate uptake and accumulation of intracellular folate has been expressed in a weight to weight system (ng folate/mg cell protein), whereas the extracellular medium folate content has been expressed in a quantity to fluid volume more commonly termed concentration (moles folate/ml of medium, or in molarity). To express the amount of accumulated folate in cells in terms of concentration, the intracellular water content of human fibroblasts was determined.

MATERIALS

As described in "Chapter 1" ^{14}C-polyethylene glycol and $^{3}H_2O$ were obtained commercially.

METHODS

DETERMINATION OF INTRACELLULAR H_2O

10^5 human fibroblasts were placed in petri dishes containing 10 ml of phosphate buffered saline to which unlabelled polyethylene glycol had been added. ^{14}C labelled polyethylene glycol and ^{3}H-H_2O were then added to the petri dish and gently shaken for twenty minutes. The supernatant was then removed, measured and prepared for radioactive analysis. The

cells were then sonicated in 10 ml of phosphate buffered saline (P.B.S.) with the supernatant again being measured and prepared for radioactive analysis.

Calculations for Intracellular Water Content were as Follows

Total Volume of suspension - Extracellular = Intracellular (Extracellular+Intracellular)

$^{3}_{H} - H_2O$ $^{14}_{C-PEG}$

Conversion Of Cell Number To Mg Protein

Human fibroblasts grown to confluence in 2.2×10^{-6} M unlabelled PteGlu were harvested from roller bottles with trypsin. The cells were then washed with 10 mls of P.B.S. and analyzed on a coulter Counter to determine cell number. The cells were then centrifuged (700 x g) and the P.B.S. removed. 2 mls of potassium-phosphate KPO_4 buffer supplemented with 10mM mercaptoethanol were then added to the cells and the mixture was shaken well for five minutes. The cells were then sonicated and a sample was taken for protein determination as described by Lowry.

Calculations

x number of cells in 1 mg of protein = conversion factor. Conversion factor used was the average of twenty individually determined conversion factors.

Results

The conversion factors determined for the techniques of cell collection and washing used were as follows:

1 mg of protein = 3.16×10^{6} (S.D. 0.8×10^{6}) confluent fibroblasts

1 ml of cell water 1.09×10^8 (S.D. $.03 \times 10^8$) confluent fibroblasts

Therefore the concentration of folate in the cell equaled:

$$\frac{\#\text{ D.P.M.'S / Mg Protein}}{3.16 \times 10^6 \text{ cells}} = \#\text{ D.P.M.'S in 1 cell}$$

$$\frac{\#\text{ D.P.M.'S cell}}{9.2 \times 10^{-9} \text{ ml of } H_2O/\text{cell}} = \#\text{ D.P.M.'S/ml cell } H_2O$$

#D.P.M.'S / ml cell H_2O x S.A. of radiolabel = # moles ml cell H_2O;

moles / ml cell H_2O x 1000 = # moles/ litre cell water = Molarity.

The correlation coefficients (r values) reported in this thesis were obtained by the method of least squares. The coefficient of variation obtained in our data was calculated to be 5.4% as derived by using the equation listed below.

$$\frac{\text{Standard Deviation}}{\text{mean}} \times 100 = \%\text{ C.V.}$$

Discussion

We have determined the parameters required to express the amount of radiolabelled folate taken up and accumulated in the cell in terms of concentration. The techniques used to determine intracellular water content of human fibroblasts were similar to those described in determining internal vesicle volume of red blood cells[1]. The advantage of expressing internal radiolabelled folate in terms of concentration was that it may be compared to the concentration of radiolabelled folate in the incubation medium.

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CHAPTER 3

EFFECT OF EXTERNAL PteGlu ON INTRACELLULAR FOLATES

INTRODUCTION

With the description of mutant Chinese hamster ovary cells auxotrophic for glycine, adenosine as well as thymidine and defective in polyglutamate synthesis[1], the importance of polyglutamates in the cell was recognized. Since this time much effort has been directed to trying to understand the factors which regulate folate polyglutamate synthesis. Although accumulation of folate in rat liver has been studied with time[2], there is not sufficient data available to determine the effect of extracellular folate concentration on the synthesis of polyglutamates in human cells.

It also was of interest to determine whether the human fibroblast would selectively accumulate a particular type of folate when placed in medium containing a great excess or minimal concentration of PteGlu. It would seem probable that a folate deficient cell might selectively accumulate or convert all intracellular folates to the type which was necessary for cell survival. In a situation when the cell was exposed to a great excess of PteGlu, it would be expected that if there was a particular folate responsible for storage, it's presence in the cell should increase.

A similar approach has been undertaken by other investigators studying folate metabolism. Regenerating rat uterine tissue[3], and rat liver[4] as well as Physarum polycephalum[3], L_{1210} cells[5], human fibroblasts[6]

and lymphoblasts[5] have been studied to determine whether the cell selectively accumulates one type of folate when regenerating. Some of these studies demonstrated a marked increase in monoglutamates[3,4] during rapid proliferation when compared to resting cells; however others showed no such shifts[5,6]. In our study we compared confluent human fibroblasts with human fibroblasts in logarithmic growth when the rate of fission had begun to decrease (late log), at different PteGlu concentrations, to determine if there was a selective accumulation of polyglutamates or monoglutamates during cellular proliferation when compared to resting cells.

Methods

Cells grown to confluence and cells in late logarithmic growth (day 5) were cultured in medium containing 2.2×10^{-6} M unlabelled PteGlu. The cells were then placed in petri dishes containing medium supplemented with different concentrations of ^{3}H PteGlu (10^{-9} -5.4×10^{-5}M) for 24 hours. At the end of 24 hours the cells were harvested by scraping with a rubber policeman, sonicated, and filtered over Sephadex G-25.[7,8] Radiolabelled polyglutamate and monoglutamate folates as well as intracellular PteGlu concentrations were then calculated by the method described in Chapter 2.

Results

At all concentrations of ^{3}H PteGlu tested in the medium polyglutamates, monoglutamates and PteGlu could be found inside the cells and these were proportional to the PteGlu in the medium. Late logarithmic cells contained more folate

than did confluent cells (Table 1). In cells in the late logarithmic phase of growth (proliferating cells), polyglutamate represented 55.9 $\pm$ 0.09% (mean $\pm$ S.D.) of total intracellular folate, and this proportion was not affected by the external PteGlu concentration ($r=0.181$, $p=0.3$). In confluent cells, although the total intracellular folate was consistently less than in proliferating cells grown in the same PteGlu concentration, the proportion of intracellular folate which was polyglutamate was proportional to the concentration in the incubation medium ($r=0.962$, $p=0.0001$). The increase in the proportion of intracellular polyglutamate in confluent cells with increasing PteGlu concentration was noted when the confluent cells were grown in PteGlu concentrations greater than that required to maintain optimal logarithmic growth.

Discussion

From the study it is apparent that PteGlu concentrations in the incubation medium affected the amount of polyglutamates, monoglutamates and PteGlu found inside the cell (See Discussion, Para. 4 for evidence that folateswere intracellular). In cells grown in suboptimal external PteGlu concentration, and also cells cultured in a great excess of PteGlu, polyglutamates, monoglutamates and intracellular PteGlu were observed, with polyglutamates being the predominant cellular form (Fig. 1 and 2). Fibroblasts in logarithmic growth accumulated more folate than did confluent cells grown in similar PteGlu concentrations (Table 1). In late logarithmic cells (proliferating), the distribution of this intracellular folate into reduced folate monoglutamate and polyglutamate

was unaffected by PteGlu concentrations (Fig. 3). In confluent cells grown in excess PteGlu (0.1 M and more) however, an increasing proportion of intracellular folate was accumulated as polyglutamate (Fig. 3). This would suggest that as external PteGlu concentration increased, confluent cells stored the excess folate as polyglutamate, whereas in growing cells requiring folate, this storage did not occur. This observation supports the ratio level of monoglutamate to polyglutamate folates seen in regenerating rat uterine tissue and Physarium polycephalum[3]. Our study indicates that although human fibroblasts utilize both polyglutamates and monoglutamates, excess folate is stored as polyglutamate.

Effect of Proliferating and Non-Proliferating Cells on Intracellular Folates.

Introduction

The identification of the various phases of cellular proliferation involved with human fibroblasts was essential in the experiment conducted on the comparison of late logarithmically growing fibroblasts with cells grown to confluence. A study was also conducted on types of folate derivatives found in the cell at different stages of cell proliferation. Based on prior experiments comparing proliferating fibroblasts with confluent fibroblasts, our theory predicts that no polyglutamate: monoglutamate shift should appear as there should be no excess folate available for storage.

Methods

4×10^6 confluent fibroblasts were subcultured at low cell density in replicate roller bottles containing 2.3×15^6 M PteGlu. On successive days, cells were placed in 0.1 M ^{3}H PteGlu for 24 h, harvested with trypsin, and the intracellular radioactive folates analyzed by filtration through Sephadex G-25.

Results

When the cells entered early logarithmic growth (days 1, 2, 3, 4 and 5) there was a rapid accumulation of polyglutamates and monoglutamate folates during 24 h of incubation in ^{3}H PteGlu (Fig. 4). As growth rate decreased the rate of accumulation of polyglutamate and monoglutamate folate decreased towards the value obtained in confluent cells. The concentration of PteGlu associated with the cells was unaffected by the proliferation phase of the cells.

Discussion

The purpose of this study was to determine the effect of proliferating and non-proliferating cells on total folate accumulation. We found that confluent cells (subcultured at low cell density) in 10^{-7}M PteGlu entered late logarithmic growth at days 6 and 7 after the initial subculture at low density.

During logarithmic growth both polyglutamate and monoglutamate folate levels increased rapidly. This response was probably due to an increased level of folate binding in the cell, perhaps caused by an increased level of folate requiring enzymes during logarithmic growth[9, 10]. The enzymes which have been noted to have increased activity during logarithmic growth are dihydrofolate reductase serine hydroxymethyl-transferase, thymidylate synthetase and 10-formyl-H_4PteGlu synthetase[9].

In the experiments the ratio of polyglutamate: monoglutamate remained constant despite proliferation phase. This might have been due to the use of 10^{-7}M PteGlu in this experiment, a concentration below that at which we observed increased diversion of intracellular folate to polyglutamate in confluent cells.

TABLE I

Effect of external PteGlu concentration on intracellular folate concentration in confluent and proliferating fibroblasts

PteGlu in Medium (nM)	Intracellular Folate (nM)					
	Confluent Cells			Proliferating Cells		
	Polyglu	Monoglu	PteGlu	Polyglu	Monoglu	PteGlu
1	.10	.07	.12	.33	.17	.07
11	.94	.66	.31	1.39	.54	.43
44	1.67	1.34	.62			
88	2.89	2.36	.58	3.23	2.8	.67
220	5.5	1.75	1.0			
550				27.0	17.0	7.8
1100	56.7	18.5	2.31			
2100	76.3	12.9	9.39	157.0	107.0	50.3
4200				237.0	190.0	170.0
10500	510.0	56.7	56.7	578.0	162.0	111.0
21000	774.0	81.9	49.3	663.0	308.0	292.0
42000	972.0	144.0	83.4	1200.0	373.0	48.7

Data represent the mean of 3 experiments with mean coefficient of variance of 5.7%.

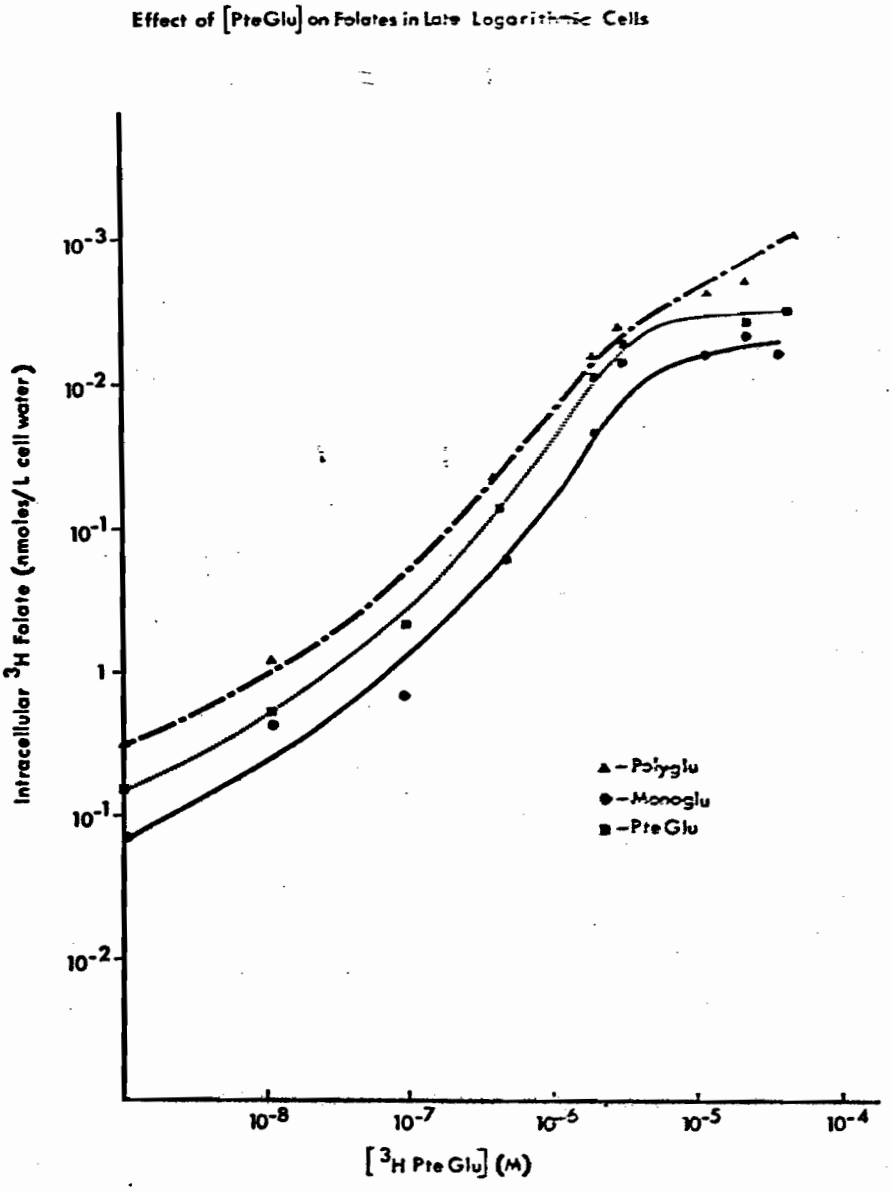


Figure 1. Fibroblasts in logarithmic growth phase were incubated in different concentrations of (^{3}H) PteGlu for 24 h. Polyglutamates, monoglutamates and PteGlu levels were determined by filtration through Sephadex G-25.

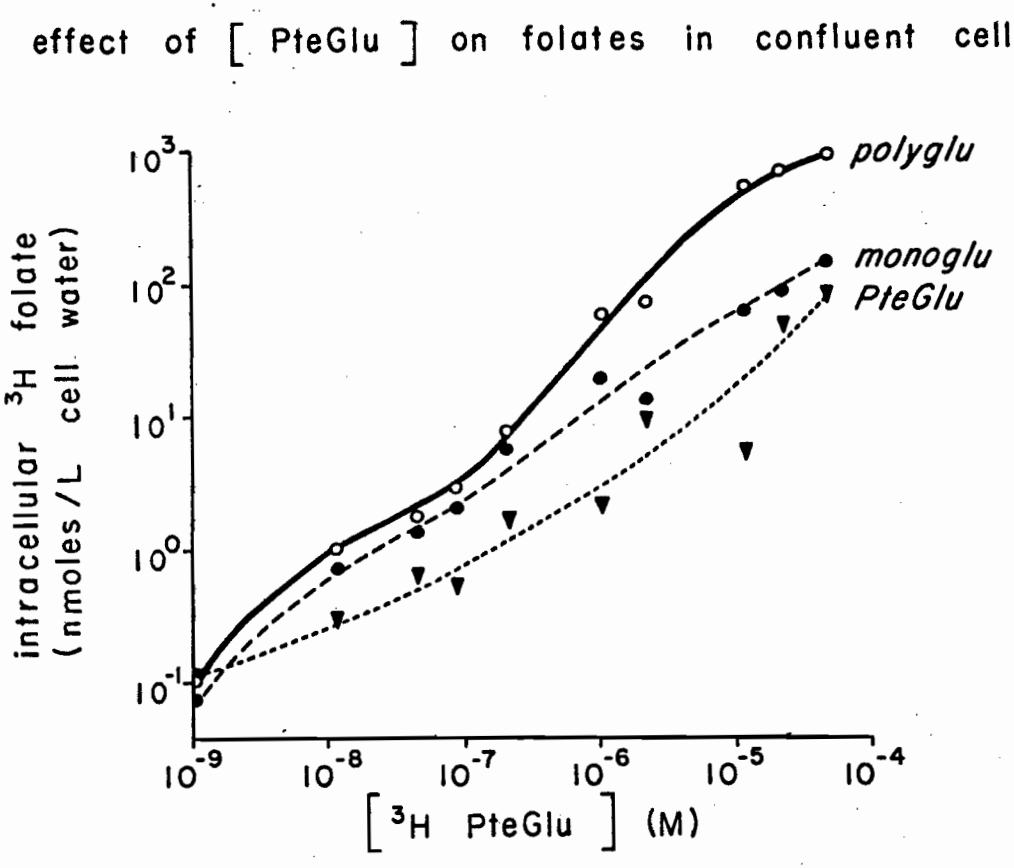


Figure 2. Confluent fibroblasts were incubated in different concentrations of (^{3}H) PteGlu for 24 h. Polyglutamates, monoglutamates, and PteGlu levels were determined by filtration through Sephadex G-25.

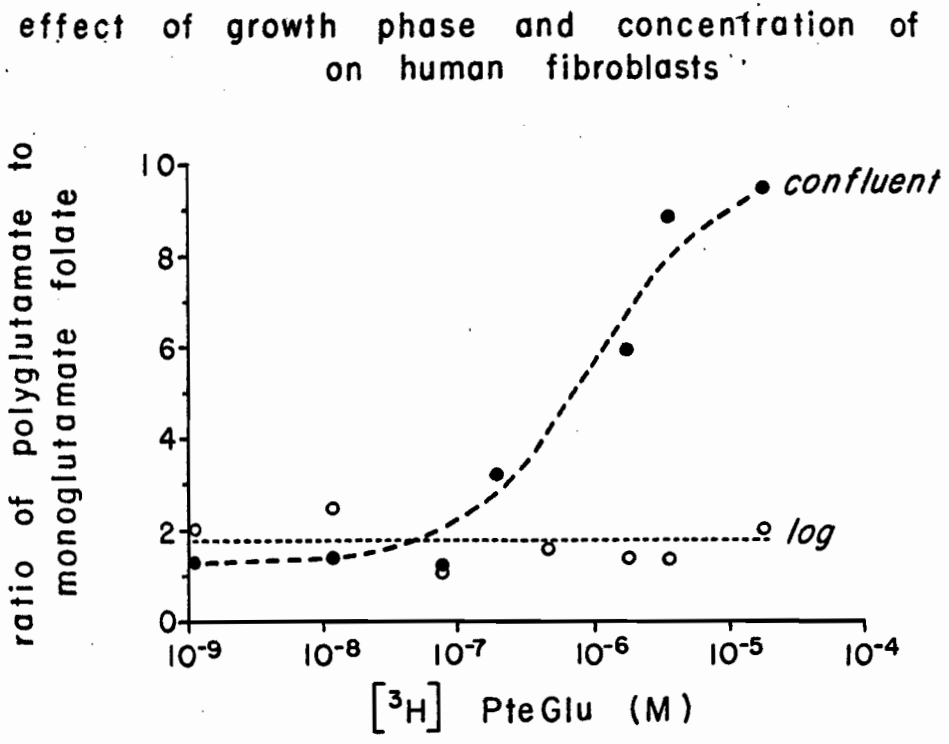
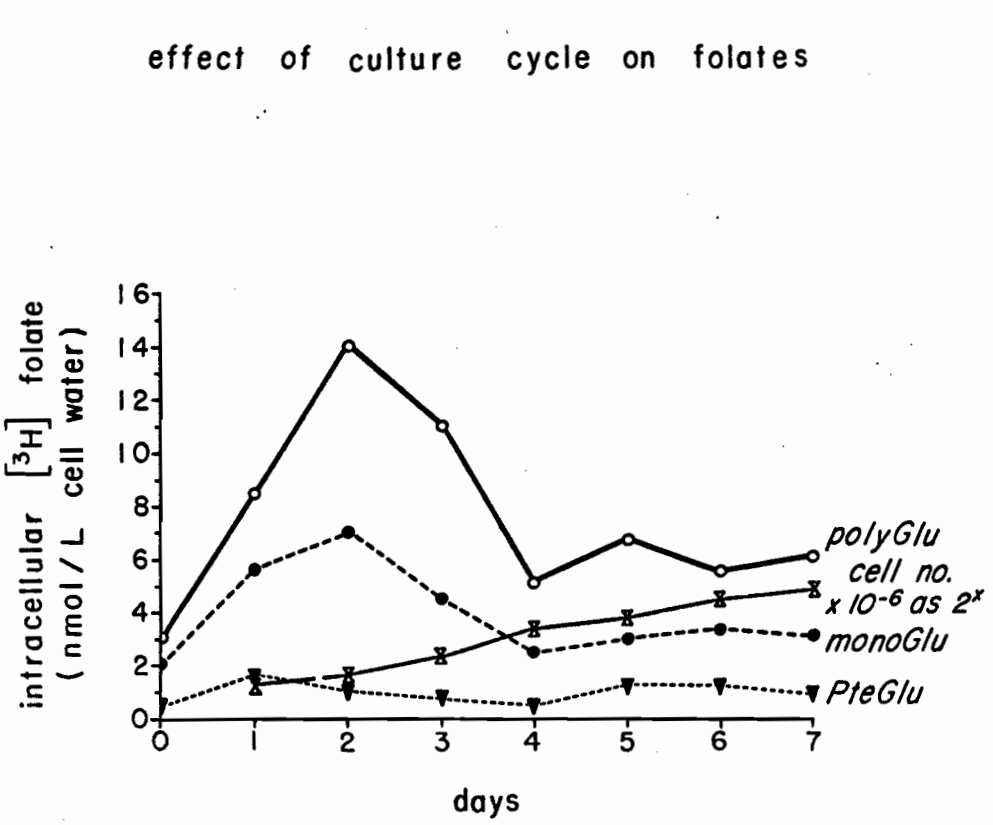


Figure 3. Fibroblasts in confluent and late logarithmic growth were incubated in different concentrations of (^{3}H) PteGlu for 24 h. Polyglutamates, monoglutamates and PteGlu levels were determined by filtration through Sephadex G-25. The ratio of intracellular concentrations of polyglutamates to monoglutamates are shown.



Data represent the mean of 2 experiments with a mean coefficient of variance of 4.1%.

Figure 4. Effect of proliferating and non-proliferating cells on folates. Confluent fibroblasts were subcultured at low density into replicate dishes and refed daily. Replications were incubated each day for 24 h in 1.0 uM (^{3}H) PteGlu. Intracellular folate was fractionated by filtration through Sephadex G-25.

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CHAPTER 4

EXCHANGEABLE FOLATE OF HUMAN FIBROBLASTS

INTRODUCTION

It has been reported that the majority of intracellular folate is bound by intracellular binders[1]. The probable source of this folate binding inside the cells has been attributed to the various folate dependent enzymes[1,2], and to other folate binders which have been described[3,4].

It occurred to our group that there might be a non-bound portion of folate inside the cell which represented folate which had not yet been bound by one of the intracellular folate binders. Since this portion of folate was not bound inside the cell, the possibility of this unbound folate being able to pass out of the cell was examined. The purpose of our study was to determine if such an unbound pool of intracellular folate existed in human fibroblasts and if so, what type of folates it contained.

Methods

Confluent fibroblasts were grown in medium containing different concentrations of ^{3}H PteGlu (10^{-9} - 10^{-5}M) for 24 h. The first incubation medium was removed and the attached cells were then washed 4 times with cold saline. A second folate-free medium was then added to the cells and incubation of the cells in the folate-free medium at 37°C was allowed to proceed for 1 h. The medium was then collected, heat treated, centrifuged (700 x g for 10 min) and filtered on Sephadex G-25. Radio-

labelled folates found in this medium were defined as exchangeable.

Results

When filtered through Sephadex G-25 the exchangeable folate obtained from cells grown in 10^{-6}-5.4 x 10^{-5}M ^{3}H PteGlu consisted of 23% polyglutamate folate, 15% monoglutamate folate, and 62% PteGlu (Fig. 1). Because of the low quantities of folate involved, determination of the folate derivatives found in exchangeable folate derived from cells grown in less than 10^{-6} M ^{3}H PteGlu was not possible. The total exchangeable folate associated with human fibroblasts represented only a minor portion of the total folate associated with the cells (about 3%). When the quantity of exchangeable folate was plotted against the concentration of ^{3}H PteGlu in which the cells were grown, a sigmoidal relationship was observed (Fig. 2).

Discussion

It would appear that there was a readily exchangeable pool of folate associated with the cell. Although not large when compared to total cellular folate in the cell (about 3%), it might have an important function, similar to the exchangeable pool of methotrexate associated with human fibroblasts, which has been suggested to be the pool utilized for the synthesis of methotrexate polyglutamates[5].

The total exchangeable folate appeared to increase in a sigmoidal fashion when compared to the external ^{3}H PteGlu in which the cells were cultured. The sigmoidal curve might be explained as the sum of two

compartments: one saturating at 0.01 and the other at 13.0 nmoles of folate per litre of cell water. Since no other data is available at present, further speculation about this would not be productive.

The exchangeable folate of cells grown in 10^{-6} M - 10^{-5} M ^{3}H PteGlu contained 62% PteGlu, 23% polyglutamate folate and 15% monoglutamate folate. This observation might indicate that polyglutamate folate could pass through the cell membrane of fibroblasts. It has been found in L.casei that an active efflux mechanism for folate polyglutamates greater than 2 glutamates does not exist[6]. If polyglutamates were in fact able to pass through the cellular membrane, it would be very difficult to attribute their function in the cell solely to folate accumulation, by making storage folate unavailable for egress.

It is possible that the exchangeable folates we observed were derived from dying cells which lost their intracellular folates into the incubation medium. This is unlikely, as the cells were washed 4 x in the cold with buffer before being placed in the folate-free medium, therefore probably washing off any folates already attached to the cells surface. In further studies it was also observed that the movement of exchangeable folate into the incubation medium was time dependent, again indicating the exchangeable folate was not surface bound and explainable by cellular death expulsion of intracellular folates into the medium. The sigmoidal curve obtained when exchangeable folate was compared with external PteGlu concentration indicates that the two were not proportional to each other, as would be expected if exchangeable folate was attributable to a constant death rate of cells. Trypan blue exclusion studies indicated that 99% of the cells placed in folate-free medium for 7 days excluded the dye.

Velocity of Exchangeable Folate Release From the Cell

INTRODUCTION

Since there was in fact exchangeable folate associated with the cell, the question became, did the exchangeable folate come from within the cell or from on it's surface. If the exchangeable folate was in fact membrane associated, the absorbed folate should leave the cell immediately and it's presence in the folate-free medium should be time independent. A different situation than this would occur if the folate had to come from within the cell and cross the cell membrane to enter the folate-free medium. The accumulation of exchangeable folate in the folate-free medium in this situation would be time dependent. The purpose of this study was therefore to study the time efflux of exchangeable folate into folate-free medium so that evidence might be presented which would indicate whether the exchangeable folate was membrane associated or not.

Methods

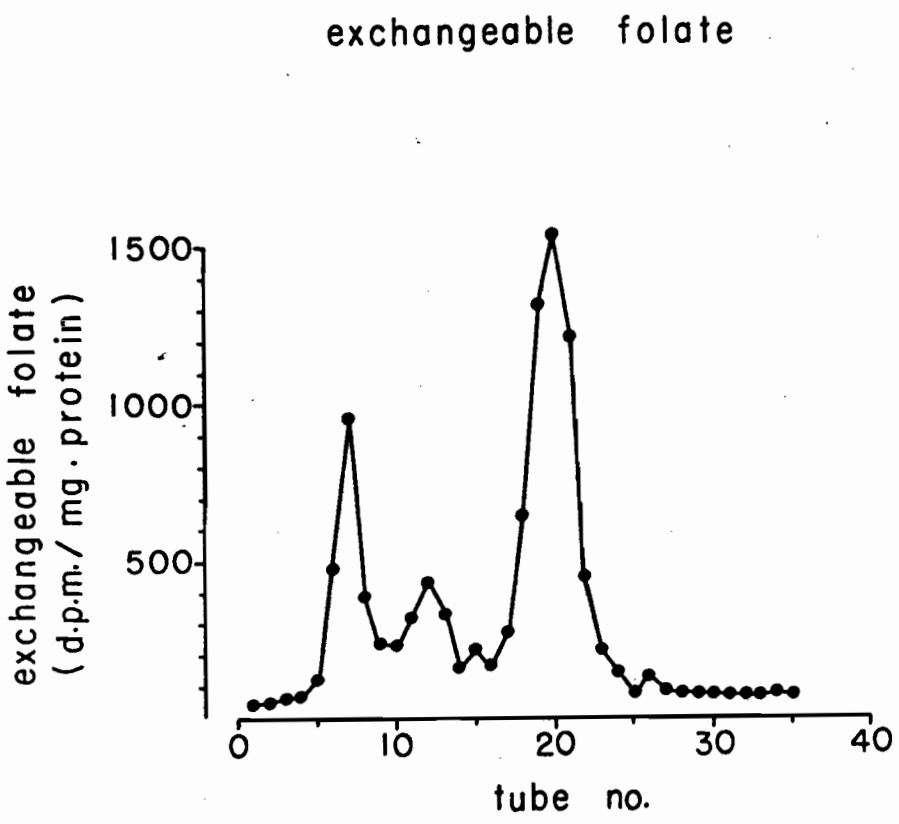
Confluent fibroblasts were grown in medium containing 10^{-7} M ^{3}H PteGlu for 24 h in replicate petri dishes. The first incubation medium was then removed, with subsequent washing of the cells 4 times with cold saline. Folate-free medium was then added to the petri dishes and allowed to incubate for different time periods both in the cold and at 37°C. Folate-free medium was analyzed for radiolabelled folates at each of the different incubation times.

Results

Radiolabel entered the folate-free medium in an exponential manner and accumulated with time, until a time period of 15 minutes, when its level in the medium became relatively constant (Fig. 3). Because of the minute amounts of the various folates leaving the cell at the time periods examined, we were not able to determine whether PteGlu left the cell more rapidly, then followed by monoglutamates and finally by polyglutamate folates. There was no significant change in folate efflux time when studied in the cold or at 37°C.

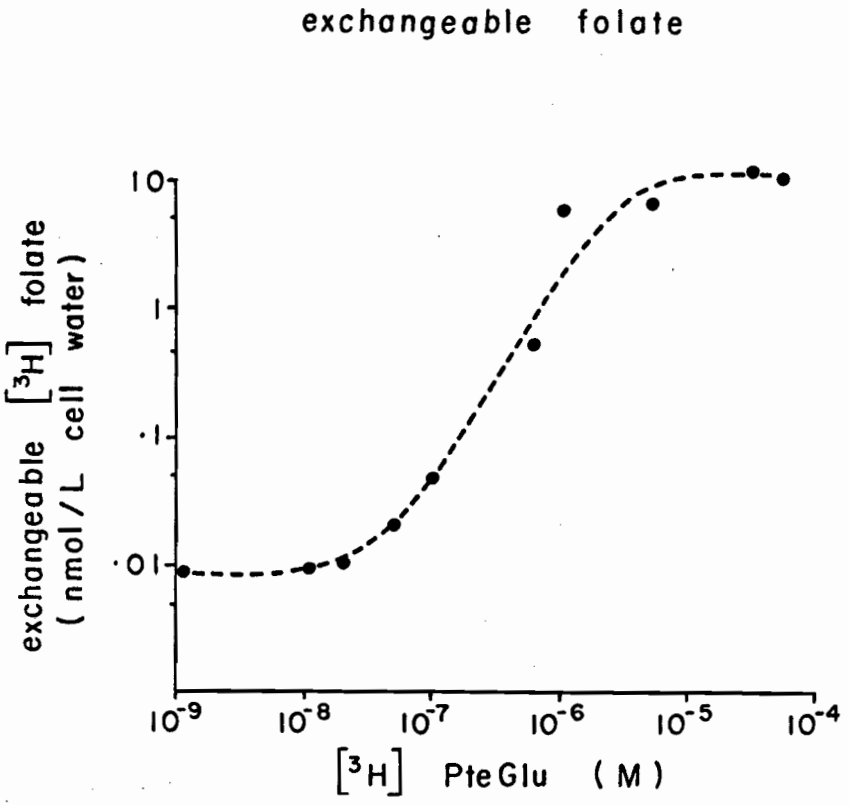
Discussion

The efflux of folate into folate-free medium was time dependent. This could indicate that the exchangeable folate was in fact coming from within the cell and penetrating the cell membrane. It is possible that there is another way of interpreting these results. Folate absorbed onto the surface of the cell located on membrane infoldings could be responsible for the time period of folate efflux. During cold treatment the cell membrane infoldings become larger. One would expect based on this, that if folate was indeed trapped on the surface of membrane infoldings, that folate efflux would be longer in the cold than at 37°C. Since there was no difference in folate efflux either in the cold or at 37°C, it is difficult to believe that the folate was located on membrane infoldings. It is however, possible that the folate entry into the folate-free medium could be accounted for by folate located in different levels of the membrane. Data are not available to justify further speculation about where the exchangeable folate may have come from.



Data represent the mean of 3 experiments with mean coefficient of variance of 4.7%

Figure 1. Exchangeable folate found in the folate-deplete medium. Following incubation in 10^{-6}-5.4×10^{-5} M (^{3}H) PteGlu for 24 h, confluent fibroblasts were washed 4 times in the cold and placed in folate-deplete medium for 1 h. The folate-deplete medium was then collected, heat treated, and filtered through Sephadex G-25.



Data represent the mean of 3 experiments with mean coefficient of variance of 5.4%.

Figure 2. "Exchangeable Folate" associated with cells after incubation for 24 h in different concentrations of PteGlu. Following incubation for 24 h, cells in dishes were rinsed four times with cold saline, and incubated in folate-free medium for 1 h at 37 deg C. Folate entering the second incubation medium is plotted against the PteGlu concentration in which the cells were incubated.

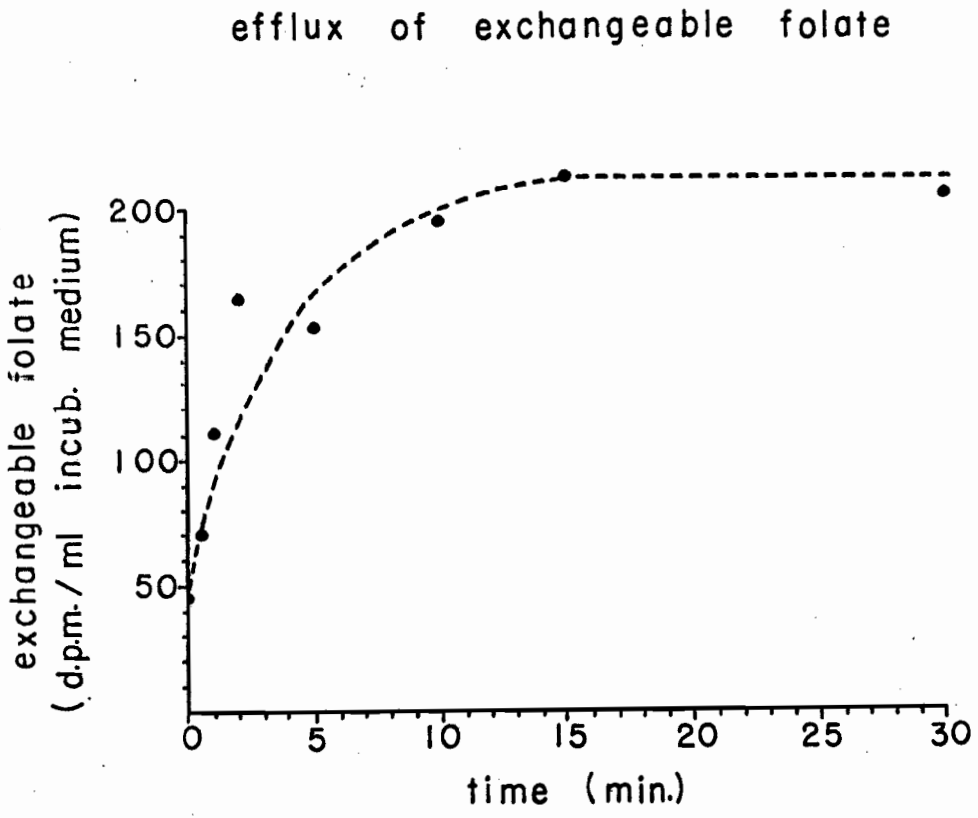


Figure 3. Appearance of exchangeable folate found in the folate-deplete medium with time. Following incubation in 10^{-7} M (^{3}H) PteGlu for 24 h, confluent fibroblasts were washed 4 times in the cold, then placed in folate-deplete medium for different time periods. The folate-deplete medium was then collected and measured for radiolabel.

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CHAPTER 5

EFFECT OF TIME ON ACCUMULATION OF INTRACELLULAR FOLATES

Introduction

It has been observed that liver cells accumulated polyglutamate folate as early as 4 hr after exposure to injected PteGlu[1]. Folate has also been found to accumulate in the cells with time when they are exposed to exogenous PteGlu[1]. Injected PteGlu reached equilibrium with endogenous liver folate polyglutamates very quickly (3 day) in guinea pig or rat liver[1,2] and very slowly in monkey kidney, where even after three days, full equilibrium had not taken place[3].

The purpose of our study was to study the accumulation of intracellular folates in human fibroblasts when exposed to PteGlu for different time periods. Intracellular folate levels were also studied with time to determine whether the cells were able to concentrate intracellular folate to a concentration above that in the external medium. Cells grown in PteGlu and in folate-free medium prior to experiments were studied to determine whether the intracellular folate level in the cell prior to experiments would affect the rate and amount of radiolabelled folates accumulated with time.

Methods

Human fibroblasts were depleted of intracellular folate by incubation of confluent cells in folate-free medium for 7 days. Fibroblasts grown in unlabelled folate replete medium (2.2×10^{-6}M PteGlu) and fibroblasts

depleted of intracellular folate were incubated in medium containing 10^{-7} M PteGlu. The cells were harvested at different time periods, with intracellular folate polyglutamates and monoglutamates as well as PteGlu determined by filtration chromatography utilizing Sephadex G-25.

Results

Folate depleted and replete human fibroblasts were found to accumulate polyglutamate and monoglutamate folate with time (Fig. 1 and 2). Folate polyglutamates could be detected as early as 4 h after subculture in ^{3}H PteGlu, and it's rate of accumulation exceeded that of accumulation of labelled monoglutamate after 4 h of incubation. PteGlu was found to accumulate in the cell until 8 h of incubation in folate depleted fibroblasts (Fig. 1)and for 24 hr in replete fibroblasts (Fig. 2), after which time it's level in the cell decreased.

Folate-depleted fibroblasts accumulated radiolabelled folate at a greater rate than did non-starved fibroblasts. Total intracellular folate concentration increased rapidly in both folate depleted and replete fibroblasts until a 3-5 days of culture, at which time accumulation increased very slowly. Neither folate depleted nor replete cells were able at any time measured to accumulate their intracellular folate to a concentration greater than that of the PteGlu in the incubation medium (Fig. 3).

Discussion

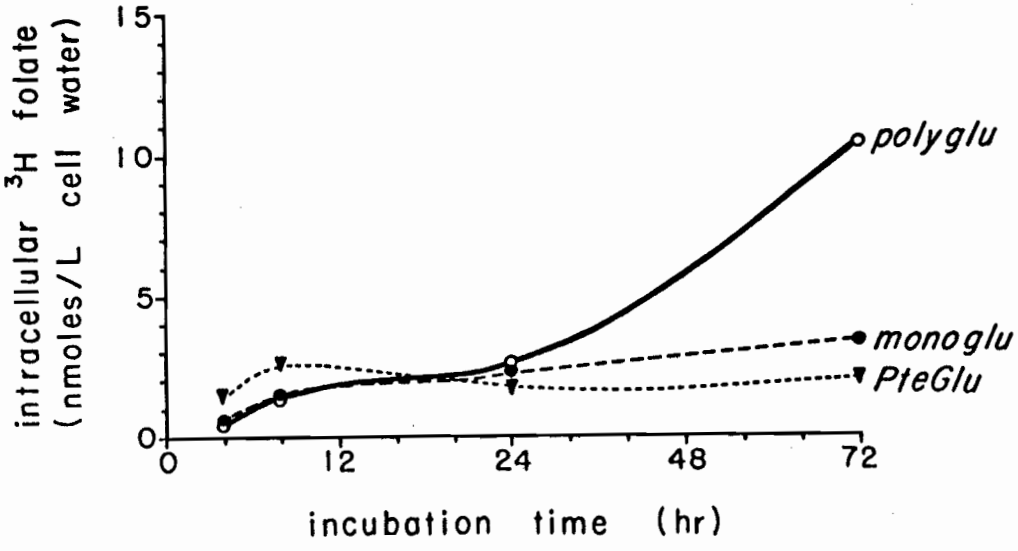
Unlike bacteria[4], lymphocytes[5], and red blood cells[6], as well as L.casei[7]

human fibroblasts cultured in vitro did not accumulate intracellular folate to a concentration greater than that of the PteGlu in the culture medium (See Discussion, Para. 4 for evidence folate was intracellular) The appearance of folate polyglutamates as early as 4 h after initial subculture was in agreement with the observations of Corrocher and co-workers who similarly found folate polyglutamates in liver at 4 h.[1]

The decrease in PteGlu levels after 8-24 h could be explained by reduction of newly transported PteGlu into reduced monoglutamates by dihydrofolate reductase. The increase in polyglutamates within the cell may be explained as the product of polyglutamate synthesis, with subsequent binding to intracellular folate binders.

Folate-depleted fibroblasts accumulated labelled folate derivatives at a greater rate than did non-starved cells. It would appear then that the intracellular folate levels of the cells had an effect on the rate of accumulation of the labelled folates intracellular derivatives, however the pattern of accumulation appeared to be similar in both starved and non-starved cells.

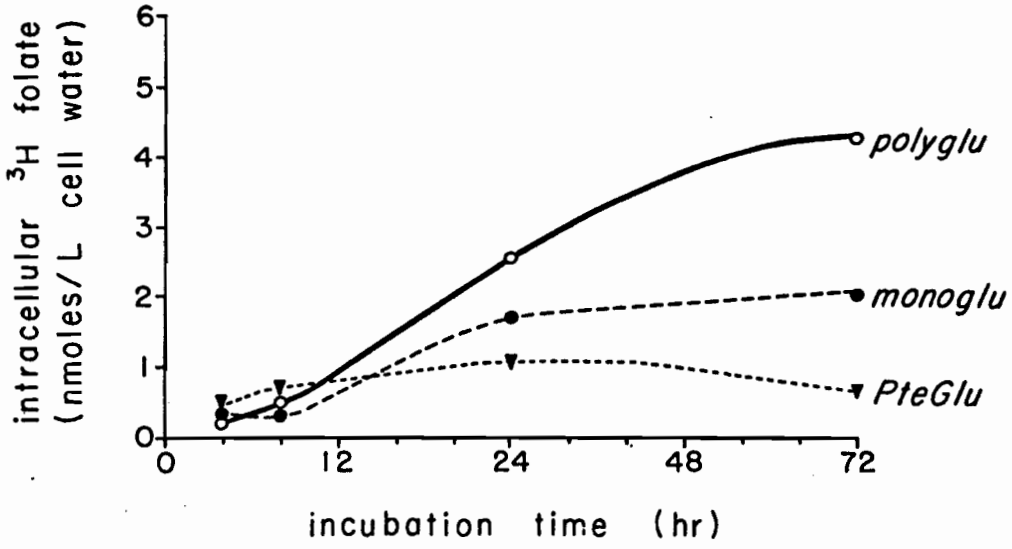
folate accumulation in starved human fibroblasts



Data represent the mean of 2 experiments with a mean coefficient of variance of 5.4%.

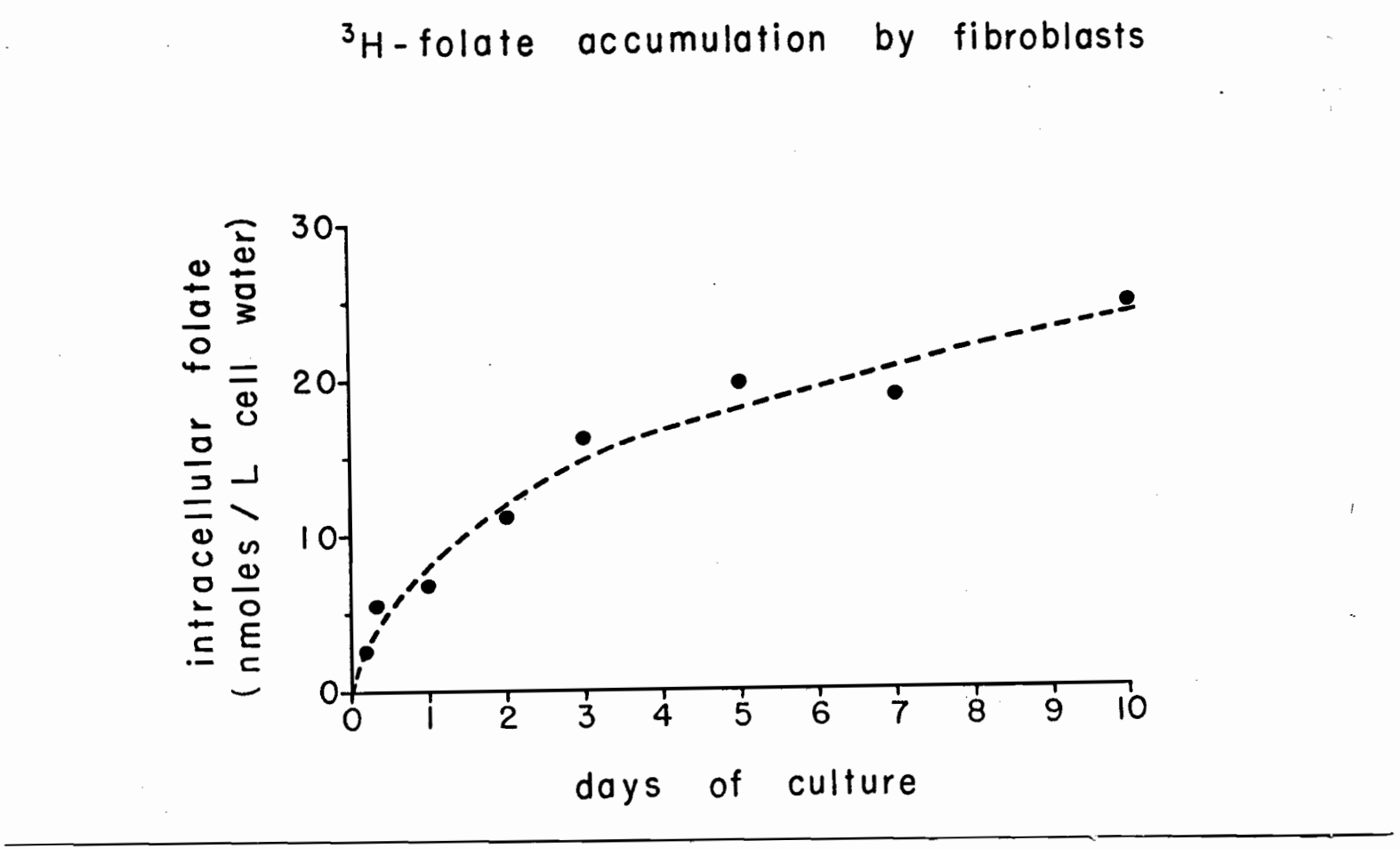
Figure 1. Accumulation of folate in fibroblasts. Following incubation in folate-free medium for 7 days, confluent fibroblasts were incubated in 10^{-7} (^{3}H) PteGlu, extracted at intervals and polyglutamates, monoglutamates, and PteGlu measured by filtration through Sephadex G-25.

folate accumulation in non-starved human fibroblasts



Data represent the mean of 4 experiments with a mean coefficient of variance of 4.2%.

Figure 2. Accumulation of folate in fibroblasts. Following incubation in folate replete medium, confluent fibroblasts were incubated in 10^{-7} M (^{3}H) PteGlu, extracted at intervals and polyglutamates, monoglutamates and PteGlu measured by filtration through Sephadex G-25.



Data represent the mean of 2 experiments with a mean coefficient of variance of 5.4%.

Figure 3. Accumulation of folate in fibroblasts. Following incubation in folate-free medium for 7 days, confluent fibroblasts were incubated in 10^{-7}M (^{3}H) PteGlu, extracted at intervals and total folate measured.

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CHAPTER 6

EFFECT OF FOLATE DEPLETION ON HUMAN FIBROBLASTS

Introduction

The time required for 1/2 the injected PteGlu to disappear in rat liver has been reported by two laboratories to be 3 and 5 days respectively[1,2]. The possibility of studying the turnover of the different folates in human fibroblasts became very interesting to us, as it could have indicated which form of folate was preferentially used by the fibroblast. The purpose of our study was to monitor the disappearance of folate from human fibroblasts cultured in folate-free medium and determine if one particular type of folate was turning over at a faster rate than others.

Methods

Confluent human fibroblasts were cultured for 10 days in medium containing 10^{-7} M PteGlu. The cells were then reincubated in folate-free medium which was changed daily. The cells were harvested at different times after subculture into the folate-free medium and their intracellular folates analyzed by filtration through Sephadex G-25.

Results

Total intracellular folate decreased with duration of time in the folate-free medium. Polyglutamate folate disappeared from the cell in an exponential fashion with a T 1/2 of 3.5 days, and represented an average loss per day of 20%. (Fig. 1). Monoglutamate folate levels remained

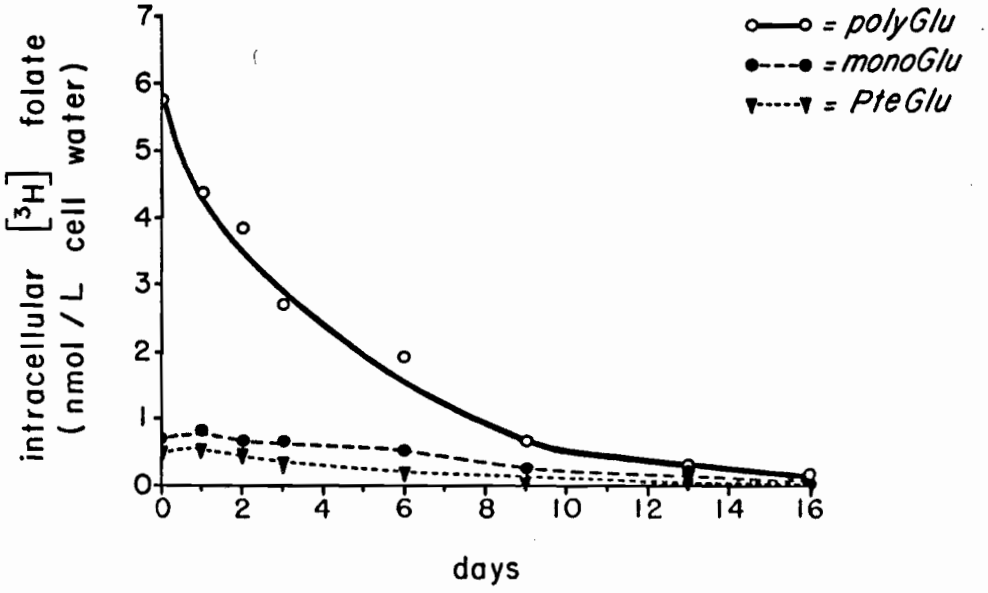
relatively constant for 3 days, after which its levels in the cell decreased. It was noted that the intracellular monoglutamate concentration began to decrease at an intracellular folate concentration of approximately 2 nmolar. This was the same intracellular concentration observed in cells grown in 50 nM PteGlu, the lowest PteGlu concentration which supported optimal growth. PteGlu levels in the cell fell after 1 day of incubation in folate-free medium. Trypan blue exclusion studies indicated that after 7 days in folate-free medium, 99% of the cells excluded trypan blue.

Discussion

From our study it was obvious that polyglutamate folates disappeared quickly from the cell (See Discussion, Para 4 for evidence folate was intracellular). It is curious that the T 1/2 of polyglutamate disappearance from human fibroblasts (3.5 days) was very similar to the total folate turnover observed in rat liver (3 days)[1]. The explanation of monoglutamates remaining constant after 3 days of deprivation was very complex. It might be explained by the fact that the cell was expelling stored polyglutamate and monoglutamate folate from the cell at a rate which was indicative of the affinity of the efflux transporting protein for the different folates. Another explanation was that polyglutamates were being broken down into monoglutamates and then expelled from the cell. However, since so little polyglutamate was broken down to monoglutamate this explanation seems inadequate.

It is difficult to determine what in fact the disappearance of the radiolabelled folate represents. It may represent turnover of the different folates on their particular enzymes with subsequent removal from the cell, or it may represent the degradation of the folate dependent

loss of folate from human fibroblasts



Data represent the mean of 3 experiments with a mean coefficient of variation of 3.2%.

Figure 1. Confluent fibroblasts were grown for 10 days in .1 μM (^{3}H) PteGlu and refed daily with folate-free medium. Replicates were harvested daily and analyzed for retained folates.

enzymes, which then released bound folate into the cytosol with subsequent removal from the cell. Because of the reasons presented above it is difficult to say whether one particular type of folate was in fact turning over faster than another.

Examination of New and Old Folate During the First Three Days of Folate Starvation

Introduction

The concept of polyglutamates being built by the addition of single glutamate residues onto monoglutamate folates by the enzyme polyglutamyl synthetase has been shown in various laboratories[3]. The reverse process has never been shown inside the cell. Murphy and coworkers have presented evidence in the rat which indicates that folate polyglutamates are cleaved at the C-9, N-10 bond, with subsequent expulsion of the remaining pteridine and $PABAGlu_n$ out of the cell[4].

Methods

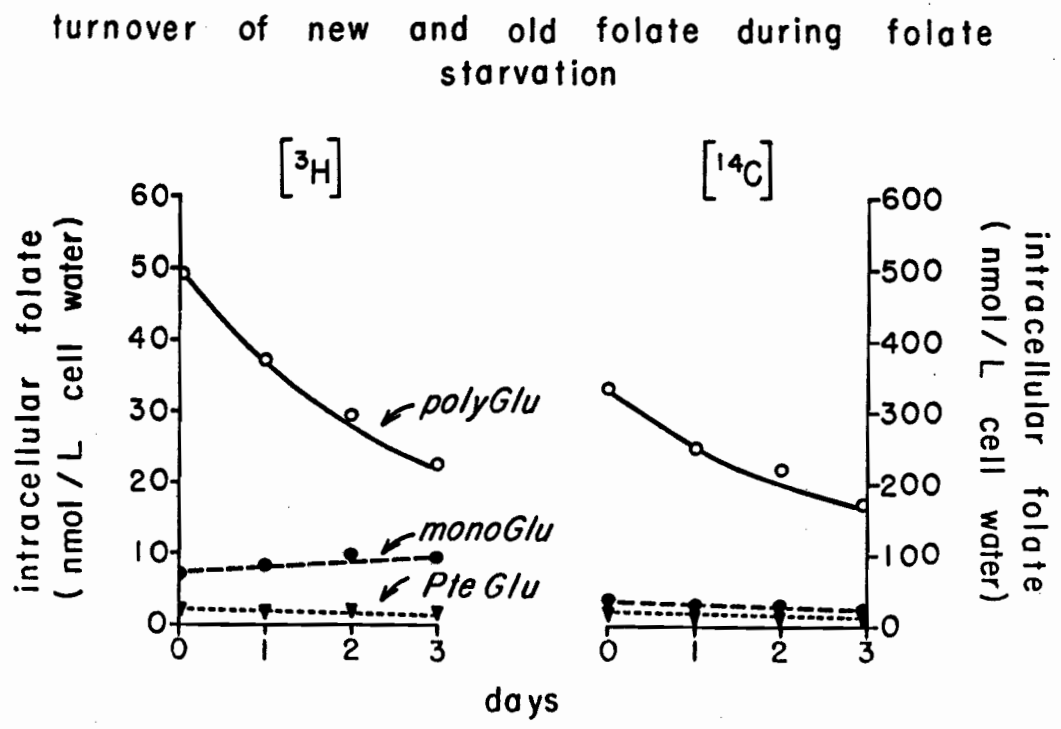
Confluent fibroblasts were grown in 1 μM ^{14}C PteGlu for 5 days. Cells were then washed 4 x in the cold with phosphate buffered saline and incubated in 2 μM ^{3}H PteGlu for 1 day, washed once again and reincubated in folate-free medium. The folate-free medium was changed daily. The cells were harvested at different time periods during folate deprivation and their intracellular folates analyzed by filtration through Sephadex G-25.

Results

Total intracellular folate decreased as observed in experiments using a single isotope. Polyglutamates labelled with both isotopes decreased at similar rates (19.1 and 22.6 per day, $p = 0.95$), as ^{3}H monoglutamate folates increased and ^{14}C monoglutamates decreased. The increase of ^{3}H monoglutamate folates (.067 nmoles per litre per day) was greater than the decrease of ^{3}H labelled PteGlu associated with the cells (0.03 nmoles per litre per day), limiting the source of newly labelled ^{3}H monoglutamate folates to ^{3}H labelled polyglutamates.

Discussion

The apparent sparing of intracellular monoglutamates during the first 3 days of folate depletion could have been due to the appearance of new monoglutamates from polyglutamates. Loss of monoglutamate folate from the cell with prolonged periods (over 3 days) of folate deprivation might have been caused by the amount of polyglutamates entering the monoglutamate pool being smaller than the concomitant loss of monoglutamates. This would result in the total amount of monoglutamates in the cell decreasing. The majority of polyglutamate folate which disappeared from the cell was however not converted to monoglutamate folate. Since the addition of the ^{14}C (old folate) and ^{3}H (new folate) gave the same folate disappearance rates as the single isotope study, it would appear that the mechanism responsible for the consistancy of the monoglutamates during the first three days of folate starvation was the same in both studies.



The data represent the mean of 3 experiments with a mean coefficient of variation of 4.2%.

Figure 2. Turnover of new and old folate in cells incubated in folate free medium. Confluent cells were incubated in (^{14}C) PteGlu for 5 days, then in (^{3}H) PteGlu for 1 day, and then in folate-free medium. Extracts were analysed at intervals by filtration through Sephadex G-25.

Efflux of Folate From the Cell During Folate Starvation

Introduction

The efflux of folate from the cell has been demonstrated in L.casei[5] and shown to include the transport of both diglutamates and monoglutamates from the cell in an energy dependent mechanism[5]. Recent studies in rat liver have indicated that polyglutamate folates are expelled from the cell by being broken at the C-9, N-10 bond into $PABAGlu_n$ and pteridine[4], with subsequent removal of both from the cell. The purpose of our study was to determine what folate derivatives, or breakdown products would be found in the incubation medium during deprivation of cells in folate-free medium. The expelled products found in the folate-free medium should provide useful information as to the mechanism involved when folate is lost from the cell.

Methods

Confluent human fibroblasts were cultured in replicate dishes with medium containing 10^{-7} M ^{3}H PteGlu for a period of one week. The cells were then washed 4 times in the cold and refed with folate-free medium. Cells were harvested at different time periods and their intracellular folates determined by filtration over Sephadex G-25.

Folate-free medium was also collected at different time periods during deprivation, heat treated, centrifuged (700 x g for 10 min) and chromatographed on a Sephadex A-25 column[7]. For elution of folate derivatives from Sephadex A-25, a 0.01-1 M KCL gradient was used. Ten ml samples were collected at a flow rate of 60 ml/h with 1 ml of

each sample prepared for radioactive determination. Sephadex A-25 colums were standardized with cellular extracts treated with and without serum conjugase, 5-methyl-H_4PteGlu, 5-formyl-H_4PteGlu, 10-formyl-H_4 PteGlu as well as PteGlu and identified by optical density. Cellular extracts of known polyglutamate and monoglutamate folate content were added to folate-free medium as well as folate-free medium containing cells for different periods of time. The medium was then collected, heat treated, centrifuged (700 x g for 10 min) and filtered on Sephadex G-25 to determine the amounts of polyglutamate and monoglutamate folate found. To determine the oxidation products of 5-methyl-H_4 PteGlu, H_2O_2 was added at pH 6 for 1 hour as described by Gapski et al[6]. Oxidation products of 5-methyl-H_4PteGlu were also examined by placing 5-methyl-H_4PteGlu in folate-free media in the presence of cells for 2 days. Oxidation products from both procedures were eluted on DEAE-Sephadex A-25 with a gradient of .01 - 1 M KCI gradient.

Results

Folate-free medium collected during deprivation of cells previously incubated in ^3H PteGlu contained both polyglutamate and monoglutamate folates. Radioactive peaks obtained from the folate-free medium when chromatographed on Sephadex A-25 eluted in the same positions as 5-methyl-H_4PteGlu, PteGlu, 5-formyl-H_4PteGlu, and a polyglutamate extract prepared from a human fibroblast extract. There was insufficient folate in each of the samples to analyze microbiologically, however it would be

anticipated that any cleaved folate (at the C-9, N-10 bond) would have been washed off the column. No such radiolabel was found washed from the column prior to the elution of folates by the KCL gradient and no radioactivity eluted in the PABGlutamate position. The largest radiolabelled peak eluted just prior to 10-formyl-H_4PteGlu. It is unknown what form this folate might be, as it did not elute from the column in the same position as did oxidation products of 5-CH_3OH_4PteGlu or 10-formyl-H_4PteGlu.

Folate-free medium supplemented with a known concentration of radiolabelled polyglutamates showed very little breakdown of the polyglutamates to monoglutamates during 2 days of incubation. Folate-free medium containing human fibroblasts and supplemented with a known concentration of radiolabelled polyglutamates showed considerable breakdown of polyglutamates to monoglutamate folates during 2 days of incubation. Folate extracted from fibroblasts refiltered as polyglutamate folate when tested without exposure to conjugase; and as monoglutamate when treated with conjugase.

Discussion

Both polyglutamate and monoglutamate folates were found in the folate-free medium during deprivation indicating that both are able to be expelled from the cell in the intact form. Because of the breakdown of polyglutamates to monoglutamates in the incubation medium with time, it is probable that the majority of the folate expelled from the cell was in the polyglutamate form. There was no PAB-glu found

bound to the column or radiolabel found in the wash, non-bound to the Sephadex A-25. It does not appear therefore that cleavage of the C-9, N-10 bond of folate with subsequent expulsion of the pteridine and $PABAGlu_n$ was the primary method of folate removal in the cell. Although it is unknown what type of folate is eluting prior to 10-formyl-H_4PteGlu, it appears to be neither an oxidation product of either 5-CH_3-H_4PteGlu or 10-formyl-H_4PteGlu. It appears to be the predominant form of folate monoglutamate in the folate-free medium, and perhaps is the same folate found in human bile[8]. Further speculation about the material should wait until sufficient amounts of the substance may be gathered to do appropriate identification studies.

Figures 3A and B. DEAE Sephadex A-25 chromatograph of folates found in folate-deplete medium. Following incubation in 10^{-7} M (^{3}H) PteGlu for 7 days, confluent fibroblasts were washed 4 times in the cold, then placed in folate-deplete medium for different time periods. The folate-deplete medium was then collected, heat treated and eluted on a DEAE Sephadex A-25 column. Folates were eluted from the column by a 0.01 - 1 M KCl gradient of KCl.

The controls indicated in Figure 3A and 3B were human fibroblast polyglutamate extracts added to folate-deplete medium, in the presence and absence of cells respectively and allowed to incubate for 2 days. The medium was then harvested, heat treated and chromatographed on DEAE Sephadex A-25.

folate-free medium containing cells pre-incubated with ^{3}H-folate

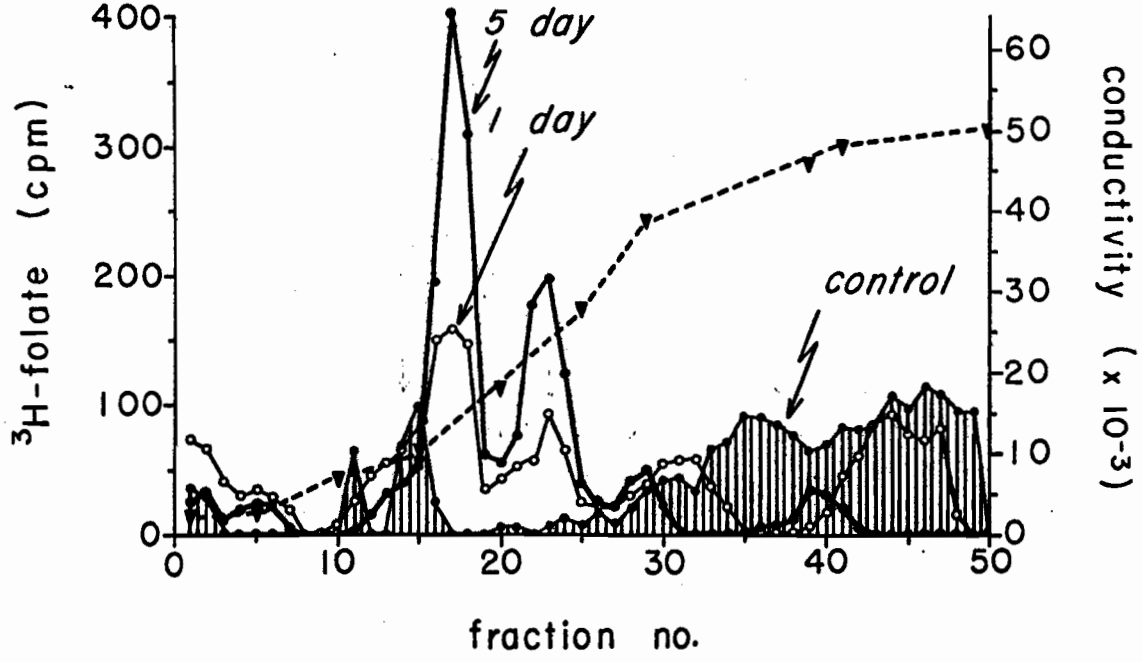


Figure 3A.

folate-free medium containing cells pre-incubated with ^{3}H-folate

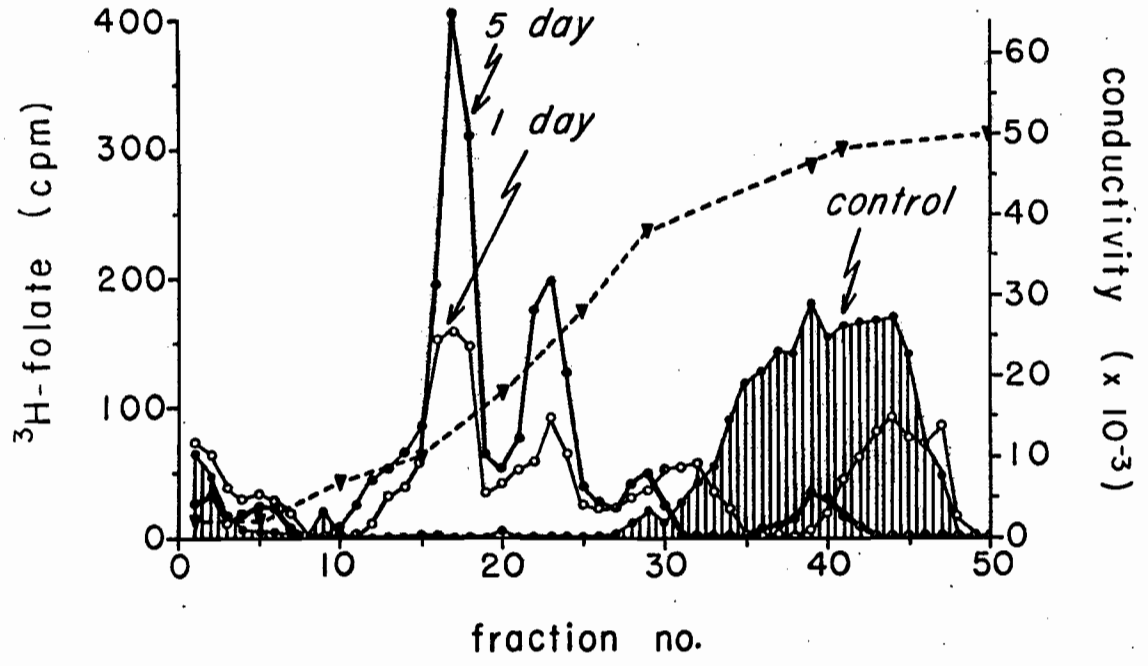


Figure 3B.

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CHAPTER 7

INTRACELLULAR FOLATE DETERMINATION IN MUTANT CHINESE HAMSTER OVARY CELLS

Introduction

Chinese hamster ovary cells have been described which are auxotrophic for glycine, adenosine and thymidine at 38°C but prototrophic at 34°C.[1] The auxotrophy of the temperature sensitive cells has been explained by a lack of biologically functional polyglutamyl synthetase[2]. Analysis of cellular extracts on DEAE-cellulose showed there was little or no long chain polyglutamate folates found in the cells after 24 h incubation in ^{3}H PteGlu presumably due to the lack of polyglutamyl synthetase[1]. It was further demonstrated that the auxotrophic cells contained a total intracellular pool of folate only 1.4% as large as the wild type cell.

The problems involved with polyglutamate determination on DEAE-cellulose has already been described in the thesis (Chapter 1). The purpose of our study was to determine the types of intracellular folates found in the mutant Chinese hamster ovary cells by filtration of the cellular extracts over Sephadex G-25. We were further interested to determine if the auxotrophic cells had the ability to accumulate folate as normal cells do.

Methods

Chinese hamster ovary cells auxotrophic for glycine, adenosine and thymidine as well as wild type Chinese hamster ovary cells grown in

medium supplemented with glycine, adenosine, thymidine and 0.1 μM ^{3}H PteGlu for different time periods. The cells were harvested by centrifugation (700 x g for 10 min), sonicated and their cellular extracts eluted over Sephadex G-25 to separate the different folate derivatives.

Results

Auxotrophic Chinese hamster ovary cells had no long chained polyglutamate folates after 24 h incubation in 0.1 μM ^{3}H PteGlu but were found to contain monoglutamate folates and intracellular PteGlu. After 4 days of incubation in medium containing 0.1 μM ^{3}H PteGlu, the auxotrophic cells were found to contain significant amounts of folate polyglutamates and the cells did accumulate folate but at a very slow rate.

Normal Chinese hamster ovary cells grown in GAT medium contained 81 and 89% of their intracellular folate in the polyglutamate form after 1 and 4 days of incubation respectively. Although monoglutamate folates and intracellular PteGlu were found in the cells, they constituted a very small proportion of the total intracellular folate. Total intracellular folate in these cells was accumulated rapidly.

Discussion

Mutant Chinese hamster ovary cells auxotrophic for GAT medium appeared to contain no intracellular folate polyglutamates after 24 hours incubation in PteGlu whether analyzed by Sephadex G-25 filtration or

DEAE-cellulose affinity chromatography as previously reported[1]. (See Discussion, Para. 4 for evidence folates are intracellular). The auxotrophic cells were able to synthesize long chained folate polyglutamates with time periods of 4 days or more. By increasing the external PteGlu concentration by one log at 24 h, no polyglutamates were found in the cell, yet incubation with time produced folate polyglutamates. It would appear then, that the ability of the polyglutamyl synthetase in the auxotrophic cells to produce folate polyglutamates was partially corrected with time.

The mutant ovary cells accumulated intracellular folate so slowly that it would be improbable to expect that their intracellular folate pool could ever become large enough to equal that of the prototrophic cells. The deficiency in folate accumulation in these cells lends evidence to the fact that polyglutamate folates have a role in folate accumulation in the cell.

As observed in human fibroblasts, Chinese hamster ovary cells when presented with excess amounts of PteGlu, stored the excesss folate as polyglutamate folate (Chapter 3). By growing the normal Chinese hamster ovary cells in GAT medium, the folate requirement of those cells became very small and therefore the excess folate in the cell was stored as polyglutamate folate.

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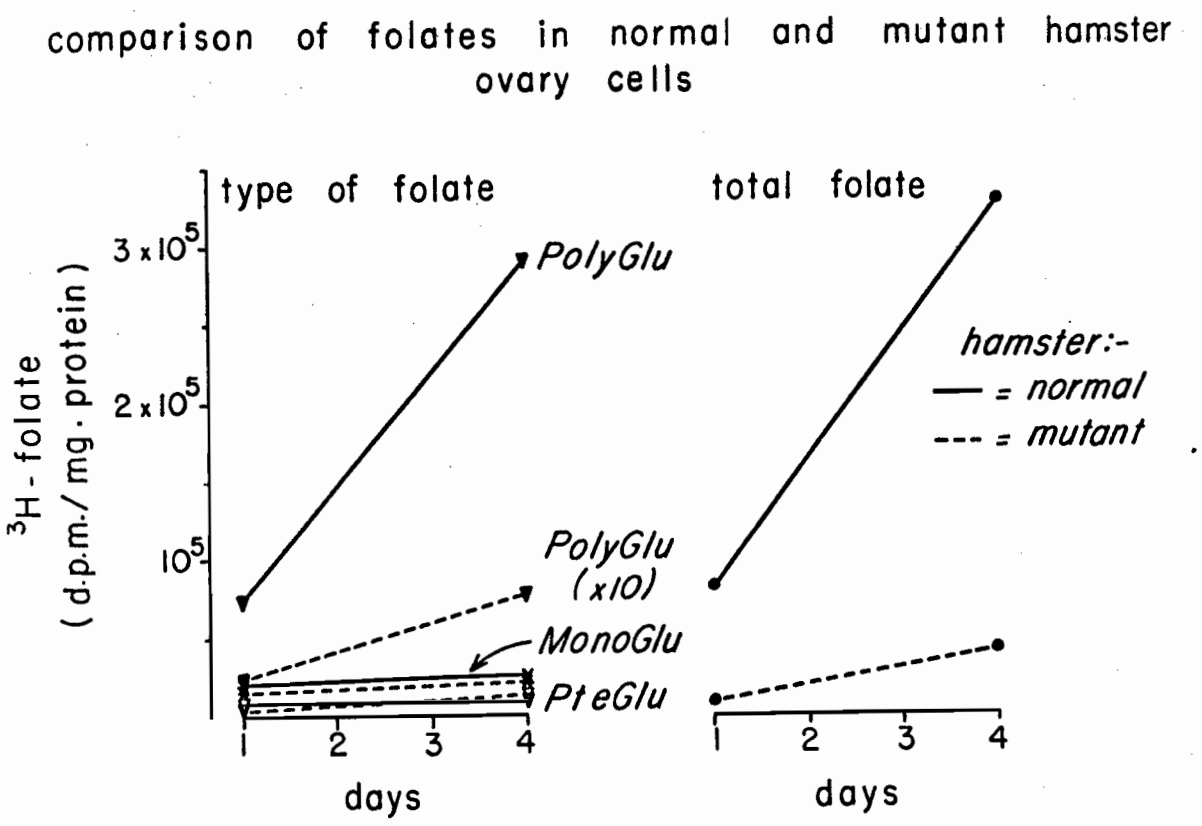

Figure 2. Comparison of total folate and types of folate accumulated in normal and mutant Chinese hamster ovary cells with time. Following incubation in GAT medium supplemented with 10^{-7} M (^{3}H) PteGlu, the cells were extracted at intervals and polyglutamates, monoglutamates and PteGlu measured by filtration through Sephadex G-25.

sephadex G-25 chromatograph of mutant hamster cells

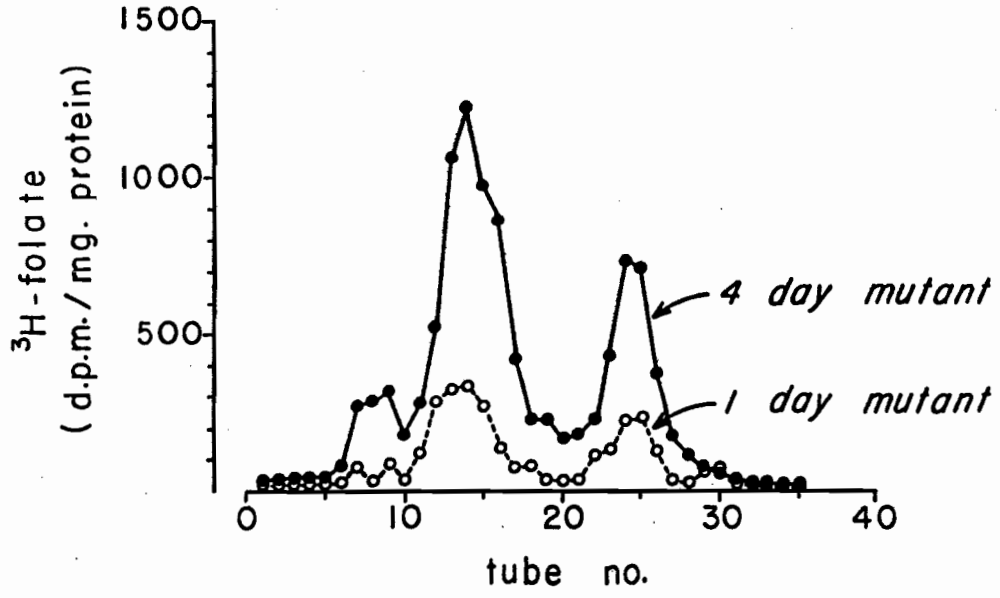

Figure 1. Sephadex G-25 chromatograph of folate derivatives found in mutant Chinese hamster ovary cells. Following incubation in GAT medium supplemented with 10^{-7}M (^{3}H) PteGlu, the mutant ovary cells were extracted at intervals and filtered through Sephadex G-25. Polyglutamates = Tubes 6-10. Monoglutamates = tubes 11-17. PteGlu = tubes 21-26.

DISCUSSION

The purpose of this study was to describe polyglutamate synthesis, turnover and folate economy in human fibroblasts cultured in vitro. The cells were grown in PteGlu, a stable, easily purified folate which is not however, a natural substrate and does not compete with reduced monoglutamates for transport into the cells[1,2].

Studies to examine the function of folate derivatives in cells have been delayed by the absence of reliable, generally accepted techniques for preservation of polyglutamates during extraction of cells, measurement of polyglutamates, and determination of chain length. The technique of gel filtration (using Sephadex) separates folates both by molecular size, and by interaction of different pteridine structure with the gel[3,4,5].

We have extended this system so that it has become a reproducible means of determining the level of intracellular folate polyglutamates, monoglutamates and PteGlu in human fibroblasts. It was possible that our folate monoglutamates were contaminated with di- and triglutamates, however the filtration position of authentic monoglutamate standards appeared to negate this possibility. Polyglutamate folate fractions were identified as polyglutamate by treatment with serum conjugase and filtration position of the chemically synthesized standard, PteGlu pentaglutamate. Elution position of the various monoglutamate standards on Sephadex G-25 were somewhat different than those previously reported[5]. This may have been due to the fact that in previous studies, the column length was much larger than the one used in our study, and therefore the filtration through their column required considerably more time.

Elution positions of our monoglutamate standards were analyzed by both spectrophotometric analysis and microbiological assay.

The polyglutamates and monoglutamates as well as PteGlu contained in the extract of washed wells were thought to be intracellular and not surface bound for the following reasons: PteGlu may only be converted to the reduced polyglutamates and monoglutamates by intracellular enzymes; the cells were washed 4 times in the cold to remove absorbed PteGlu; folate efflux from the cell was time dependent; PteGlu found in the cellular extract was not directly proportional to the exogenous PteGlu concentrations when measured at high concentrations. Although this evidence indicates that the folates found in the cellular extracts were intracellular, we are unable to prove this to 100% certainty.

Oxidation or reduction of the C-9, N-10 bond of folate was not used in our studies to determine actual folate glutamate chain length. Our studies showed that a large fraction of the intracellular folate found in human fibroblasts was not reproducibly cleaved at the C-9, N-10 bond by such treatment and so identification of the eluted material on DEAE-cellulose would be very difficult. Even with a modification of the oxidation procedure described by Scott and co-workers[6] involving a lowering of pH to break the bond in 5-methyl-H_4PteGlu, reproducible cleavage of all of the fibroblast's intracellular folate at the C-9, N-10 bond was not attained.

The small fraction of polyglutamate breakdown observed during the extraction procedure assured us that our method of analyzing intracellular

folates did not hydrolyse the polyglutamates to monoglutamates.

The folate requirement of human fibroblasts for PteGlu was found to be between 4.4 and 22 ng per ml of incubation medium. This was very close to the folate limiting concentration in human serum which has been reported to be 4 ng per ml of serum[7]. Although the human serum is comprised mainly of 5-methyl-H_4PteGlu, it was encouraging for our studies using PteGlu that the cellular requirement for PteGlu was approximately the same as that of 5-methyl-H_4PteGlu.

These studies demonstrated that fibroblasts accumulated folate when grown in PteGlu, but not to a concentration which was higher than the folate content of the exogenous medium. We note that unlike some bacteria[8], erythroid[9] and granulocyte precursors[10], and other cells[11,12] as well as L.casei[13], fibroblasts did not accumulate intracellular folate above the concentration of PteGlu in the medium, despite prolonged incubation. This was confirmed by microbiological assay of cells grown for many weeks in non-radioactive PteGlu. It was shown that cells grown in PteGlu over the first hours of incubation had a very high level of intracellular PteGlu, followed by accumulation of monoglutamate folates, followed in turn by a more rapid accumulation of polyglutamate folates.

We suggest that fibroblasts in culture allow entry of PteGlu by facilitated diffusion as described for other cells[14], that intracellular PteGlu then binds to dihydrofolate reductase and is reduced to H_4PteGlu, which is available for polyglutamate synthesis[15,16], or for conversion to the different co-enzyme forms of monoglutamate folate. We believe that

our short chain folates were in fact monoglutamates and that most intracellular folate appeared to be either long chain polyglutamates (mostly penta)[17] or monoglutamate. As polyglutamate accumulated in the cell, they appeared to displace PteGlu from it's binders (probably dihydrofolate reductase), reducing the intracellular concentrations of PteGlu.

Although mammalian polyglutamyl folate synthetase (ligase) appears to add glutamates one at a time in vitro,[15,16] the pattern of intracellular folate during gel filtration suggest that most intracellular reduced folate was either short chain (probably monoglutamate) or long chain (probably mostly pentaglutamate)[17]. If true this would suggest that addition of the 3rd, 4th, and 5th glutamates was facilitated over adding a 2nd glutamate, as suggested previously[15].

Fibroblasts in logarithmic growth accumulated more folate than did confluent cells grown in similar PteGlu concentrations. The greatest rise in folate accumulation in human fibroblasts occurred in early logarithmic phase. In proliferating cells, the distribution of this intracellular folate into reduced folate monoglutamate and polyglutamate was uneffected by external PteGlu concentrations. In confluent cells grown in excess PteGlu (0.1 μM and more) however, an increasing proportion of intracellular folate was accumulated as polyglutamate. This would suggest that as external PteGlu concentration increased, confluent cells stored the excess folate as polyglutamate,

whereas in growing cells requiring folate, this storage did not occur. This observation supports folate monoglutamate: polyglutamate shifts observed in Physarum polycephalum, and in rat uterine tissue[18]. These data suggests that although these cells utilize both polyglutamate and monoglutamate folate, excess folate is stored as polyglutamate.

These data also demonstrate the large capacity of the polyglutamyl synthetase (ligase) to synthesize folate polyglutamates. When subjected to external PteGlu concentrations ranging from 1 - 40,000 nM, the intracellular ligase showed no indication of saturation by the folate substrates.

PteGlu levels remaining constant throughout the culture cycle was represented by the sum of PteGlu entering the cell, and the loss of PteGlu being converted to reduced folates.

A small proportion of folate associated with cells in culture was freely exchangeable with folate-free medium. This exchangeable folate consisted of polyglutamates, monoglutamates and PteGlu. It is possible that some of the exchangeable PteGlu could have been loosely bound to the surface of the cell, but this would be an improbable source of the polyglutamate folate. The small proportion of exchangeable, metabolically active folate could have been derived from injured cells, although our failure to remove it with 4 cold rinses with saline suggests that at least some of this might represent folate moving freely through the cell membrane.

The relationship between the quantity of exchangeable folate and the concentration in which the cells were incubated was sigmoidal. The sigmoidal curve might have represented the sum of two compartments: one saturating at 0.01 and the other at 13.0 nmoles of folate per litre of cell water. Because of limitations of specific activity, it was not possible to determine the proportion of PteGlu in the exchangeable folate found in the second incubation medium of cells grown in less than 1 μM PteGlu. The exchangeable folate in medium from cells grown in this concentration of PteGlu or more (50 μ M) was composed of 62% PteGlu, 23 and 15% of polyglutamate and monoglutamate folates respectively. Data are not available to justify further speculation about this material.

The timed efflux of folate from the cell was very difficult to interpret. Because efflux from the cell was time dependent, it was probable that the folate which left the cell was indeed folate which had been inside the cell and transported out. The possibility did exist however that the timed efflux of folate from the cell may merely represent folate trapped in the cell membrane and it's time dependent transport out of the cellular membrane. At present we do not have sufficient evidence to either confirm or eliminate either of the above possibilities.

Cells exposed to a situation of folate starvation lost folate with a T 1/2 of 3.5 days. Polyglutamates and intracellular PteGlu were lost throughout starvation, however monoglutamate folates remained constant during the first three days of starvation then decreased after that time.

The level of intracellular folate found in the cell at the point when the monoglutamate folates began to decrease was approximately 2 nmoles per litre of cell water, a concentration of similar to that found in cells growing in folate deficient medium.

Recently accumulated polyglutamate folate decreased during folate starvation, while monoglutamate bearing the same label increased. Polyglutamate folate labelled with isotope for 1-8 days before starvation also decreased, while the monoglutamate fraction bearing the "old" label did not increase. The intracellular folate concentration appeared to be the sum of folate entry, reduction and polyglutamate formation and folate loss. Folate loss from the cell appeared to be first order, while folate entry has been reported to differ for different folates. In fibroblasts, the velocity of the reactions involved in polyglutamate formation (transport of PteGlu, reduction and polyglutmate synthesis) appeared to be too low to overcome the rate of folate loss, resulting in intracellular folate concentration below that of the medium. Although the double label experiment illustrated some exchange of recently formed polyglutamate with monoglutamate folates, the majority of intracellular folate appeared not to be in equilibrium with the monoglutamates. The majority of the loss of intracellular polyglutamate during folate starvation probably represented loss or destruction of polyglutamate folate without conversion to intracellular monoglutamate.

Polyglutamate and monoglutamate folates were found to be expelled from the cell during folate starvation. Once in the medium the polyglutamate folates were exposed to severe hydrolysis of the polyglutamate side chains. Since polyglutamate extracts were exposed to medium conditions

for the same period as medium containing cells with insignificant hydrolysis of the folate polyglutamate chains, one can only assume the cells were responsible for polyglutamate hydrolysis, probably by means of excreted lysosomal conjugase. Since quantities were too low of the expelled material into the folate-free medium, microbiological analysis on the radioactive peaks was not possible. Chemical standards were run on the DEAE-Sephadex A-25 column with their elution positions measured. These data are constent with an efflux mechanism for folate monoglutamate and polyglutamate folates from the cell. Since polyglutamate folates were calculated to be present in the folate-free medium in greater quantitites than the folate monoglutamates, it is probable that the efflux folate carrier has a higher affinity for the polyglutamate form of folate.

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