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APPLICATION OF FLOW MICROCALORIMETRY TO THE STUDY OF DEHYDROFOLATE REDUCTASE ACTIVITIES IN CRUDE TISSUE HOMOGENATES

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Calorimetric methods are becoming important analytical tools in several areas of biochemical and biological research. In this work, a flow microcalorimetric method has been applied to the determination of dihydrofolate reductase (DHFR) activities in rat and human malignant tissue homogenates. In contrast to other commonly used DHFR analytical assays, the sensitivity of flow microcalorimetry allows direct measurements of this enzyme in crude tissue preparations. Our experimental data from rat tissue homogenates show that liver has the highest level of enzyme activity, while lung and brain have lower amounts of DHFR activity. The liver enzyme has a higher activity at *pH* 4.5, but the optimum *pH* for the lung and brain enzymes is 6.8. The substrate/cofactor molar ratio which gives the highest levels of DHFR activity is 1/1.5 for the liver and lung enzymes and 1/2.5 for brain DHFR. The DHFR in these rat tissue homogenates is activated by KCl or NaCl: in the presence of these salts (0.6 *M*), the values of enzyme activity are 1.5–3 times higher than in their absence. Using flow microcalorimetry, very low levels of DHFR activity were also measured in human bone tumour homogenates, demonstrating the potential of the technique in the analysis of this enzyme in malignant tissues.

Keywords: biochemical research, enzymes, flow microcalorimetry

Introduction

Microcalorimetry has been recognized as a valuable analytical tool for the study of a wide variety of biochemical and biological systems with different levels of complexity including, in particular, the enzymatic systems [1–3].

In this paper we report the application of flow microcalorimetry to the study of dihydrofolate reductase (DHFR) activities in rat and human malignant tissue homogenates. The enzyme DHFR plays an essential role in maintaining the intracellular tetrahydrofolate pools which, in turn, are an absolute requirement for the biosynthesis of purines and thymidylate and hence for DNA synthesis and

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cell replication [4, 5]. For this reason, DHFR is the molecular target of several antifolate compounds such as methotrexate (MTX), a widely used anticancer drug [5, 6]. Hence, the quantification of DHFR activity in mammalian tissues may be of clinical and pharmacological value in defining MTX therapeutic regimens.

In spite of the major antoneoplastic properties of MTX, its clinical application is, however, frequently limited by the ability of malignant cells to develop resistance to this drug [7, 8]. It is known that increased levels of DHFR [8] or increased activity of this enzyme [9] are two of the main mechanisms of acquired cellular resistance to MTX. Thus, determination of DHFR activity in malignant tissues may also be of interest as a predictive test of MTX sensitivity.

Several analytical methods have been devised for the determination of DHFR activity [4], but very few are sensitive enough to allow direct measurements of this enzyme in crude tissue preparations. It was felt that flow microcalorimetry could be an adequate method for this type of analysis, since it is a very simple and sensitive analytical technique, also well-suited for automatization. In order to analyse the potential use of flow microcalorimetry for the direct assay of DHFR activities in crude tissue homogenates, we have performed a flow microcalorimetric study of DHFR activity in three types of rat tissue preparations. The effects of *pH*, substrate concentration and ionic composition of the medium on the enzyme activity were also analysed using this technique. In order to obtain information about a possible application of flow microcalorimetry to the analysis of malignant tissues, this technique was subsequently applied to the determination of DHFR activities in human bone tumour homogenates.

Experimental

Materials and equipment

NADPH, dihydrofolic acid (DHF), methotrexate (MTX) and the enzyme dihydrofolate reductase from bovine liver (EC 1.5.1.3, DHFR), used in calibration experiments, were obtained from Sigma Chemical Co (St. Louis) and used without further purification. Stock solutions of 10 *mM* NADPH and of 10 *mM* dihydrofolate were made in a 10 *mM* Tris-Maleate buffer (*pH* 6.8) and kept at 4°C during the experiments. Methotrexate (stock 0.1 *M*) was used as an aqueous solution and stored in the dark.

Dihydrofolate reductase activities were determined in three types of rat (Sprague-Dawley) tissue homogenates, namely, liver, lung and brain. The tissues

were homogenized in 5–10 volumes of 10 *mM* Tris-Maleate buffer (*pH* 6.8) containing 1.5×10^{-7} *M* PMSF, with a Potter-Elvejhem homogenizer.

Human bone tumours were obtained from biopsy and either stored at –80° until used or immediately processed for the experiments. The tissues were cleaned of adipose material and cut into small pieces, homogenized in 5–10 volumes of 10 *mM* Tris-Maleate buffer (*pH* 6.8) containing 1. 5×10^{-7} *M* PMSF, using a Polytron homogenizer. The homogenates were filtered through glass-wool and eventually centrifuged at 18000×g for 30 min.

Protein was determined by the biuret method using bovine serum albumin as a standard [10].

The calorimetric experiments were performed in a LKB 2277 model microcalorimeter ('Bioactivity Monitor') operating in the flow-through mode and with a sensitivity of 10 μW, full scale. The reaction mixtures were kept under continuous magnetic stirring in the reaction vessel outside the calorimeter and were pumped into the measuring cell with an LKB 2232 microperspex-S peristaltic pump at a flow rate of 20 ml/h. Electrical calibrations of the instrument were always performed prior to the experiments.

Experimental procedure

Dihydrofolate reductase assays were performed at 37°C and *pH* 6.8 in a buffer mixture containing 10 *mM* Tris-Maleate, NADPH and dihydrofolate. The concentrations of NADPH and dihydrofolate used are given in the text or in the legends to the figures.

After establishing the instrument baseline by pumping buffer, substrate and cofactor through the calorimeter, an aliquot of tissue homogenate was added to the medium. When a steady-state heat flow was reached, the DHFR specific inhibitor methotrexate was added to the reaction vessel in order to block the enzyme activity. Heat flow was recorded until another lower steady-state level was reached. The decrease in heat effect produced by methotrexate constitutes a direct measure of DHFR activity. These heat power values were converted into enzyme activity units by using a purified bovine liver DHFR as a standard. One unit of DHFR activity (*U*) is defined as the amount of enzyme which hydrolyses 1 μ*M* of dihydrofolate per minute, at 37°C and *pH* 6.8.

Rat and human tissue homogenates were submitted to polyacrylamide gel electrophoresis in order to separate their constituent proteins and analyse their DHFR content. Electrophoresis were carried out in slab gels containing 10% acrylamide and 0.25% bisacrylamide, 0.1% SDS, 0.12% ammonium persulfate, 0.025% TEMED and 0.05 *M* Tris-glycine, *pH* 8.8. Samples were prepared by dissolving 1 mg/ml of protein in a mixture containing 1% SDS, 8 *M* urea, 1%

mercaptoethanol and 0.1 *M* Tris-glycine, *pH* 8.8, and heating for 2 min at 100°C. Marker proteins were subjected to identical treatment. Electrophoretic runs were carried out at 50 mA/gel for 4 h using 0.05 *M* Tris-glycine (*pH* 8.8) with 0.1% SDS as the electrode buffer. Gels were stained with 0.25% Coomassie Blue (in 40% methanol and 10% acetic acid) and destained in a 25% methanol and 5% acetic acid mixture.

Results and discussion

In the present study, the microcalorimetric measurements of DHFR activities in rat and in human neoplastic tissue homogenates were based on the inhibition of the enzyme activity by methotrexate. As shown in Fig. 1A, this procedure yields activity values (Δ_A) identical to the ones obtained by performing three independent measurements of heat-output rates (Figs 1B, C, D): two measurements of the heat flows produced independently by the tissue homogenate in the presence of NADPH (Δ_1) and in the presence of dihydrofolate (Δ_2), and one measurement of the heat flow generated when both substrate and cofactor are added to the tissue extract (Δ_3). Under these conditions, DHFR activity is proportional to the heat flow Δ_A, with

$$\Delta_A=\Delta_3-\Delta_2-\Delta_1$$

The simultaneous presence of NADPH and DHF is, in fact, an absolute requirement for the DHFR to the catalytically active. Addition of methotrexate to a reaction mixture containing only one of these compounds (DHF or NADPH) proved to have no influence on the heat effect signal produced by the tissue extract (data not shown).

In our experiments, MTX was added to the reaction mixture in a concentration which entirely blocked the DHFR activity in the sample (0.5 *mM*). In fact, increasing the concentration of this compound above that level did not further increase the fraction of the total heat flow which is reduced by MTX.

Our experimental results also showed a linear relationship between the fraction of total heat power reduced by excess MTX and protein concentration (Fig. 2).

Based on these results and on the assumption that MTX is a specific inhibitor of DHFR [4], we conclude that the inhibitory effects of MTX upon heat production by the tissue extracts constitute a specific measure of the total DHFR activity present in the sample.

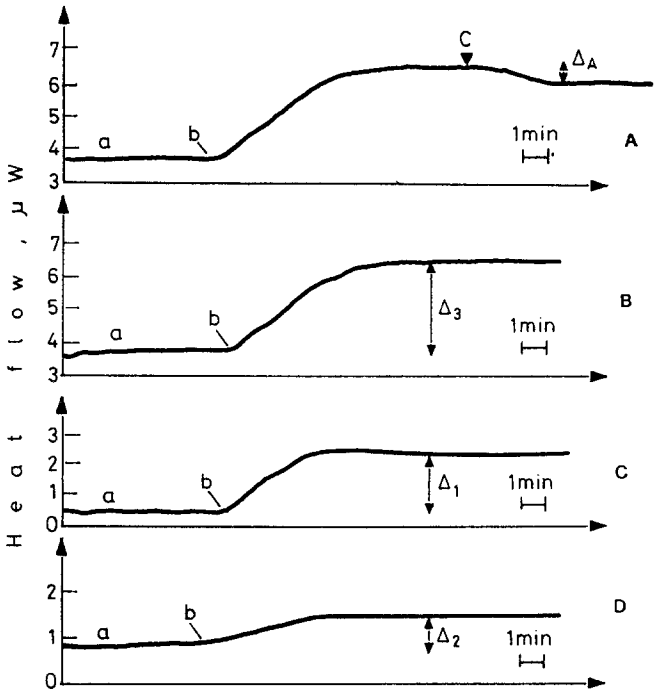

Fig. 1 Comparison of the two procedures for the microcalorimetric determination of DHFR activity in a rat liver homogenate
A. The tissue (0.5 mg protein) was added to the experimental medium (*a*) containing 10 *mM* Tris-Maleate buffer, *pH* 6.8, 0.4 *mM* DHF and 0.6 *mM* NADPH at the time indicated by the arrow at (*b*). MTX (0.5 *mM*) was added as shown in (*c*). The decrease in the heat effect representing DHFR activity is 0.6 μW (Δ_A)
B. Similar to A, except for the addition of MTX. The total heat power produced by the tissue extract is 3.2 μW (Δ_3)
C. Rat liver (0.5 mg protein) was added to the medium (*a*) containing 10 *mM* Tris-Maleate buffer, *pH* 6.8, and 0.6 *mM* NADPH at the time indicated by the arrow at (*b*). The heat-output rate is 1.7 μW (Δ_1)
D. As in C, but changing NADPH for DHF (0.6 *mM*). Heat flow = 0.8 μW (Δ_2). Sensitivity 10 μW full scale; flow rate 20 ml/h; temperature 37°C

When DHFR activity was assayed in the rat tissue homogenates, the highest levels of this enzyme were found in liver, the brain being the tissue with the lowest amount of DHFR activity (Fig. 3).

Flow microcalorimetry was also used to study the influence of substrate concentration, *pH* and ionic composition of the medium on the enzyme activity. The experimental results which are depicted in Fig. 4 indicate that the DHFR activity in rat liver and lung is higher for a 1/1.5 substrate/cofactor molar ratio. In contrast, higher levels of DHFR activity in rat brain homogenates were obtained when the [dihydrofolate]/[NADPH] molar ratio was equivalent to 1/2.5. These

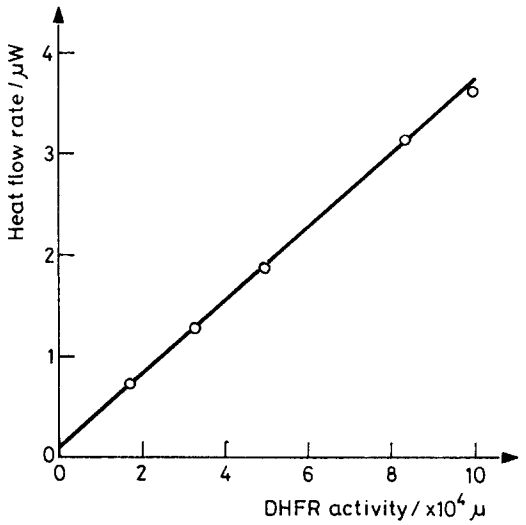

Fig. 2 Linearity of the microcalorimetric method for the assay of DHFR activities. Aliquots of the enzyme suspension containing increasing amounts of DHFR activity were added to the reaction medium composed of 10 *mM* Tris-Maleate buffer(*pH* 6.8), 0.4 *mM* DHF and 0.6 *mM* NADPH. DHFR activities were evaluated by the addition of MTX (0.5 *mM*), as referred under Experimental. The results shown are means of three independent determinations

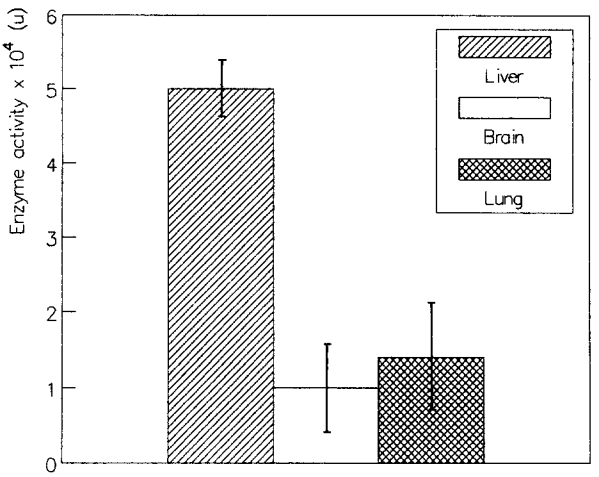

Fig. 3 DHFR activity in rat tissue homogenates. Aliquots of the tissue extracts (0.1–0.2 mg protein/ml of experimental medium) were added to the reaction medium containing 10 *mM* Tris-Maleate (*pH* 6.8), 0.4 *mM* DHF and 0.6 *mM* NADPH, DHFR activities were evaluated by the addition of MTX (0.5 *mM*), as referred under Experimental. The results shown are means ±SD of three independent determinations

differences might be related to different affinities of the enzymes in each type of tissue for the substrate and/or the cofactor. In fact, it is known that values of K_m for dihydrofolate and for NADPH vary considerably among dihydrofolate reductases from different sources [4, 10].

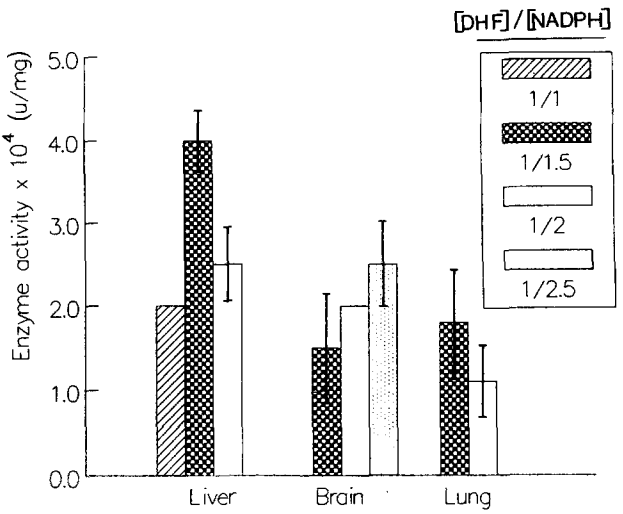

Fig. 4 Effect of substrate and cofactor molar ratio concentrations on DHFR activity. The experimental conditions are the same as those reported in Fig. 3, except that NADPH and DHF concentrations varied between 0.23–1.0 *mM* and 0.15–0.6 *mM*, respectively. The values shown are means ± SD of triplicates

Our data concerning the effect of *pH* on the enzyme activity show that the highest levels of liver DHFR activity are obtained at *pH* 4.5. However, in rat brain and lung the enzyme is not detectable at *pH* values below 6.0. The DHFR activity in these tissues is higher at *pH* 7.0. On the other hand, *pH* values above 8.0 inhibit DHFR in all the tissues studied (Fig. 5). Since the mechanism of DHFR inhibition by MTX involves binding of the inhibitor in its protonated form in N-1 to the active site of the enzyme, and considering the $pK_a = 5.7$ for the ionization of the pteroyl group of MTX [12], we could expect that MTX had no inhibitory effect upon DHFR in alkaline medium. However, this hypothesis was ruled out, since in the present work similar results were obtained when both types of calorimetric procedures were used for measuring DHFR activities as a function of *pH* (Fig. 1 for a description of these two procedures). Furthermore, ^{13}C-NMR studies performed by Cocco *et al.* [12] have shown that the binding of MTX to DHFR remains unchanged over the *pH* range 6–10, that is, the MTX

pteridine ring remains protonated at these *pH* values when the inhibitor binds to the enzyme active site.

The DHFR activity is also dependent on the ionic composition of the experimental medium. As shown in Fig. 6, when 0.6 *M* KCl or 0.6 *M* NaCl are added to the reaction mixture, there is a 1.5–3 fold increase in the DHFR activity of rat

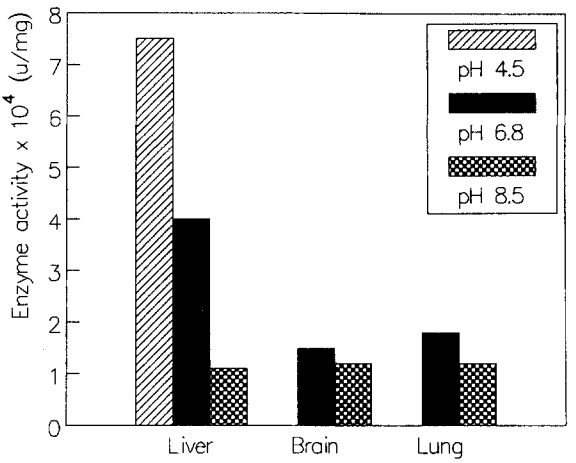

Fig. 5 DHFR activity in rat tissue homogenates as a function of *pH*. Experimental conditions are similar to the ones reported in Fig. 3. Assays at *pH* 6.8 and *pH* 8.5 were performed in a 10 *mM* tris-Maleate buffer, while 10 *mM* tris-Acetate was the buffer used at *pH* 4.5. The results are means of 3–5 determinations

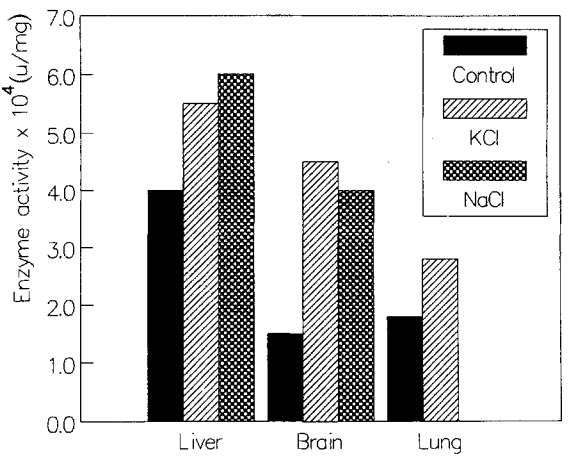

Fig. 6 Effect of salts on DHFR activity in rat tissue homogenates. Control experiments were performed as described in Fig. 3. The ionic composition of the experimental medium was modified, as indicated, by the addition of 0.6 *M* KCl or 0.6 *M* NaCl. The results are means of triplicates

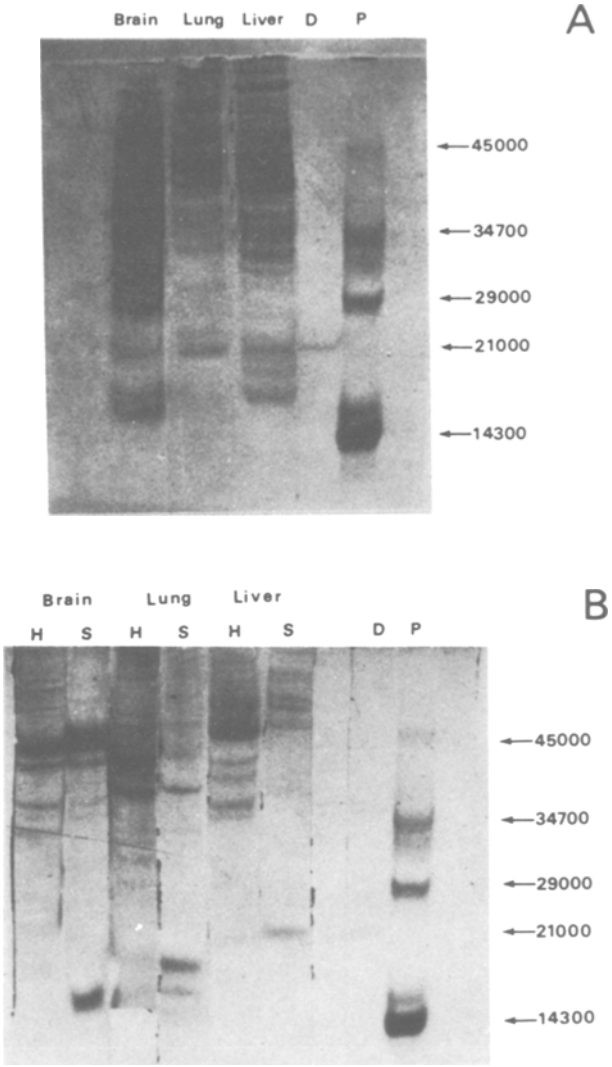

Fig. 7 Protein gel electrophoresis of rat tissue homogenates

A. Electrophoresis of tissue extracts whose DHFR activity levels were measurable by microcalorimetry

B. Electrophoresis of tissue homogenates in which no DHFR activity was detectable by microcalorimetry; electrophoresis of supernatants obtained after centrifugation of these homogenates. Electrophoresis were performed in SDS-polyacrylamide gels, as described under Experimental. Each tissue sample in the gel contains 50 μg of protein. Column P contains marker proteins (from top to bottom: egg albumin, 45000; pepsin, 34700; carbonic anhydrase, 29000; lisozyme, 14300). Column D corresponds to a purified bovine liver DHFR (4 μg protein). H-homogenate; S-supernatant

tissue homogenates. As stated by Kamen and coworkers [13], the extent of inhibition of DHFR by MTX is independent of the presence of 0.1– 1.0 *M* KCl in the reaction medium. These results validate the experimental procedure adopted in the present work for measuring DHFR activity in media with high salt concentrations.

Some of the rat tissue preparations studied by flow microcalorimetry had no detectable levels of DHFR activity. In order to obtain further information about

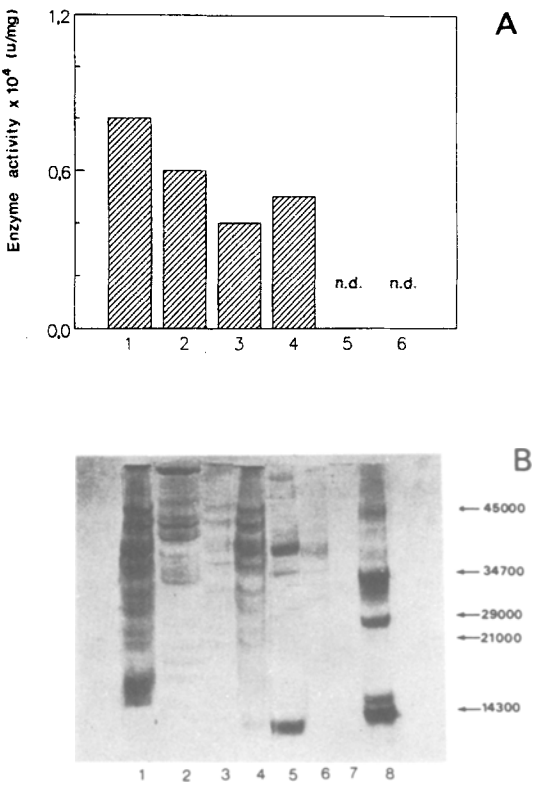

Fig. 8 A. DHFR activity in human malignant tissue homogenates
B. Protein gel electrophoresis of malignant tissue homogenates. Microcalorimetric measurements of enzyme activity were performed by adding the tissue extract (2–5 mg of protein) to the reaction medium containing 10 *mM* Tris-Maleate buffer (*pH* 6.8), 0.4 *mM* DHF and 0.6 *mM* NADPH. MTX was added in a final concentration of 0.5 *mM*. The values are means of triplicates. Electrophoresis were performed in SDS-polyacrylamide gels, as described under Experimental. Each sample in the gel contains 50 μg of protein (columns 1 to 6: asteosarcoma, Ewing's sarcoma, osteoid osteoma, mesenchymal chondrosarcoma, multiple myeloma and synovial sarcoma). Columns 7 and 8 contain a purified bovine liver DHFR and marker proteins, respectively. n.d. not detected

the presence or absence of the enzyme in the tissue homogenates, these samples were also analysed by SDS-polyacrylamide gel electrophoresis. Those tissue preparations with DHFR activity levels which are detectable by microcalorimetry also produce, on electrophoresis, a protein band with a molecular weight of 21000, characteristic of this enzyme (Fig. 7A). In contrast, the DHFR protein band was not visualized in gels obtained from electrophoresis of lung and brain homogenates which had no detectable levels of DHFR activity, when assayed by microcalorimetry. Similarly, the supernatants obtained after centrifugation of these homogenates neither show the DHFR protein band nor have DHFR activity (Fig. 7B). On the other hand, electrophoresis of a liver homogenate with no measurable DHFR activity levels indicated the presence of the enzyme in this sample. Centrifugation of the liver homogenate yielded a partially purified supernatant fraction which was enriched in DHFR, as shown by the more intense DHFR band on electrophoresis (Fig. 7B). In this fraction, a low level of DHFR activity was subsequently measured using the flow microcalorimetric method.

The application of low microcalorimetry to the assay of DHFR activities in human bone tumour homogenates yielded the results illustrated in Fig. 8A. Although in some tissues DHFR activity was not detectable by microcalorimetry (multiple myeloma and synovial sarcoma-columns 5 and 6, respectively), other samples showed small levels of this enzyme, which varied between 4×10^{-5} and 8×10^{-5} U/mg protein (from column 1 to 4, osteosarcoma, Ewing's sarcoma, osteoid osteoma and mesenchymal chondrosarcoma). These results are consistent with data from Kamen and coworkers [14] on the radiometric measurement of DHFR activities in several types of human tissues.

In contrast to the enhancement of DHFR activity by salts found with the rat tissues studied, no significant changes in enzyme activity were observed, under these conditions, with malignant tissues. Structural differences between dihydrofolate reductases from normal and neoplastic tissues may account for the observed differences in enzyme activity in the presence of inorganic ions [15].

Protein gel electrophoresis of these neoplastic tissues yielded results which are in agreement with the microcalorimetric data (Fig. 8B), demonstrating the potential of microcalorimetry for measuring DHFR activities in malignant tissue homogenates.

Conclusions

Flow microcalorimetry constitutes a very simple and sensitive method for the direct assay of dihydrofolate reductase activities in tissue homogenates.

Flow microcalorimetric measurements of dihydrofolate reductase activities in crude tissue preparations can be simplified by using the enzyme inhibitor methotrexate. With this procedure one obtains a specific measure of the total DHFR activity in crude biological samples. Using flow microcalorimetry, very low levels of DHFR activity are also measured in human bone tumour homogenates, demonstrating the potential of the technique in the analysis of this enzyme in malignant tissues.

The high specificity and the simplicity of this analytical procedure make possible the wide spread application of flow microcalorimetry in the pharmacological area.

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Zusammenfassung — Kalorimetrische Verfahren werden immer mehr zu wichtigen Hilfsmitteln in einigen Gebieten der biochemischen und in der biologischen Forschung. Es wird hier eine Wärmefluß-Mikrokalorimetermethode bei der Bestimmung der Aktivität von Dihydrofolatreduktase (DHFR) in Homogenaten bösartiger Gewebe bei Ratten und beim Menschen angewendet. Im Gegensatz zu anderen, allgemein benutzten DHFR-Bestimmungen, erlaubt die Empfindlichkeit der Wärmeflußmikrokalorimetrie direkte Messungen

dieses Enzymes in Rohgewebepräparaten. Die experimentellen Daten von Rattengewebehomogenaten zeigen, daß die höchste Enzymaktivität in der Leber zu finden ist, während Lunge und Hirn geringere DHFR-Aktivitäten aufweisen. Das Leberenzym hat eine höhere Aktivität bei *pH* 4,5, das *pH*-Optimum für die Enzyme in Lunge und Hirn liegt jedoch bei 6,8. Das Molverhältnis Substrat/Kofaktor für die höchste DHFR-Aktivität liegt für Leber- und Lungenenzyme bei 1/1,5 und bei 1/2,5 für Gehirn-DHFR. In diesen Rattengewebehomogenaten wurde DHFR durch KCl oder NaCl aktiviert: in Gegenwart dieser Salze (0.6-molar) liegt die Enzymaktivität um das anderthalb- bis dreifache höher als normal. Unter Anwendung der Wärmeflußmikrokalorimetrie konnten auch in menschlichen Knochentumorhomogenaten sehr niedrige DHFR-Aktivitäten gemessen werden,was die Leistungsfähigkeit dieser Methode in der Analyse dieser Enzyme in bösartigen Geweben demonstriert.