Specific and non-specific folate binding protein in normal and malignant human tissues

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SUMMARY   Binding of tritiated folic acid by supernatants prepared from extracts of normal and leukaemic leucocytes, normal mucosa, and malignant tumours from different parts of the gastro-intestinal tract has been measured using Sephadex-gel filtration and albumin-coated charcoal techniques. Non-specific binding (measured by Sephadex G-75 gel filtration) was almost invariably greater than specific binding measured by albumin-coated charcoal separation of bound and un-bound folate. In nine normal leucocyte extracts, binding measured by Sephadex G-75 filtration ranged from 1·3 to 18·2 (mean 8·2) pg/mg protein and by albumin-coated charcoal from 1·0 to 14·8 (mean 6·7) pg/mg protein. Raised specific binding was found in the extracts from leucocytes of eight of 14 patients with chronic granulocytic leukaemia, in four substantially so (389, 121, 108, 59·7 pg/mg protein), but was only marginally increased in one of eight cases of acute myeloid leukaemia and in two of five cases of chronic lymphocytic leukaemia. Binding was normal in the extracts of all three cases of acute lymphoblastic leukaemia tested.

Among the tissues of the gastrointestinal tract binding was greatest by the duodenal mucosa and liver. Extracts of carcinoma of the stomach and colon bound greater amounts of ^{3}H-folic acid than the corresponding normal mucosal extracts but the differences were not large. Sephadex G-200 gel chromatography showed more than one binding peak in the extracts of liver and duodenum but only one peak in the other tissues of the gastrointestinal tract, and only one peak, of molecular weight either about 50 000 or over 200 000, in the leucocyte extracts.

The presence of a folate binding protein (FABP) in human tissues was first demonstrated by Rothenberg (1970) in the leucocytes of some patients with chronic granulocytic leukaemia. Subsequently, a folate binding protein was found in granulocytes of women who were pregnant or taking oral contraceptives (Da Costa and Rothenberg, 1974a) and in the sera of normal subjects and patients with a variety of diseases (Rothenberg, 1973; Waxman and Schreiber, 1973; Zettner and Duly, 1974; Colman and Herbert, 1976; Gorst *et al.*, 1976; Rothenberg and Da Costa, 1976).

In these studies it was not always clear whether the binding of ^{3}H-folic acid (^{3}H-PteGlu) was specific or non-specific in nature. As recently pointed out by Rothenberg and Da Costa (1976), at physiological pH the glutamic acid part of the folate molecule is negatively charged ($-COO^-$) so that it tends to bind non-specifically to molecules with a positive radical (as NH_3+). This non-specific binding is characterised by low affinity and fails to demonstrate saturation kinetics. In contrast, specific binding has a high affinity for the ligand and demonstrates saturation kinetics. It depends on the characteristic structure of the molecule and it involves, in the case of folate, the pteridine moiety. This specific binding, in contrast to the non-specific binding, cannot be dissociated by short exposure to albumin-coated charcoal (Rothenberg and Da Costa, 1976).

The aim of the present study was to detect and quantitate the unsaturated folate binding activity in various normal and malignant human tissues, and to compare specific and non-specific folate binding capacities of these tissues. A preliminary report of some of this work has already been published (Corrocher *et al.*, 1976).

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Material and methods

LEUCOCYTES AND TISSUES

Leucocytes of 14 patients with chronic granulocytic leukaemia (CGL), eight patients with acute myeloblastic leukaemia (AML), five patients with chronic lymphocytic leukaemia (CLL), three patients with acute lymphoblastic leukaemia (ALL), and nine normal adult subjects were recovered by sedimentation (2 h at room temperature) of heparinised blood mixed with Emagel (1:1 v/v), and washed three times with cold (4°C) physiological saline. The clinical and haematological data on these patients with leukaemia are listed in Tables 1 and 2. Tissues from different

Table 1 *Clinical data and tritiated folic acid (^{3}H-PteGlu) binding capacity of peripheral blood leucocytes from normal subjects and from patients with acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL)*

Case		Age	Sex	Hb (g/dl)	WBC × 10⁹/l	% Blasts	Diagnosis	Treatment	^{3}H-PteGlu binding		Cold PteGlu inhibition (using ACC) (%)
									pg/mg Protein S-G75	pg/mg Protein ACC	
1	(RC)	24	M	13·9	7·5	—	Normal	—	18·2	14·8	100·0
2	(BG)	26	M	14·7	5·8	—	Normal	—	10·3	7·4	98·2
3	(AN)	29	F	13·5	8·2	—	Normal	—	9·7	6·7	—
4	(FG)	62	M	12·8	7·6	—	Normal	—	8·4	6·5	—
5	(OP)	35	F	13·1	6·9	—	Normal	—	8·2	5·8	99·1
6	(VT)	20	M	14·8	9·3	—	Normal	—	6·7	7·6	—
7	(PM)	48	F	12·2	6·4	—	Normal	—	6·6	6·5	—
8	(BF)	47	M	15·6	6·9	—	Normal	—	4·5	4·1	—
9	(MG)	33	F	13·0	5·8	—	Normal	—	1·3	1·0	—
1	(LA)	27	M	8·7	24	97	AML	No	20·9	14·8	99·4
2	(RS)	55	M	7·4	120	92	AML	No	17·0	18·0	86·4
3	(BP)	26	M	8·8	13	38	AML	No	17·0	10·2	—
4	(TG)	71	M	4·6	54	93	AML	No	13·8	7·1	—
5	(NI)	48	M	9·2	27	75	AML	No	7·4	6·7	—
6	(BM)	52	M	10·5	18	88	AML	No	4·9	1·7	—
7	(FV)ᵃ	67	M	8·8	38	87	AML	No	3·7	0·9	90·1
	(FV)ᵇ			9·0	46	88	AML	Yes*	3·7	2·8	—
8	(SG)	69	M	5·3	120	88	AML	No	—	0·76	—
1	(MC)	31	F	9·1	131	80	ALL	No	37·6	7·9	97·3
2	(GL)	34	F	10·6	170	96	ALL	No	14·1	12·0	89·9
3	(ZL)	29	M	81	38	95	ALL	No	—	0·0	—

S-G75 = Sephadex G-75; ACC = albumin-coated charcoal (also in Tables 2 and 3)
*Treatment = thioguanine, daunorubicin, cytosine, arabinoside, prednisone (TRAP)

Table 2 *Clinical data and tritiated folic acid (^{3}H-PteGlu) binding capacity of peripheral blood leucocytes from patients with chronic granulocytic leukaemia (CGL) and chronic lymphocytic leukaemia (CLL)*

Case		Age	Hb (g/dl)	WBC × 10⁹/dl	PMN (%)	Blasts (%)	Diagnosis	Treatment	pg/mg Protein S-G75	pg/mg Protein ACC	Cold folate % inhibition (using ACC)
1	(PS)	24	7·6	79	54	—	CGL	No	689	108	98·8
2	(PM)	17	11·6	155	50	4	CGL	No	450	389	87·5
3	(VT)	70	8·8	120	49	15	CGL	No	218	122	—
4	(ZM)ᵃ	45	9·5	104	27	24	CGL	No	71·5	59·7	—
	(ZM)ᵇ	46	10·5	62	46	5	CGL	Busulphan	129	103	—
5	(FV)ᵃ	53	8·4	160	65	—	CGL	No	48·9	9·3	—
	(FV)ᵇ	54	8·0	125	66	—	CGL	Busulphan	13·1	—	—
	(FV)ᶜ	54	9·3	103	44	4	CGL	Busulphan	7·1	10·3	—
6	(MN)	53	9·4	87	64	7	CGL	No	38·6	9·2	84·9
7	(DS)	20	13·5	116	63	—	CGL	No	25·4	18·9	—
8	(TA)	50	8·6	138	66	—	CGL	No	24·4	13·7	100
9	(PV)	52	7·6	132	72	6	CGL	No	16·1	18·7	92·2
10	(SF)	18	9·5	350	28	8	CGL	No	14·7	20·1	93·7
11	(SP)	27	8·4	52	42	17	CGL	No	9·3	8·7	—
12	(CI)	33	9·0	85	54	12	CGL	No	7·0	7·9	100
13	(RO)	52	11·5	49	85	—	CGL	No	4·4	1·8	—
14	(PA)	37	8·1	34	25	37	CGL	No	2·1	1·7	—
					PMN (%)	Lympho-cytes (%)					
1	(BF)	76	10·2	90	2	98	CLL	No	38·7	23·7	—
2	(LA)	50	9·7	100	1	99	CLL	No	32·7	17·2	—
3	(RA)	70	11·8	52	6	94	CLL	No	22·6	8·6	—
4	(TR)	62	8·4	93	11	89	CLL	No	—	0	—
5	(BG)	69	13·4	138	5	95	CLL	No	—	5·4	—

Table 3 *Tritiated folic acid (^{3}H-PteGlu) binding of supernatants prepared from normal and malignant tissues of the gastrointestinal tract*

Case		Age	Sex	Tissue	^{3}H-PteGlu binding		Cold PteGlu inhibition (using ACC) (%)
					pg/mg Protein S-G75	pg/mg Protein ACC	
1	(CR)	58	M	Oesophagus	22·5	10·1	—
2	(VB)	49	M	Stomach (fundus)	15·2	10·1	—
3	(GB)	49	M	Duodenum	67·2	46·8	94·3
4	(RT)	61	M	Colon	52·8	31·4	—
5	(GF)	54	M	Liver	72·1	51·4	97·5
6	(BL)	57	M	Adenocarcinoma of oesophagus	77·5	29·1	—
7	(BM)	53	M	Carcinoma (squamous) of oesophagus	77·4	—	—
8	(GG)	61	M	Adenocarcinoma of stomach (cardia)	28·3	18·0	—
9	(DG)	49	M	Adenocarcinoma of stomach (fundus)	21·3	11·1	—
10	(PA)	54	M	Adenocarcinoma of colon	60·7	33·8	—
11	(SC)	59	M	Adenocarcinoma of rectum	63·8	33·8	—
12	(MC)	58	M	Carcinoma (mucus secreting) of rectum	37·2	23·8	—

parts of the gastrointestinal tract were obtained during laparotomy with the informed consent of the patient as follows. Histologically normal mucosa of oesophagus and colon was taken at operations to remove cancer of these organs (Table 3). Duodenal mucosa was taken at operation for a benign duodenal ulcer; gastric and cardia mucosae were taken at operation for peptic ulceration of the pylorus. Liver biopsy was obtained during cholecystectomy for calculi.

The tissues were plunged into cold (4°C) phosphate buffered saline (PBS) and washed free of blood, and, where appropriate, the mucosa was scraped onto a cold glass slide.

PREPARATION OF SUPERNATANTS

The leucocytes and mucosal samples were suspended in sodium citrate buffer, pH 8·5, 0·05 M (Rothenberg, 1970), and lysed after thawing and freezing three times (leucocytes) or homogenised (tissues) in the same buffer using a Teflon-coated Potter-Elvejehm homogeniser, and centrifuged at 110 000 g for two hours. The particle-free supernatants were removed and stored at −20°C until used.

CHROMATOGRAPHIC STUDIES

To study the unsaturated folate binding capacity (UFBC), 0·2 ml of each supernatant was incubated in 0·3 ml of 0·05 M sodium citrate buffer, pH 8·5, with 20 μl (5·96 ng) of generally labelled ^{3}H-folic acid (^{3}H-PteGlu), specific activity 40 mCi/mmol (Radiochemical Centre, Amersham), for 15 min at 37°C and then chromatographed on a Sephadex G-75 column (0·5 × 31 cm). Thirty 0·5-ml fractions were collected in counting vials containing 0·4 ml of Instagel (Packard). The radioactivity of each sample was determined in a liquid scintillation beta-counter (Packard). Aliquots of the supernatants incubated with ^{3}H-PteGlu were also chromatographed on a Sephadex G-200 column (90 × 2·0 cm) previously

equilibrated with the sodium citrate buffer. One hundred and twenty 5-ml fractions were collected, and the radioactivity of 0·5-ml aliquots was measured and plotted against protein concentrations.

The columns were calibrated with blue dextran (MW 2 000 000), albumin (MW 64 000), or haemoglobin (MW 68 000) and ^{3}H-PteGlu (MW 441) by measuring the optical density (280 or 560 nm) or the radioactivity.

CHARCOAL ADSORPTION STUDIES

Albumin-coated charcoal was prepared by mixing equal volumes of a Norit A suspension (5 g in 100 ml distilled water) and 1% aqueous solution of bovine albumin (Sigma). 0·5 ml of each supernatant was incubated with 20 μl (290 pg) of ^{3}H-PteGlu in distilled water for 1 h at 37°C; then 0·7 ml of the albumin-coated charcoal was added to the samples and stirred for 10 min. The coated charcoal was removed by centrifugation (10 000 rpm, 10 min) and the radioactivity of 0·5 ml of the remaining supernatant was measured; the radioactivity of a charcoal control, in which sodium citrate instead of supernatant was incubated with ^{3}H-PteGlu, was subtracted from each result. Each experiment was performed in triplicate, and the results between tubes over all experiments varied by a mean of 5·6% with a maximum of 12·6%.

INHIBITION OF BINDING OF TRITIATED FOLIC ACID BY COLD FOLIC ACID

Aliquots of the supernatants were preincubated for 30 min at 37°C with 2 μg of cold PteGlu before the addition of 290 pg of ^{3}H-PteGlu; then albumin-coated charcoal was added, and the experiment described above was repeated.

KINETIC STUDIES

0·2 ml (306 μg of protein) of the peak folate binding fraction on Sephadex G-200 of a CGL supernatant

was incubated with increasing amounts of ^{3}H-PteGlu, ranging from 5·9 to 5960 pg. The amount of folate bound was determined using coated charcoal.

Results

SEPHADEX G-75 CHROMATOGRAPHY

The chromatographic profile, after addition of ^{3}H-PteGlu to leucocyte supernatants, was invariably characterised by two radioactive peaks, the earlier peak being due to protein bound folate, the later peak being free folate (Fig. 1). Tables 1 to 3 show the amount of ^{3}H-PteGlu bound per milligram of protein by supernatants from various tissues using this technique. The values for the UFBC of nine normal leucocyte preparations ranged from 1·27 to 18·7 (mean 8·2) pg/mg protein (Table 1). Similar results were obtained using supernatants from eight leucocyte preparations from AML, only one sample being slightly above normal (Table 1). On the other hand, using supernatants from two patients with ALL and three with CLL, the mean values were higher, being 25·8 and 31·3 pg/mg protein respectively (Tables 1 and 2).

The results obtained using supernatants from peripheral leucocytes of 14 patients with CGL are given in Table 2. Two of them (FV and ZM) were studied before (a) and after (b, c) therapy. In eight of the 14 patients the amount of folate bound was above the normal range, and in four the amount of folate bound was over 100 pg/mg (Table 2).

Among the tissues from the gastrointestinal tract, UFBC was highest in the duodenal mucosa (67·2 pg/mg protein) and liver (72·2 pg/mg), and was progressively lower in the colon (52·8 pg/mg), oesophagus (22·5 pg/mg), and stomach (15·2 pg/mg). In tumours derived from different parts of the gastrointestinal tract, the UFBC was invariably greater

(although not markedly so) than that of the corresponding normal mucosa (Table 3).

SEPHADEX G-200 CHROMATOGRAPHY

Using this system, there was a wide variation in the pattern of elution peaks between different tissues. Indeed, the chromatographic profile of ^{3}H-PteGlu incubated with the cytoplasm of CGL leucocytes from two patients showed different patterns: in the first case (FV) (Fig. 2) there was a single small peak in the exclusion peak, suggesting a binder of MW greater than 200 000, whereas in the second case (PM) (Fig. 3) a single large peak eluted just later than haemoglobin, suggesting an MW of about 50 000. On the same column, liver and duodenal
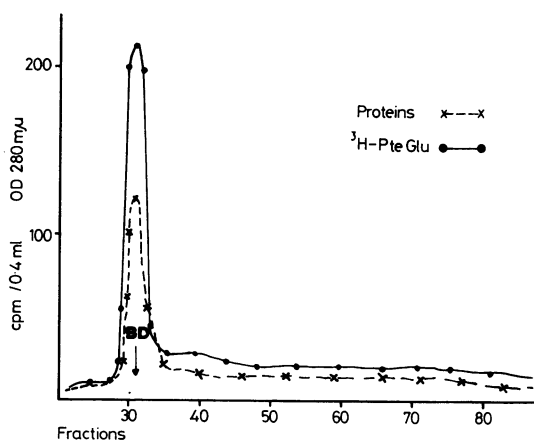
Fig. 2 Elution pattern of ^{3}H-PteGlu incubated with the supernatant of leucocytes from a patient with CGL (before treatment) (FV). BD = blue dextran. Free ^{3}H-PteGlu eluted from the Sephadex G-200 column with a peak at fraction 120 and is therefore not shown in Figs 2, 3, or 4.
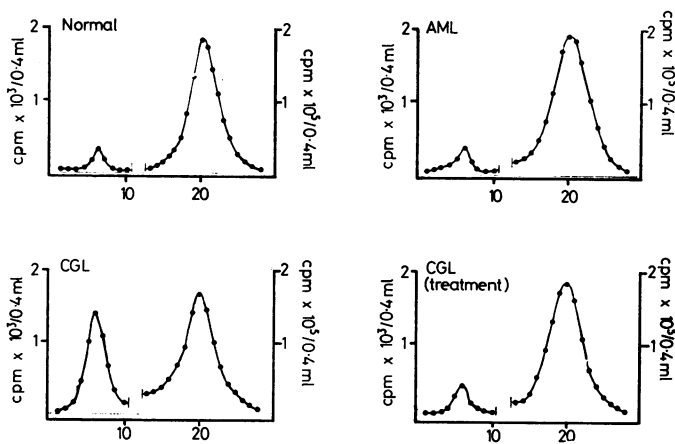
Fig. 1 Chromatographic patterns on Sephadex G-75 after addition of ^{3}H-PteGlu to lysates prepared from peripheral leucocytes of a typical normal subject and patients with acute myeloblastic leukaemia (AML) and chronic granulocytic leukaemia (CGL). This CGL patient (FV) was restudied after treatment. The first peak represents the bound folate, whereas the second represents the free folate. Horizontal scale = fraction number. Left-hand ordinate scale (cpm × 10^3/0·4 ml) refers to counts in peak 1, and right-hand ordinate scale (cpm × 10^5/0·4 ml) refers to counts in peak 2.
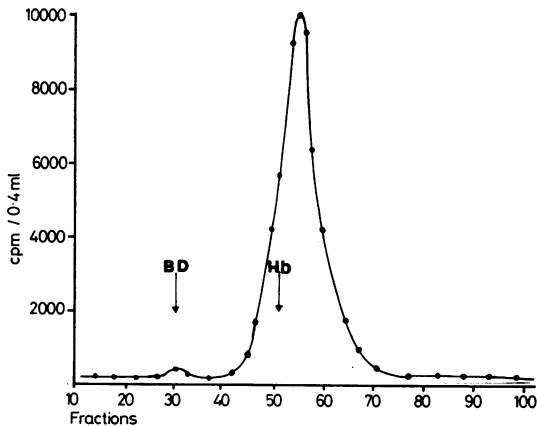
Fig. 3 *Elution pattern of ³H-PteGlu incubated with the supernatant of an untreated patient (PM) with CGL. BD = blue dextran; Hb = haemoglobin.*
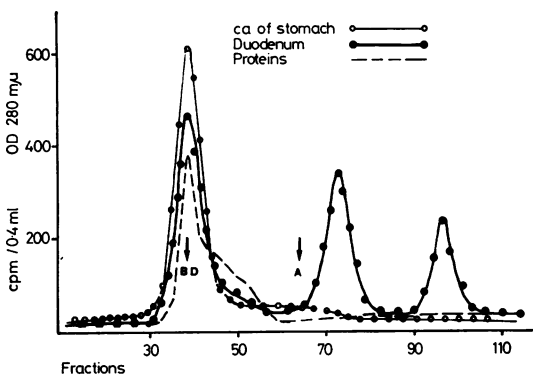

Fig. 4 *Elution of ³H-PteGlu incubated with supernatants prepared from normal duodenum and from a carcinoma of stomach (case 10, Table 3). Protein pattern is for duodenal supernatant. The protein pattern for the carcinoma supernatant was similar. BD = blue dextran; A = albumin.*

ranged from 1·0 to 14·8 (mean 6·7) pg/mg protein. In four cases of CGL, the binding capacity measured by coated charcoal was particularly high, whereas in seven it was normal and in the other three it was slightly above the normal range. In the other kinds of leukaemia (ALL, AML, and CLL), the coated charcoal-resistant folate binding was normal or slightly raised in three cases.

In the gastrointestinal tract, the highest albumin-coated charcoal-resistant folate-binding capacity was found in liver and duodenum (51·4 and 46·8 pg/mg protein respectively), but some charcoal-resistant binding capacity was found also in other parts of the gastrointestinal tract. In all cases tested, albumin-coated charcoal-resistant binding could be inhibited by cold folate (Tables 1 to 3). Moreover, the amount of ³H-PteGlu bound per milligram of protein in one case of CGL was proportional to the amount of ³H-PteGlu added to the incubation mixture for the first 100 pg; it then decreased progressively (Fig. 5).
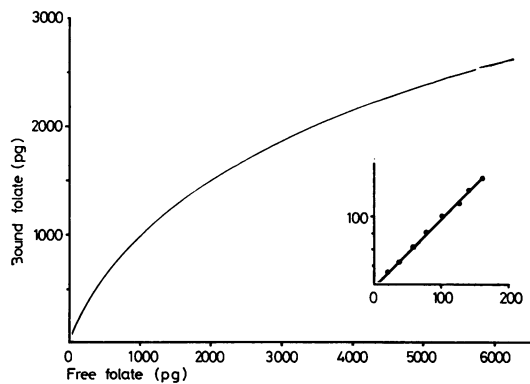

Fig. 5 *Saturation curve after addition of increasing amounts of ³H-PteGlu to a supernatant from peripheral blood leucocytes in a case of CGL (case 9, Table 2). Inset shows the early part of the saturation curve.*

supernatants showed three radioactive peaks, the first being in the exclusion peak of protein (Fig. 4). These patterns differed from those obtained using a carcinoma of stomach supernatant or supernatant from other tumours which showed only one peak.

ALBUMIN-COATED CHARCOAL STUDIES
The amount of ³H-PteGlu bound by the different extracts after treatment with albumin-coated charcoal is shown in Tables 1 to 3. The UFBC using coated charcoal was almost always lower than the binding capacity measured by Sephadex filtration. Among the nine normal leucocyte preparations, binding

Discussion

The chromatographic studies carried out using Sephadex G-75 have shown that supernatants from all the tissues studied bind a proportion of added radioactively labelled folic acid. As the cytoplasm of the different tissues contains endogenous folate, the amount of ³H-PteGlu bound only represents the unsaturated folate binding capacity, provided there is no exchange between the endogenous and added folate. This folic acid binding may be non-specific to proteins normally present in the cell cytoplasm or to proteins derived

from membranes or other organised structures obtained during the preparation of supernatants.

The results obtained with activated charcoal and the inhibition of this binding by the cold folate suggest that at least in the leucocytes of some cases of chronic granulocytic leukaemia and in some of the other tissues studied, especially duodenum and liver, binding is specific. In the one case of CGL studied (PM) the saturation curve of the binder showed the typical behaviour of specific binding over the range of ^{3}H-PteGlu used in these experiments (Fig. 5).

A folate-binding protein was first identified in the cytoplasm of leucocytes by Rothenberg (1970). Recently, Rothenberg and colleagues have shown that the binder, which is eluted on Sephadex-gel chromatography as a single peak, is probably a mixture of two binders with a similar molecular weight (Fischer *et al.*, 1975). Moreover, these authors demonstrated that a number of folic acid analogues (H$_2$-folate; triglutamate of folic acid; N^{10} methylfolate; diglutamate of folic acid; and N^5-methyl-H$_4$-folate) all inhibit the binding of ^{3}H-PteGlu. This supports the hypothesis that the binder of folic acid, itself an unnatural form of folate, may have biological significance in the metabolism of endogenous natural folates, including polyglutamate forms (Da Costa *et al.*, 1972). On Sephadex G-200 the supernatant from the leucocytes of a patient with CGL (FV) showed a small peak of bound folate in the exclusion peak, suggesting an MW of more than 200 000 (Fig. 2). A binder of about 50 000 MW was not found in this case but was present in the supernatant of the leucocytes of another case (PM) (Fig. 3). The large MW peak may be a different binder; a folate binder of 200 000 MW has been isolated from human milk (Waxman and Schreiber, 1974), or it may be due to non-specific binding by protein in the supernatant. It could also be a polymerised form of the smaller MW binders.

The amount of specific (albumin-coated, charcoal-resistant) folate-binding protein in some CGL leucocytes was particularly high, whereas in the other types of leukaemia studied, the amount of folate bound in leucocytes did not differ significantly from the normal range. These findings suggest that mature granulocytes and their immediate precursors may be the source of the binder. Previous studies have shown that folate-binding protein may be actively synthesised at a greater than normal rate by chronic granulocytic leukaemia cells (Da Costa and Rothenberg, 1974b). In AML leucocytes, the level of folate binding was invariably normal, even though Gorst *et al.* (1976) found folate binding to be raised in the sera of patients with AML. The exact cause of this increased serum binding in AML

remains to be determined.

In the normal tissues of the gastrointestinal tract, the highest concentration of binder was found in duodenal mucosa and in liver cells. On Sephadex G-200 the radioactivity after incubation of labelled folic acid with duodenal mucosa supernatant was characterised by three peaks of MWs approximately 200 000 or more, 50 000, and 20 000, a pattern similar to that described for human, rat, and rabbit liver (Corrocher *et al.*, 1974; Zamierowski and Wagner, 1974; Markkanen *et al.*, 1975). High molecular weight binding has also been found in the intestine of rat and in the brush border of small intestine in rat (Leslie and Rowe, 1972; Zamierowski and Wagner, 1974).

The supernatants prepared from some solid tumours were found to contain higher concentrations of folate binder than the corresponding normal tissues (Table 3). Sephadex G-200 chromatographic analysis in the case of carcinoma of the stomach showed a peak of radioactivity in the exclusion peak of proteins, and a similar pattern was found using a colonic mucosa but whether the high MW protein binder is the main binder at all sites in the gastrointestinal tract remains to be shown.

The different kinds of folate binder suggest that these proteins may have different functions in folate metabolism. Ingested folates are specifically absorbed by duodenal mucosa, and a specific folate malabsorption has been documented in which there is malabsorption of all forms of folate, including the unphysiological folic acid (Lanzkowsky *et al.*, 1969). It is possible to speculate that the high concentration of binder in duodenal mucosa may be involved in some way in the folate absorption mechanism, and a fault in this binding mechanism is responsible for specific malabsorption of folates (Waxman, 1975). The exact physiological function of the folate-binding proteins, however, remains uncertain, and further studies of their *in vitro* binding of natural (reduced) folates and of their *in vivo* function are needed to elucidate this.

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