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CORRELATION OF INCREASED ACETATE BINDING WITH ALKYLATING AGENT RESISTANCE IN WALKER AND YOSHIDA TUMOR CELLS

SETH D. PINSKY*
Department of Biochemical Pharmacology, Institute of Cancer Research, Sutton, Surrey, England

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Abstract—The utilization of acetate is fundamental to numerous cellular processes. At the level of chromatin, histone acetylation is thought to regulate transcription, and acetate is a major source of cellular energy as it is the substrate for the citric acid cycle. The present work investigates the incorporation of [1-^{14}C]sodium acetate in alkylating agent sensitive (WS) and resistant (WR) Walker 256 carcinosarcoma cells. WR bound the labeled acetate four to six times faster than WS cells, as determined by incorporation of [1-^{14}C]sodium acetate into trichloroacetic acid precipitable material. This difference was consistently observed in both nuclear and cytoplasmic fractions of the cells and in cells permeabilized prior to incubation with radioactive acetate. WS and WR cells did not differ from each other in content of either reduced or acetylated CoA. Since adding exogenous CoA-SH to cell lysates did not alter acetate binding or reduce the differences between WS and WR, increased acetylation in WR cells was independent of CoA levels. Using [1-^{14}C]acetyl CoA to label lysolecithin-permeabilized WS and WR cells revealed no difference between the sensitive and resistant lines, in contrast to the 5-fold greater binding of [1-^{14}C]sodium acetate in permeabilized WR cells. This suggests that WR cells formed acetyl CoA more rapidly than did the WS cells from acetate plus endogenous CoA. Chlorambucil treatment (24 hr) did not affect acetylation of nuclear proteins in log phase cells. Finally, 3-fold greater acetylation in a line of Yoshida sarcoma cells that is resistant to alkylating agents, compared to the sensitive line, supported the generality of the phenomenon.

Acquired resistance to alkylating agents is a formidable clinical problem. In his review of the topic in 1974, Connors [1] concluded that, in contrast to methotrexate and asparaginase resistance, alkylating agent resistance is slower to develop and that no single biochemical event could be identified as the cause. This was taken to imply that a summation of several biochemical factors yields a high degree of resistance. Diminished drug uptake, increased catabolism of the drug, alteration of the DNA target, enhanced DNA repair capacity and increased production of target nucleophiles were all suggested as participants in alkylating agent resistance. Thiols have been the center of attention in the latter category. However, the total increase in thiol levels was too small to account for the degree of alkylating agent resistance [2].

Tisdale and Philips [3, 4] treated Walker ascites carcinosarcoma 256 cells in culture with sequentially increasing doses of melphalan to produce a highly drug resistant line. This line exhibited cross-resistance to other bifunctional alkylating agents, including chlorambucil. They found that in response to chlorambucil the sensitive (WS)†, but not the resistant (WR), line showed increased cyclic AMP levels. Likewise, chlorambucil induced nonhistone nuclear protein phosphorylation in alkylating agent sensitive Yoshida sarcoma cells (YS) but not in cells with acquired resistance (YR) [5]. Furthermore , the steroid, prednisolone, yielded loss of condensed chromatin and enhanced chlorambucil cytotoxicity in YR and WR cells [6].

Post-synthetic acetylation of histones, like increased nuclear protein phosphorylation, is associated with loss of condensed chromatin and increased transcription [7]. The present study was initiated to examine in detail acetate disposition in WS and WR cells. A fundamental difference in acetate metabolism between alkylating agent sensitive and resistant cell lines is reported. Unlike the above studies, this difference is demonstrable in the absence of drug treatment.

A preliminary report of this work was given at the annual meeting of the American Association for Cancer Research, Washington, DC in April 1981.

MATERIALS AND METHODS

Materials

Dulbecco's minimum essential medium (MEM), Fischer's medium and donor serum were from Flow Laboratories Ltd. (Irvine, Scotland). [1-^{14}C]Acetic acid, sodium salt (sp. act. 55.5 mCi/mmole) and [1-^{14}C]acetyl CoA (sp. act. 5.5 mCi/mmole) were purchased from Amersham Ltd. (Amersham, England). Electrophoretic reagents, including acrylamide, were from BioRad UK (Watford, Herts, England). Boehringer UK (London, England) supplied

* Present address: Department of Cell Biology, The Rockefeller University, 1230 York Ave., New York, NY 10021, U.S.A.

† Abbreviations: WS, alkylating agent sensitive Walker 256 carcinoma cells; WR, alkylating agent resistant Walker 256 carcinoma cells; YS, alkylating agent sensitive Yoshida sarcoma cells; YR, alkylating agent resistant Yoshida sarcoma cells; TCA, trichloroacetic acid; and MEM, minimum essential medium.

enzymes used for the CoA assay and the trilithium salt of acetyl CoA. Chromatopure CoA-SH was purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). Lysolecithin (L-α-lysophosphatidylcholine) Type I was from Sigma UK. All other chemicals were of analytical grade. Chlorambucil was a gift from The Burroughs Wellcome Foundation (Beckenham, Kent, England).

Methods

Tissue culture and animals. Two lines of Walker rat carcinosarcoma 256 cells, one a descendant of the Tisdale and Philips alkylating agent resistant line [4], were grown in Dulbecco's MEM supplemented with 10% donor horse serum in static suspension culture under 5% CO_2. All experiments were performed on cells in logarithmic growth phase. Resistance has been maintained by periodic exposure to chlorambucil monitored with growth curves and colony forming assays. Figure 1 is a typical growth curve. For one experiment, the cells were grown as ascitic tumors in 200 g female Wistar rats and were used 4 days after inoculating 10^6 cells intraperitoneally. Fischer's medium with 10% donor horse serum was used for suspension culture of Yoshida sarcoma cells.

Acetate labeling. Cells were routinely grown to 2–3 × 10^5/ml (log phase), harvested by centrifugation, and resuspended at 10^7 cells/ml. [1-^{14}C]Sodium acetate (sp. act. 55.5 mCi/mmole, Amersham) was then added to a final concentration of 90 μM. For the brief incubations (15 sec to 10 min series: Fig. 2), cells were divided into 0.35-ml aliquots, and the reaction was terminated by a 30-sec spin in a Beckman microfuge. Medium containing labeled acetate was decanted, and the last traces were removed with an absorbent tissue. The pellet was immediately resuspended in 0.2 ml of phosphate- buffered saline at 4°; 0.1 ml was transferred to a vial for an estimate of total cellular acetate uptake by liquid scintillation spectrometry. TCA (100%) was rapidly added to the remaining 0.1 ml of the sample to a final concentration of 10%, and this was filtered through glass fiber discs for determination of acid-insoluble radioactivity. An analogous experiment was performed adding labeled acetate to six different final concentrations: 18, 36, 90, 180, 360 and 720 μM. Data were obtained for 2- and 10-min incubations.

Cellular fractionation. Nuclei were prepared by homogenization of the cells in hypotonic buffer according to the method of Sporn *et al.* [8], modified in that a motor-driven Thomas Teflon/glass homogenizer was used. Early supernatant fractions were retained to give an estimate cytoplasmic acetylation. Pelleted nuclei were extracted twice with 1 ml of 0.4 N H_2SO_4 for 1 hr, and these supernatant fractions were pooled. Histones and other acid-soluble nuclear proteins were precipitated by adding TCA to a final concentration of 20% TCA, and the pellets were washed with acidified acetone (10 mM HCl) and pure acetone. These were dissolved in buffer containing 1 M Tris–HCl (pH 6.8), 1% 2-mercaptoethanol and 6 M urea. A Bio-Rad protein determination [9] was performed so that identical amounts of each sample could be applied to a 15% sodium dodecylsulfate polyacrylamide electrophoresis slab gel [10], which was stained for 1 hr with 0.1% Coomassie blue in 50% methanol, 7% acetic acid and destained in 20%

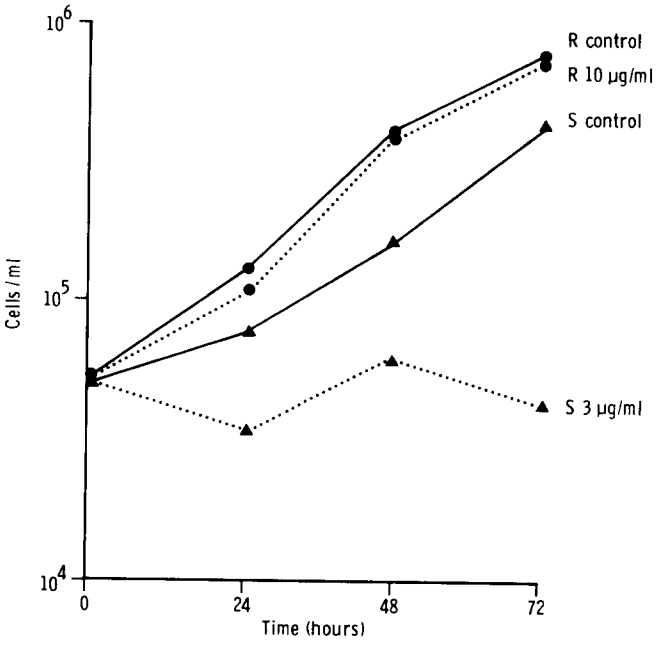

Fig. 1. Effect of chlorambucil on the growth of WS and WR cells. Cells were maintained in static suspension culture in Dulbecco's MEM. Chlorambucil in ethanol (final ethanol concentration less than 0.1%) was added to cultures for 2 hr. Cells were then harvested by centrifugation and resuspended in drug-free medium. Key: (●——●) WR control; (● · · · ●) WR 10 μg/ml chlorambucil; (▲——▲) WS control; and (▲ · · · ▲) WS 3 μg/ml chlorambucil.

methanol, 7% acetic acid. Slices corresponding to histones H1 and H4 were combusted in an Intertechnique Oxymat for scintillation counting.

Permeabilizing cells prior to addition of CoA and acetyl CoA. For the addition of exogenous CoA to the [1-^{14}C]sodium acetate incubation, cells were suspended in hypotonic (RSB) [8] buffer at 10^8/ml for 30 min and then subjected to five strokes with a Thomas (Teflon/glass) homogenizer. Lysolecithin was used instead of hypotonic lysis in the experiment comparing binding of [1-^{14}C]acetate with [1-^{14}C]acetyl CoA, according to Miller *et al.* [11]. Briefly, cells were resuspended at 10^8/ml, 4° in solution A [11]; 1/3 volume of lysolecithin at 1 mg/ml was added for 1 min; cells were immediately diluted 1:10 with 37° medium for radioactive labeling. The specific activity of [1-^{14}C]acetate was adjusted to 5.5 mCi/mole for this experiment only, to be equal to the specific activity of the [1-^{14}C]acetyl CoA (Amersham). Reactions in both experiments were stopped by the addition of 100% TCA to a final concentration of 10%, and precipitable radioactivity was counted.

CoA assay. Cellular CoA concentrations were determined exactly as described by Michal and Bergmeyer [12] by the catalytic assay method with phosphotransacetylase.

RESULTS

Figure 2 characterizes the differential binding of [1-^{14}C]sodium acetate in WS and WR cells. Experiments were conducted at both 0° and 37°, and radioactivities in both total and acid-insoluble fractions were determined. Several facts can be discerned from the figure. First, there was a virtual absence of binding at 0°. Second, uptake at 15 sec was approximately equal in WS and WR cells, and remained so in the absence of binding at 0° for 10 min. Third, as binding increased with time at 37°, so did total intracellular acetate. Finally, the rate of binding of [1-^{14}C]acetate in the resistant cells was approximately five times greater than in the sensitive, as measured by TCA precipitable radioactivity. Changing the concentration (and consequently the specific activity) of the labeled acetate from 18 to 720 μM did not alter the ratio of labeled acetate binding in WR and WS cells. That is, the ratio remained between 4.1:1 and 5.2:1 for 2- and 10-min incubations (data not shown).

By fractionating the cells into nuclear and cytoplasmic components (Fig. 3), it can be seen that the difference between WR and WS, shown in Fig. 2, was present in both cellular compartments. Furthermore, histone H4, which was rapidly acetylated,

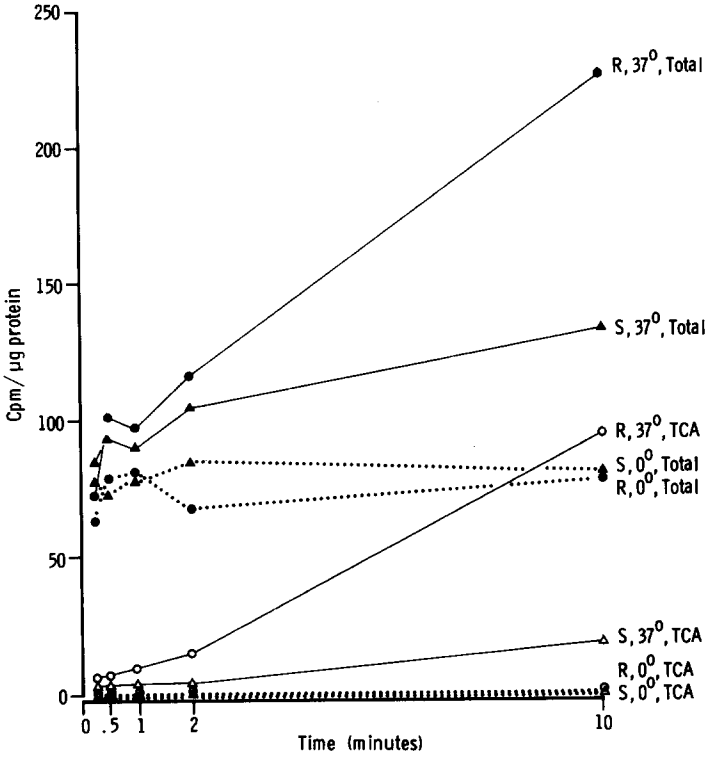

Fig. 2. Uptake and binding of [1-^{14}C]sodium acetate in WS and WR cells. Logarithmically growing cells were concentrated to 10^7/ml in MEM at 37°. [1-^{14}C]Acetate was added, and the reaction was terminated by centrifugation in a Beckman microfuge followed by resuspension in ice-cold PBS. Half of the cells were immediately transferred to vials containing scintillation fluid, and TCA was added to the remainder to count bound radioactivity. Points are means of three experiments. The maximum standard error was less than 10%. Key: (●——●) WR 37°; (● · · · ●) WR 0°; (▲——▲) WS 37°; and (▲ · · · ▲) WS 0°. Closed symbols = TCA insoluble cpm, and open symbols = total cpm.

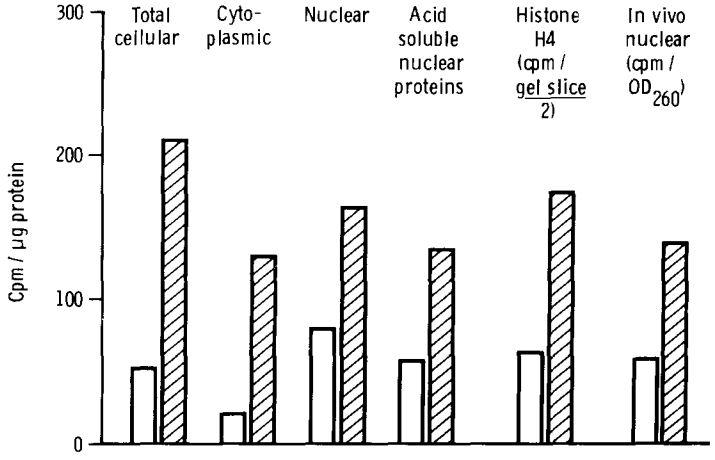

Fig. 3. Binding (TCA precipitable counts) of [1-^{14}C]sodium acetate in fractions of WS and WR cells. Cells were resuspended as in Fig. 2 and incubated for 10 min with labelled acetate. Nuclei were isolated by homogenization in hypotonic RSB and washing in sucrose/Triton X-100. Histones were extracted from nuclei in 0.4 N H_2SO_4. The last columns pertain to cells grown in Wistar rat ascites prior to assay. Key: (□) WS, and (▨) WR.

similarly showed more incorporation of label in WR than in WS cells. No binding to H1 could be detected in either WS or WR cells during the 10-min incubations. By 1 hr of incubation with labeled acetate, widespread, non-specific accumulation of label was seen in most nuclear proteins (data not shown). The final set of bars in this figure refers to an experiment in which Walker S and R cells were grown *in vivo* as an ascitic tumor in rats. Results were substantially the same in that greater acetylation in WR cells was observed.

CoA is an intermediate in cellular acetylation reactions. Therefore, the experiments in Fig. 4 and Table 1 were performed to determine if CoA levels contributed to acetylation differences between WS and WR cells. The figure shows that CoA levels did not limit the acetylation rate in lysed cells since the addition of exogenous coA did not measurably alter acetate binding. Furthermore, the enzyme assay (Table 1) for CoA levels in reduced and disulfide or acetylated forms did not reveal a difference between WS and WR cells.

Transacetylases which transfer acetate from acetyl CoA to proteins tend to be relatively specific enzymes. Therefore, it seemed unlikely that a phenomenon demonstrable in nuclear and cytoplasmic compartments could be the result of alterations in numerous specific enzymes. Since CoA levels were likewise ruled out as the locus of differential binding it was necessary to determine if the difference in acetate binding was based on differential formation of acetyl CoA from the labeled acetate. This was done by using [1-^{14}C]acetyl CoA, by-passing the formation of acetyl CoA from [1-^{14}C]acetate + CoA. The incorporation of label into WS cells increased such that no difference in ^{14}C binding between WS and WR cells was seen with labeled acetyl CoA in permeabilized cells compared to 5-fold greater binding in WR and WS using labeled acetate (Fig. 5).

Experiments shown in Fig. 6 were designed to measure the effect of chlorambucil treatment on acetate binding. Neither 2- (data not shown) nor 24-hr chlorambucil treatment at 1 or 3 µg/ml significantly modified acetate binding in WS or WR cells.

Finally, the generality of the relationship between acetate binding and alkylating agent resistance was probed by testing another cell line, the Yoshida sarcoma. These YR cells showed a pattern of resistance to alkylating agents similar to that of the WR cells. Likewise, the YR cells bound acetate approximately three times faster than the YS cells (Fig. 7).

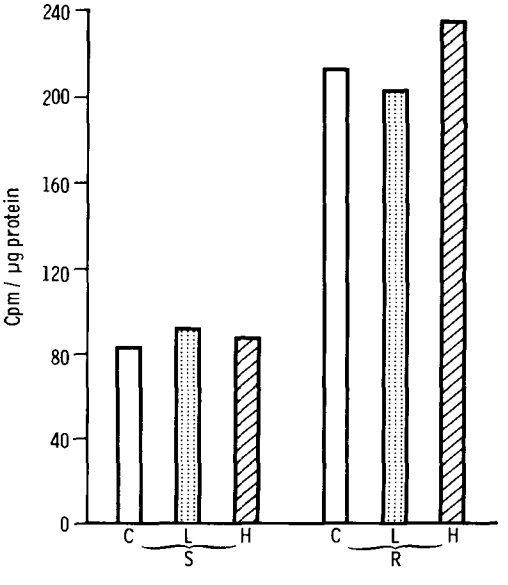

Fig. 4. Effect of exogenous CoA on binding (TCA precipitable counts) of [1-^{14}C]sodium acetate in Walker tumor cell lysates. Ten-minute incubations with labeled acetate in cell lysates at 5×10^7 cells/ml were terminated by adding TCA to a final concentration of 10%. Key: C = no external CoA-SH; L = + 175 µg/µl CoA-SH; H = + 500 µg/ml CoA-SH; S = sensitive; and R = resistant.

Table 1. CoA levels in WS and WR cells*

	CoA (μg/4.75 × 10^8 cells)		
	CoA-SH +CoA-SS-CoA +CoA-S-AcO	CoA-SS-CoA +CoA-S-AcO	% Available (in SH form)
S	1.82 ± 0.125	1.46 ± 0.124	20.1
R	1.71 ± 0.130	1.37 ± 0.100	19.9

* The catalytic phosphotransacetylase enzyme assay of Michal and Bergmeyer [12] was used.

DISCUSSION

It can be concluded that the binding of acetate is on the order of five times more rapid in alkylating agent resistant than sensitive Walker tumour cells. This difference is probably a function of the rate of formation of acetyl CoA from free cellular CoA plus exogenous labeled acetate.

By limiting incubation of cells with labeled acetate to a maximum of 10 min, it is probably safe to assume that TCA insoluble radioactivity is in the form of acetylated macromolecules and does not include substantial amounts of ^{14}C incorporated into newly synthesized molecules. This contention is supported by the fact that, following 10-min incubations, histone H4 was heavily labeled whereas H1 was free of detectable radioactivity. However, it is not feasible to use incubations longer than 15–20 min since by 1 hr extensive, non-specific incorporation of ^{14}C into proteins occurs.

Although Fig. 6 indicates that chlorambucil had no effect on acetylation of histones, additional experiments would be required to validate this conclusion. First, it might be necessary to perform the experiment in synchronized cells since histone acetylation is cell cycle dependent [7]. And it might also be important to fractionate the histones into individual species since inclusion of mixed proteins could obscure small effects.

The lack of binding of labeled acetate at 0° (Fig. 2) suggests that acetylation is enzymatically mediated. It can also be inferred from that figure that the uptake of labeled sodium acetate into WS and WR cells is approximately equal and does not, therefore, contribute to the observed S/R difference. A possible explanation for the increase in total radiolabeled intracellular acetate concentration (which parallels increased binding) in both WS and WR cells during the 10-min incubation at 37° is: (1) uptake is complete by 15 sec since there was no change in intracellular radioactivity at 0° from 15 sec to 10 min; (2) that same value for uptake was observed at 37° for the initial (15 sec) time point; (3) endogenous levels of free acetate were low with respect to the concentration of acetate added to the incubation; and (4) it is therefore likely that the rapid binding

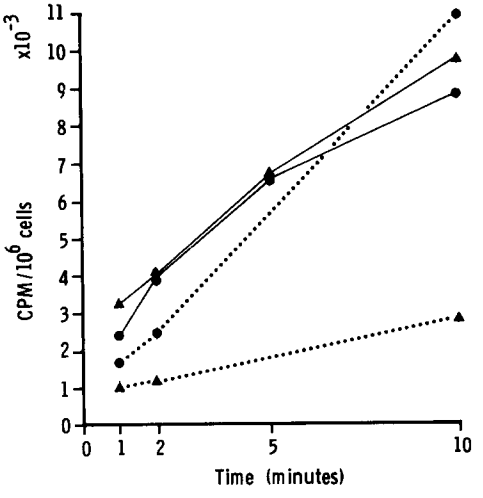

Fig. 5. Comparison of binding of [1-^{14}C]sodium acetate and [1-^{14}C]acetyl CoA in WS and WR cells. Incubation of labeled compounds was as in Fig. 2. Reactions were stopped by adding TCA to a final concentration of 10%. Key: (●) WR, and (▲) WS. Solid lines = [1-^{14}C]acetyl CoA, and dotted lines = [1-^{14}C]$Na^+CH_3COO^-$.

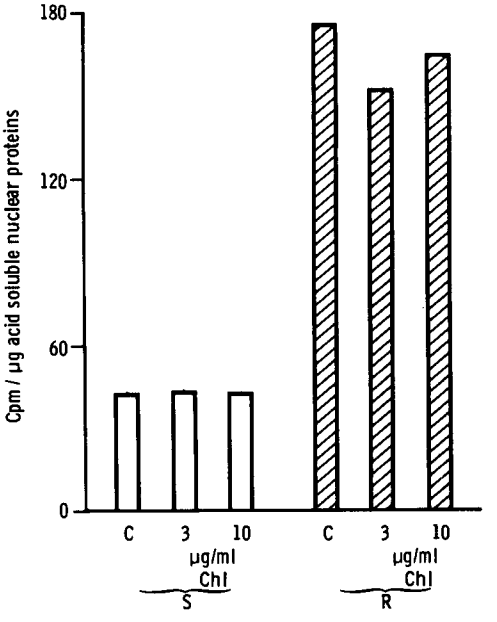

Fig. 6. Effects of chlorambucil on acetylation of acid-soluble nuclear proteins in WS and WR cells. Cells were exposed to chlorambucil for 24 hr prior to a 10-min incubation with labeled acetate and subsequent nuclear protein isolation as in Fig. 3. Key: C = control; R = resistant; and S = sensitive.

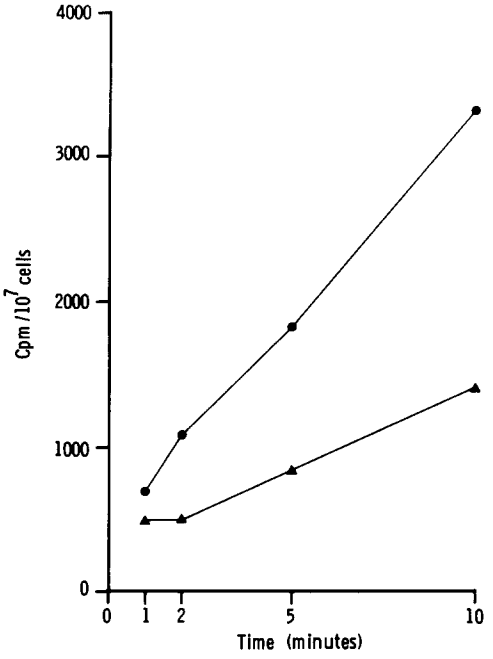

Fig. 7. Binding of [1-^{14}C]sodium acetate in alkylating agent sensitive and resistant Yoshida tumor cells. [1-^{14}C]Sodium acetate incubations were as in Fig. 2. Key: (▲) YS, and (●) YR.

of acetate observed at 37°, but not at 0°, led to the influx of additional labeled acetate into the cells such that intracellular free acetate levels remained in equilibrium with extracellular free acetate.

Another observation suggesting that differential uptake cannot be responsible for differential binding in WS and WR cells is that differential binding was observed in permeabilized cells. Furthermore, this binding difference is most likely not the result of different acetate pool size because the addition of different amounts of labeled acetate (5–200 μCi) gave a constant ratio of binding of approximatly 5:1 in WR compared to WS cells. Nor can the difference in acetate binding between WS and WR cells be accounted for on the basis of CoA levels in the two cell lines, since levels of available (reduced) CoA as well as acetyl CoA plus CoA-SS-CoA are the same in both lines. Additionally, supplementing the incubation with reduced CoA did not alter incorporation of CoA into macromolecules.

Taken together with Fig. 5, which shows that there was no difference between WS and WR cells in macromolecular acetate binding when [1-^{14}C]acetyl CoA was used in plase of [1-^{14}C]acetate, these data point to the following reaction as being an important determinant of the S/R difference:

$$\text{Acetate} + \text{ATP} + \text{CoA-SH} \rightarrow \text{acetyl-S-CoA} + \text{AMP} + \text{PP}_i.$$

This reaction is catalyzed by acetyl CoA synthetase (EC 6.2.1.1) [13], an enzyme which shows variability in subcellular localization across mammalian organs. In rabbit liver it is primarily cytosolic compared to bovine liver and heart where it is mitochondrial [14]. Experiments underway are aimed at characterizing this enzyme in WS and WR cells.

The question of how the observed difference in acetate metabolism might be related to alkylating agent resistance is difficult to resolve. One possibility is that the drug reacts with the thiol group in CoA, similar to acetate, and that this reaction has a higher turnover rate in WR than WS cells, with CoA-SH being regenerated in the process. This interpretation is unlikely because the alkylating agent-S-CoA complex might not readily dissociate as does acetyl-S-CoA. Another possibility is enhanced metabolism of chlorambucil in resistant cells. Chlorambucil undergoes fatty acid oxidation in a series of reactions in which an α,β-dehydrochlorambucil-S-CoA complex is formed and then yields phenylacetic mustard + CoA-SH [15]. However, chlorambucil is unique among the alkylating agents in utilizing this pathway. It is possible that the rapid rate of formation of acetyl CoA confers the resistant cells with a more general ability to cope with alkylating agent challenge in that the cell can respond with an increased capacity for oxidative metabolism. This is consistent with the theory [1] that alkylating agent resistance is the product of diverse cellular processes. Perhaps future work will clarify the relationship between acetylation, acetyl CoA formation and alkylating agent resistance, but it is tantalizing that two different paired lines, the Yoshida and Walker tumors, show similar patterns of alkylating agent resistance as well as enhanced acetate binding.

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