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Metabolism of Pyrimidine Deoxyribonucleosides and Heat-resistivity of CdR-aminohydrolase* in the Mouse Small Intestine**

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생쥐 小腸에서의 Pyrimidine Deoxyribonucleoside代謝와 CdR-aminohydrolase의 熱抵抗性

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摘 要

생쥐 小腸의 deoxycytidine-2-^{14}C (CdR-2-^{14}C)와 deoxyuridine-2-^{14}C (UdR-2-^{14}C)의 代謝를 관계酵素의 熱處理에 대한 영향과 관련해서 in vitro에서 고찰하였다.

CdR-2-^{14}C는 CdR-aminohydrolase의 作用에 의해서 먼저 nucleoside level에서 급속히 deamination된 후, nucleosidase의 作用에 의해 uracil로 分解된다. 생쥐小腸에서는 nucleosidase가 CdR과 CR에는 親和力이 없기 때문에 이들 cytosine nucleoside의 N-pentose 結合을 分解하지 못하는 것으로 보인다.

CdR-aminohydrolase는 80°C의 높은 不活性化溫度를 나타냈으며, 이에 반해서 nucleosidase는 60°C에서 不活性化되었다. 品種이 다른 생쥐의 여러 組織에 있는 CdR-aminohydrolase는 모두 80°C에서 不活性化됨이 관찰되었으나, 토끼組織에서는 80°C에서도 不活性化가 일어나지 않는 점으로 미루어 不活性化溫度에는 "屬"特異性이 있는 것으로 짐작된다.

哺乳類의 分化된 組織에서 CdR-aminohydrolase가 出現하는 生理學的 意義는 주로 分解過程과 有關한 것으로 생각된다.

INTRODUCTION

Reichard and Estborn (1951) established that deoxycytidine (CdR) is incorporated into deoxyribonucleic acid (DNA) without prior degradation. However, in the study of the metabolism of CdR-2-^{14}C by hepatoma cells (Schneider and Rotherham, 1961),

*The name, CdR-aminohydrolase, instead of nucleoside deaminase is adopted for a reason of using CdR as a substrate for deamination.

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it was shown that most of added CdR is converted to deoxyuridine (UdR) and uracil (U), and that some of added CdR is incorporated into DNA. The metabolism of cytosine derivatives by mouse kidney was investigated by Creasey (1963), showing that the cytosine derivatives are converted to uracil derivatives by CdR-aminohydrolase which does not attract cytosine (C) and cytidine/deoxycytidine monophosphate (CMP/dCMP).

The CdR-aminohydrolase activity has been measured in various tissues of mouse, rat, rabbit (Creasey, 1963), calf and human (Shejbal, 1970). The enzyme activities of the livers in various species of mammals were compared with one another (Zicha and Buric, 1969). These findings indicated that there is no correlation between tissue specificity among species and levels of the enzyme activity.

The conversion of CdR to UdR occurs at the nucleotide level in the mouse hepatoma cells (Schneider and Rotherham, 1961), and CdR-aminohydrolase activity was not observed in the rat hepatoma cells (Maley and Maley, 1960). Even when normally present in tissues such as mouse and rat livers (Zicha and Buric, 1969; Shejbal, 1970; Kang, 1972), CdR-aminohydrolase was shown to disappear by inducing regeneration. Camiemer and Smith (1965) demonstrated that CdR-aminohydrolase activity which is very high in adult human liver is not detectable in prenatal liver. In the study of mouse blood, Rothman et al.(1970) observed that the enzyme is not associated with normal erythroid cells at the stage of development. These observations strongly suggested that the enzyme is not associated with a proliferation of cells.

Deamination of cytosine derivatives by the heat-resistant CdR-aminohydrolase was observed in the human leukocytes (Silber, 1967) and human liver (Shejbal, 1969). Although the enzyme has been revealed as one of the heat-resistant enzymes, it appears that no report has so far been made on its activity change by heat treatment and on the inactivation temperature. As to the effect of radiation on this enzyme, the radiosensitivity of several tissues of the rat with special regard to CdR-2-^{14}C metabolism was reported (Kang, 1972). In the present study, the metabolism of CdR-2-^{14}C and resistivity of CdR-aminohydrolase to heat treatment have been investigated using mouse small intestine for further insight into the pyrimidine deoxyribonucleoside metabolism *in vitro*.

MATERIALS AND METHODS

Pyrimidine derivatives and radioactive compounds were purchased from the Sigma Chemical Co. and the Département des Radioéléments, France, respectively.

Most experiments were performed with male C3Hf mice, aging about 2 months, supplied from the Radiation Medical Research Institute. The animals were sacrificed by exsanguination while anesthetized with ether. The whole small intestines were

excised, incised, cleaned with tris buffer (pH 8, 0.05M) and homogenized in a Teflon homogenizer with a three-fold greater volume of tris buffer for 2 minutes. The homogenates were centrifuged at 8,000 G for 20 minutes. The supernatant was used as an enzyme source and adjusted to a proper dilution with tris buffer. All the procedures were carried out at 4°C otherwise noted.

The amount of proteins in the supernatant after centrifugation and dilution, was determined by spectrophotometry at 540 nm after reaction of modified Weichselbaum's reagent, with bovine serum albumin as a reference protein.

In the metabolism experiment, 5 m*l* of homogenate was placed in a shaking water bath at 37°C for 10 minutes and the reaction was initiated by addition to the homogenate of 1 m*l* of CdR as a substrate which was previously enriched with 2 μCi of CdR-2-^{14}C. At various times after reaction, 0.6 m*l* of reactant was recovered with an auto-pipette and immediately inactivated in a boiling water bath for 5 minutes. After removal of the precipitate, the products were isolated by paper or thin layer chromatography and their radioactivities were counted by using a gas-flow type windowless G-M counter equipped with automatic sample changer.

In the heat treatment experiment, 1 m*l* each of homogenate was treated at 50, 60, 70, 75, 80, and 100°C in a water bath for 5 minutes, respectively, and the precipitates were removed by centrifugation at 8,000 G for 20 minutes. One half m*l* each of supernatant was collected for direct determination of enzyme activities.

The enzyme activities were determined by a modified method of Zicha et al. (1969). To 0.5 m*l* of homogenate was added 0.1 m*l* of CdR (1mM) at a final concentration of 167 μM, and the system was enriched with 0.2 μCi of CdR-2-^{14}C (specific activity, 26 mCi/mM). The system was incubated in a shaking water bath at 37°C for 30 minutes and inactivated in a boiling water bath for 5 minutes. After removal of precipitate by centrifugation at 11,000 G for 30 minutes, the supernatant was stored at 4°C before being used. Isolation of pyrimidine derivatives in the supernatant was carried out by paper chromatography and by thin layer chromatography using cellulose powder MN 300. Three solvent systems were employed to correct the deviation in chromatographic patterns; isopropanol-HCl-water (17 : 4.1 : 3.9, v/v), ethylacetate-formic acid-water (60 : 5 : 35, v/v), and 4% boric acid-normal butanol (28 : 172, v/v). After development, the spot was detected under the UV-lamp. The radioactive spots were cut out and transferred to test tubes. To this test tube was added 3 m*l* of distilled water and left at 30°C for 2 hours. Two m*l* each of eluate was dried in a planchet and counted.

RESULTS

Deamination of CdR-2-^{14}C was followed by incubation of 0.5 m*l* homogenate with 1 mM CdR enriched with CdR-2-^{14}C in a final volume of 0.6 m*l* at 37°C for the period of up to 1.5 hours, during which time deamination proceeded linearly (Fig.

1). As is evident from Fig. 1, it is meaningless for measuring deamination over 0.5 and 1 hour incubations in case of homogenates containing 5 and 2.5 mg proteins, respectively.

Even if the final substrate concentration was elevated up to 15 mM, deamination velocity itself was not reduced, indicating that much higher ability for deamination is being kept in the mouse small intestine (Fig. 2).

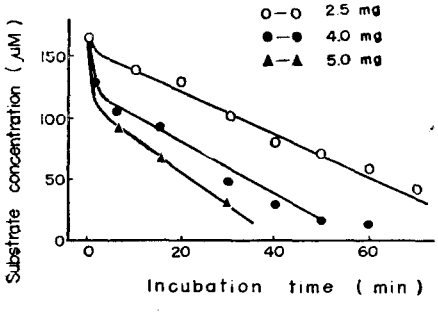

Fig. 1. The relationship between enzyme activity and protein concentrations in supernatant as expressed in the decrease of substrate concentration with incubation time.

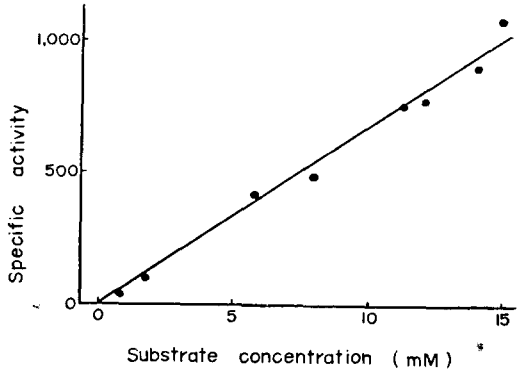

Fig. 2. The linear increase of specific activity of CdR-aminohydrolase up to 15 mM of substrate concentration indicating high deamination velocity. Specific activity is expressed as mμM/mg protein/30 min.

Metabolism of CdR-2-^{14}C

In Table 1 is expressed the amount of products formed at various time intervals in 0.6 m*l* of recovered incubation mixture. At the initial stage of reaction, a certain amount of UdR appeared and formation of U started within 10 minutes. Along with the incubation time, ratio of increase of UdR was much lower than that of U which exhibited a relatively constant increase.

The radioactivity originating from CdR-2-^{14}C appeared less than 3% in pyrimidine deoxyribonucleotides and was not found in CR and C. Using an unlabeled CR as a substrate, it was observed spectrophotometrically that most of added CR is converted to U via UR, but not CdR or C. De Veridier and Potter (1960) also

Table 1. Formation of UdR and U from CdR-2-^{14}C in mouse small intestine. Amounts of products formed at various time intervals are expressed in terms of millimicromoles formed in each recovered incubation mixture containing 2.5 mg proteins.

Product	Incubation time (min)									
	0	10	20	30	40	50	60	70	90	120
UdR	0	13.6	15.4	24.3	22.8	26.8	27.7	30.2	29.9	28.1
U	0	6.2	7.6	10.8	11.1	27.7	34.8	41.1	50.5	60.5

observed that CR and CdR are resistant to cleave N-pentose bond. Therefore, it could be ascertained that interconversion between CdR and CR is not operated in the mouse small intestine, and that added CdR or CR is not degraded into simple pyrimidine bases, showing that some of these are utilized in nucleic acid biosynthesis. Creasey (1963) obtained a similar result as ours and also observed that dUMP is formed in the presence of ATP, but not dCMP. It indicates that CdR is deaminated first to UdR and degraded into U or phosphorylated to dUMP thereafter.

As shown in Fig. 3, metabolism of CdR-2-^{14}C was confirmed by change in the Product formation by secondary addition of CdR-2-^{14}C 30 minutes after the first

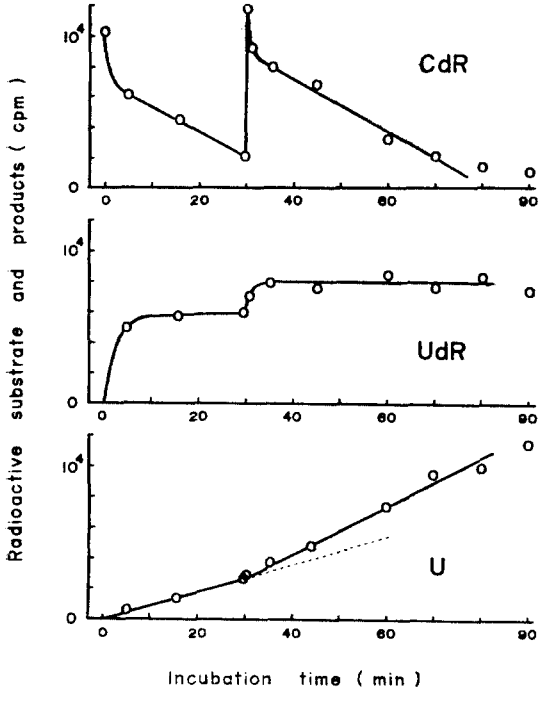

Fig. 3. The relation between the substrate addition to the incubation mixture and the appearance of UdR and U at various incubation times, as expressed in terms of radioactivity of the products. In view of the pattern of UdR and U formation after the secondary substrate addition, it could be concluded indirectly that CdR is rapidly deaminated into UdR first and then degraded into U constantly.

incubation. As might have been expected, most of added CdR was consumed within 30 minutes. Amount of UdR formed from CdR was shown to increase abruptly within 5 minutes and then maintained a certain level because of a gradual decrease in UdR formation as a consequence of low CdR concentration, followed by a steady increase in UdR degradation to U. Rapid deamination was also observed in hepatoma cells (Schneider and Rotherham, 1961), human leukocytes (Shejbal et al., 1968), and human plasma (Tseng, Barelkovski and Gurpide, 1971). When the second addition was made of CdR-2-^{14}C 30 minutes after the initial incubation, UdR was found to increase at the same rate as the first incubation, but not doubled before reaching a plateau level. On the other hand, the velocity of U formation is elevated follow-

ing secondary addition. The elevated velocity might be attributable to the increase of UdR as a substrate for the enzyme which catalyses degradation of UdR into U. In view of these changes in the products after secondary addition of substrate, it was shown that CdR-2-^{14}C is degraded into U via UdR.

Heat Resistivity of CdR-aminohydrolase

The effect of heat treatment on the metabolism of CdR-2-^{14}C and UdR-2-^{14}C is shown in Fig. 4, as observed by the change in chromatogram scanning patterns. As is evident from Fig. 4(a), most of added CdR was converted to UdR and U in the control, confirming earlier observation (Creasey, 1963). When the heat treatment was elevated to 60°C, increase of UdR and decrease of U were observed in comparison to the control. The appearance of increased amount of UdR at both 50 and 60°C is attributable to the fact that the conversion of UdR to U is blocked as the temperature increases, remaining much UdR to be accumulated. The amount of UdR formed at 70°C showed a little difference from that at 60°C, and was doubled as compared to that at 75°C. No deamination of CdR was observed at 80°C, and UdR is not degraded at 60°C, which was confirmed by using UdR-2-^{14}C as shown in Fig. 4(b).

From these results it is easily shown that the CdR-aminohydrolase maintains

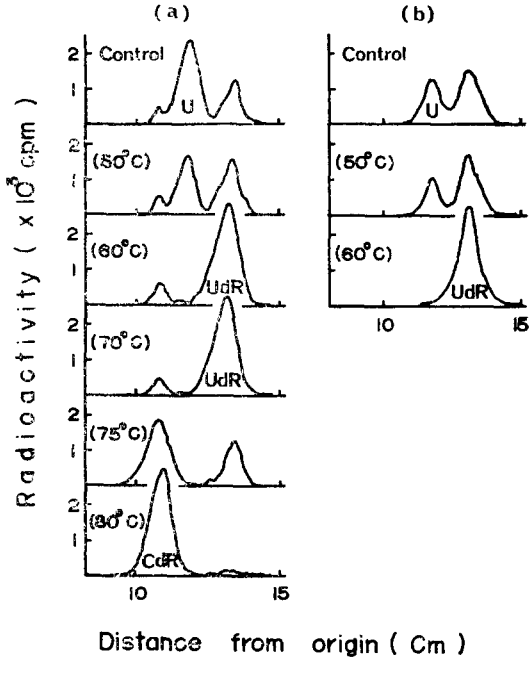

Fig. 4. The effect of heat treatment on the metabolism of CdR-2-^{14}C (a) and UdR-2-^{14}C (b), as observed by the change in paper chromatogram scanning patterns which are separated by solvent system of isopropanol-HCl-water at 30°C for 11 hours. Temperature in the parentheses indicates previously treated temperature to homogenates of mouse small intestine for 5 minutes. (a) and (b) scanning patterns were obtained with homogenates containing 5 and 2.5 mg proteins, respectively.

close to its normal level of enzyme activity up to 70°C and inactivated at 80°C, while the enzyme which catalyses degradation of UdR to U has an inactivation temperature of 60°C. Therefore, it can be concluded by use of different inactivation temperature of the two specific proteins that CdR is deaminated first to UdR and degraded to U thereafter.

It was observed that various tissues of mice have a same inactivation temperature for the enzyme. As expressed in terms of percent activity to the control (Table 2), CdR-aminohydrolase of mouse tissues such as liver and small intestine was lost its activity at 80°C, but that of rabbit tissues was not inactivated at the same temperature. Although the present result is limited only to two species of different order, mouse and rabbit, an interesting suggestion might be explored, such as some correlation between order specificity and inactivation temperature of the enzyme.

Table 2. The change of CdR-aminohydrolase activity in percent of the control of mouse and rabbit with change in treated temperatures. Inactivation temperature of the enzyme was found to be 80°C for mouse and above 80°C for rabbit.

Heat treatment	Mouse		Rabbit	
	Liver	Intestine	Liver	Spleen
Control	100	100	100	100
50°C	97.9	89.9	—*	—
60°C	87.5	91.6	—	—
70°C	78.7	85.9	104	105
80°C	4.2	3.6	101	96.7

*Not determined.

DISCUSSION

The metabolism of CdR-2-^{14}C has been studied in connection with the effect of heat treatment to enzymes concerned.

It has been doubted that whether the conversion of CdR to UdR occurs at nucleoside level or at nucleotide level in the mouse small intestine. To separate CdR-aminohydrolase from dCMP-aminohydrolase, Silber (1967) used 65°C treatment which resulted in a complete loss of dCMP-aminohydrolase activity but no effect on CdR-aminohydrolase. It indicates that dCMP-aminohydrolase is a heat labile enzyme. Therefore, conversion at nucleotide level can be blocked by inactivating dCMP-aminohydrolase with heat. As shown in Table 2, deamination of CdR was not blocked by heat treatment to 70°C in mouse small intestine. It might, therefore, be concluded that homogenate of mouse small intestine deaminates CdR at nucleoside level. The significance of this finding lies in the fact that it provides further evidence to support the suggestion (Creasey, 1963) that CdR is converted to dUMP

after deamination at the nucleoside level.

In the explanation for splitting deoxyribose off from UdR in mouse small intestine, three possible ways may be considered ; phosphorolysis, transfer and hydrolysis of N-pentose bond. Zimmerman and Seidenberg (1964) observed that intestinal mucosa of mouse has an ability for phosphorolysis, but not for transfer of deoxyribose. Since phosphorolysis is not expected in the incubation mixtures which-lack in available inorganic phosphates, it probably is believable that deribose reaction in the mouse small intestine is carried out by hydrolysis of nucleosidase. However, drawing an inference from being a deficiency of deribose reaction from CdR/CR, it might be assumed that nucleosidase at least in mouse small intestine has a substrate specificity for UdR/UR, but not for CdR/CR.

The name of enzyme which catalyzes deamination of cytosine nucleosides has been used in three ways; CdR-aminohydrolase (Shejbal, 1970; Zicha and Buric, 1969; Kang, 1972), cytidine-aminohydrolase (Wisdom and Orsi, 1969) and nucleoside deaminase (Silber, 1967; Creasey, 1963). The name aminohydrolase instead of deaminase is used after the suggestion of the Report of the Commission in Enzymes of the International Union of Biochemistry. It was observed that 46-fold purified protein deaminates 5'-dCMP derivatives, but not other cytosine and adenosine derivatives in the monkey kidney (Scarano, Bonadue and Petrocellis, 1962). Having a substrate specificity, the enzyme was referred to 2'-deoxyribosyl 4-aminopyrimidone-2, 5'-phosphate aminohydrolase. In the deamination of cytosine nucleosides, Silber(1967) suggested that CR and CdR are deaminated by a single enzyme with reference to the same rate of deamination by ammonium sulfate fractionation. Analogous finding was obtained by Creasey(1963), showing that 107-fold purified protein has an equal activity with ribonucleosides or deoxyribonucleosides of cytosine derivatives. Being a lack of information concerning deamination of adenosine derivatives, it is very difficult to mention that deamination at nucleoside level occurred with a single enzyme, and that which specific name should be used. Therefore, it remains to be solved that substrate specificity of this enzyme after purification has to be investigated, and then proper name of this enzyme could be proposed.

Rothman et al.(1970) mentioned that the physiological significance of the appearance of CdR-aminohydrolase could be regarded as two possible functions for this enzyme in pyrimidine nucleosides metabolism; "salvage" pathway involving in the formation of nucleoside triphosphates (Wisdom and Orsi, 1969) and catabolic pathway for further degradation to β-alanine (Schulman, 1961), and also offered a suggestion that physiological role of this enzyme is a degradation of nucleic acids in the "stress" erythroid cell. In the study of human leukocytes, Silber (1967) proposed that deamination catalyzed by CdR-aminohydrolase may be part of catabolic

sequence, because mature granulocytes containing numerous lysosome have a deamination ability, but immature granulocytes or lymphocytes which contain little lysosomes do not have one. Creasey (1963) demonstrated that deamination of cytosine nucleosides may be a main function of CdR-aminohydrolase to prevent the formation of abnormal cytosine derivatives, in addition to regulate the supply of normal cytosine nucleosides, CR and CdR. Although sufficient information is not yet available for deciding whether CdR-aminohydrolase function in a biosynthetic or in a catabolic process, conversion of most of added CdR to U and high substrate saturation concentration for this enzyme could be explained by the catabolic function in mouse small intestine, as judged by the present results. In view of the above facts, it might be proposed in mammals that CdR-aminohydrolase appearing in differentiated tissues has a main function in catabolism of cytosine nucleosides, derived from the break-down of nucleic acids and from unusual bases.

SUMMARY

The metabolism of CdR-2-^{14}C and UdR-2-^{14}C in mouse small intestine has been studied in connection with the effect of heat treatment on the enzymes concerned *in vitro*.

CdR-2-^{14}C is deaminated rapidly by CdR-aminohydrolase at nucleoside level and then degraded into U by the action of nucleosidase which is quite resistant to cleave N-pentose bond of cytosine nucleosides, CdR and CR.

High inactivation temperature of 80°C was observed for CdR-aminohydrolase, while nucleosidase has an inactivation temperature of 60°C. CdR-aminohydrolases in various tissues of mouse were inactivated at 80°C, but not one in tissues of rabbit. It might be assumed that there are correlations between order specificity and inactivation temperature of the enzyme.

A physiological significance of the appearance of CdR-aminohydrolase in differentiated tissues of mammals possibly be regarded as a main function in catabolic pathways.

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