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Phosphorylation of "tricyclic nucleoside" by adenosine kinases from L1210 cells and HEp-2 cells

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"Tricyclic nucleoside" (TCN) [6-amino-4-methyl-8-(β-D-ribofuranosyl)-(4*H*, 8*H*)pyrrolo[4, 3, 2-*de*]pyrimido [4, 5-*c*]pyridazine] is a nucleoside of novel structure synthesized by Schram and Townsend [1]. This compound is active against experimental animal tumours [2] and is currently in Phase I clinical trial. The active form of TCN is its 5'-phosphate, the formation of which is catalyzed by adenosine kinase [3, 4]. Several years ago we reported an apparent Michaelis constant of 215 μM for the phosphorylation of TCN by a partially purified adenosine kinase from HEp-2 cells [4]. More recently we have had occasion to study the phosphorylation of TCN by kinase preparations from both HEp-2 cells and L1210 cells and have found Michaelis constants for both enzymes two orders of magnitude less than that previously reported for the enzyme from HEp-2 cells.

For this study we used an adenosine kinase preparation purified 134-fold from HEp-2 cells [5] and a preparation purified to apparent homogeneity from L1210 cells [6]. The assays measured the conversion of [^{14}C-methyl]TCN (obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) to its 5'-phosphate. The conditions of the assays, which have been reported earlier [5–7], differed for the enzymes from the two sources. For the enzyme from HEp-2 cells [5] the assays were performed at 25°; the incubation mixture contained 2.5 mM ATP, 0.25 mM $MgCl_2$, and 50 mM potassium phosphate buffer; and the nucleotide formed was isolated by paper chromatography and assayed for radioactivity in a chromatogram scanner. For the enzyme from L1210 cells [6, 7], the assays were performed at 37°; the incubation mixture contained 1.25 mM MgATP, $MgCl_2$ in 0.4 mM excess of that of MgATP, 50 mM Tris–HCl, pH 8.0, and bovine serum albumin (6 μg); and the nucleotide formed was isolated on Whatman DE-81 paper discs which were assayed for radioactivity by liquid scintillation spectrometry. The conditions for assay of the HEp-2 enzyme were not changed to those in current use because we were attempting to determine the reasons for the discrepancy between our present and earlier results.

Double-reciprocal plots for the phosphorylation of TCN by the two enzyme preparations are shown in Fig. 1. For the HEp-2 enzyme the apparent K_m for TCN was 1.8 μM and the V_{max} was 42% that for adenosine; the apparent K_m for adenosine (plot not shown) was 1.7 μM. For the L1210 enzyme the apparent K_m for TCN was 1.3 μM and the V_{max} was 110% that of adenosine; the apparent K_m for adenosine (plot not shown) was 0.5 μM [7].

We have examined our earlier data with the HEp-2 enzyme and have found no apparent error, and we are therefore unable to explain the discrepancy between our earlier and present results. In our former study, we used a different assay for product formation (determination of formation of [^{32}P]TCN 5'-phosphate when the incubation mixture contained [γ-^{32}P]ATP and unlabeled TCN), but this method of determining kinase activity is unlikely to be

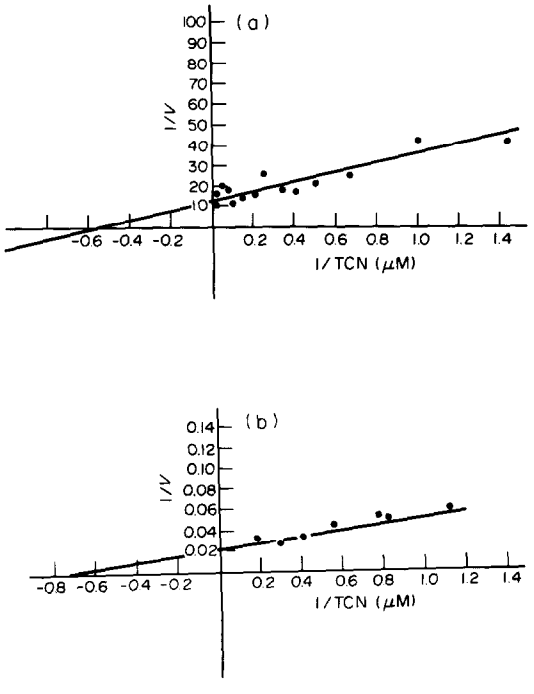

Fig. 1 Double-reciprocal plots of initial TCN concentration vs the reaction velocity (nmoles TCN-phosphate per min per mg protein) for the phosphorylation of TCN by adenosine kinase preparations from HEp-2 cells (A) and L1210 cells (B). See text for the conditions for the assays and the kinetic constants calculated from these plots.

at fault because it has been thoroughly evaluated and found to give the same results as the assay employing the labeled nucleoside as substrate [5]. An alteration in the cells and consequent alteration in the kinase cannot be ruled out. However, this is also unlikely because the K_m for adenosine has remained the same over the 16 years that we have worked with the HEp-2 enzyme. The presence of impurities in TCN could have affected the results but, even if present, impurities are unlikely to account for a difference of two orders of magnitude unless they themselves were substrates or good inhibitors. The purity of the non-radioactive sample used earlier could not be re-examined because none of it remains; however, the [^{14}C]TCN had a high degree of radiopurity (97% as determined by paper chromatography). We therefore think that our reported results for TCN were in error for reasons that cannot now be explained. It is likely that the kinetic constants found for the phosphorylation of TCN by the L1210 and HEp-2 adenosine kinases are representative of those of adenosine kinases from mammalian cells, for among the many substrates that have been studied no large differences were found with enzymes from HEp-2 cells [5], L1210 cells [6] and rabbit liver [8, 9].

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Covalent binding and the mechanism of paracetamol toxicity

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Paracetamol is a popular, over-the-counter, analgesic drug which is safe and well tolerated at therapeutic doses, but causes chiefly centrilobular hepatic necrosis when taken in overdose [1, 2]. The specific mechanism for the toxicity of paracetamol is a matter of controversy. Covalent binding of paracetamol metabolites to essential cellular macromolecules has been most extensively studied, particularly by Mitchell and colleagues [2, 3], and the received concept stemming from the work of these authors is that generalized covalent binding is the trigger that leads to cell necrosis. This view has received wide support [4–6] but some evidence contrary to the covalent binding theory has also been presented [7, 8]. Also we have recently reported that covalent binding of paracetamol metabolites to cell protein is not sufficient to account for cell injury as a consequence of paracetamol toxicity [9]. In this communication, we expand on our findings on the role of the covalent binding mechanism, and report a simple technique capable of separating and investigating cells which have been either damaged or undamaged after paracetamol treatment under exactly the same incubation conditions in the same flask. These studies provide evidence against the 'general covalent binding' theory and reinforce our previous observation that, on its own, general covalent binding of paracetamol is not sufficient cause for the cell necrosis observed in paracetamol-induced injury.

Materials and methods

Isolated rat hepatocytes were prepared and used as previously described [9]. [^{14}C]Paracetamol used for the covalent binding studies was purchased from Amersham International (Amersham, U.K.), and purified by a high-performance liquid chromatography (HPLC) technique [10] before use. Percoll was purchased from Pharmacia (Great Britain) Ltd. (Hounslow, U.K.). Ten times concentrated Hank's balanced salt solution (HBSS) was obtained from Gibco Bio-Cult (Uxbridge, U.K.). Damaged and undamaged cells were separated using Percoll which was prepared and used as follows.

Percoll solution was mixed with 10 × HBSS in the ratio of 9:1 (v/v) to give an isotonic stock solution of density 1.13 g/ml. This was further diluted with normal strength HBSS in the ratio of 42.5:57.5 (v/v) to give a working solution of density 1.06 g/ml.

Aliquots (15 ml) of the working Percoll solution, in universal containers, were used as 'columns' for separation of the damaged and undamaged cells in a given cell suspension. Usually 3–4 ml of the cell suspension was loaded on top of a Percoll solution column, taking care to avoid mixing the cell suspension into the Percoll, and then centrifuged at 40 *g* for 10 min in an angle rotor. At the end of the centrifugal run, mixing was again avoided and the cells were recovered. The damaged dead cells which remained at the top of the Percoll column were recovered by sucking off with a plastic bulb pipette, and the undamaged, apparently live cells which had migrated through the Percoll solution and pelleted were recovered after decanting the Percoll solution. Cell viability of both the damaged and the