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EFFECT OF THIOCETAMIDE ON THE ACTIVITY OF ALKALINE RIBONUCLEASE AND RIBONUCLEASE INHIBITOR IN RAT LIVER*

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Abstract—Administration of thioacetamide in a dose of 50 mg/kg body wt./day results in an increase in the alkaline ribonuclease activity of rat liver particulate fractions. There is a change in the distribution of the enzyme among these fractions inasmuch as the total ribonuclease in the lysosomal fraction remained constant or decreased while the ribonuclease in the nuclear, mitochondrial, and microsomal fractions markedly increased. A decrease in the amount of 'latent' ribonuclease was found in the cytoplasmic particulate fractions. No concomitant increase in phosphodiesterase activity could be detected. It was established earlier[1, 2] that the increased ribonuclease activity in the hepatic nuclei and nucleoli after thioacetamide treatment is not simply an accompaniment of increased nuclear size. The present study indicates that in the thioacetamide-treated liver, the augmented ribonuclease activity in the particulate fractions is associated with a decrease in the concentration of ribonuclease inhibitor in the cell-free supernatant fraction.

PREVIOUS studies in this laboratory indicated that administration of thioacetamide results in an eight-to tenfold increase in the ribonuclease activity of rat liver nuclear[1] and nucleolar[2] fractions within 24–48 hr. Along with this increase in ribonuclease activity, there is a marked stimulatory effect of thioacetamide on the synthesis and accumulation of nuclear RNA and protein.[3–6] Since it was found that there is a concomitant decrease in ribosomal RNA,[4, 7] it seemed possible[1] that this increased ribonuclease activity might produce degradation of the precursors of ribosomal RNA, which are known to be synthesized in the nuclei.[8–10] The present experiments were designed to explore the changes in the ribonuclease activity of rat liver particulate fractions in relation to the ribonuclease inhibitor[11] after thioacetamide administration. These studies indicate that the increased ribonuclease activity of rat liver particulate fractions is accompanied by a change in the distribution of the enzyme among these fractions, a decrease in the amount of latent ribonuclease, and a decrease in the level of ribonuclease inhibitor in the cell-free supernatant fraction.

MATERIALS AND METHODS

Bovine pancreatic ribonuclease (polyribonucleotide: 2-oligonucleotidotransferase, cyclizing; (EC 2.7.7.16), venom phosphodiesterase (orthophosphoric diester phosphorylase; EC 3.1.4.1.), and high molecular weight RNA prepared by the method of

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Crestfield *et al.*[12] were purchased from Worthington Biochemical Corp. and the calcium salt of bis(*p*-nitrophenyl)phosphate was obtained from Sigma Chemical Co.

Animals. Male albino rats weighing 170–250 g were used. Thioacetamide-treated animals received i.p. injections of a 1% solution of thioacetamide in 0·15 M NaCl in a dose of 50 mg/kg body wt daily. Control rats were similarly injected with 0·15 M NaCl alone.

Isolation of subcellular components. All operations were done in the cold laboratory (0–4°). Subcellular particulate fractions were isolated from the liver of control and thioacetamide-treated rats according to the method of Murray *et al.*[13] by subjecting a 10 per cent (w/v) homogenate of the tissue in 0·25 M sucrose to differential centrifugation at 600 *g* (nuclear fraction), 5000 *g* (mitochondrial fraction), and 15,000 *g* (lysosomal fraction) successively. The centrifugation time was 10 min at each speed. The 15,000 *g* supernatant fraction was centrifuged at 100,000 *g* for 1 hr in the No. 30 rotor of the Spinco model L preparative ultracentrifuge to obtain the microsomal fraction. Essentially similar results were obtained when these pellets were washed once by recentrifugation with 0·25 M sucrose or when the inside wall of the centrifuge tubes was carefully wiped after draining off the liquid after the first centrifugation. Each of the pellets thus obtained was dispersed in 0·25 M sucrose with a glass homogenizer.

The high-speed supernatant fraction containing ribonuclease inhibitor was prepared by the method of Shortman,[14] modified as follows. The tissue was homogenized in 4 vol. of 0·44 M sucrose and the homogenate was passed through 6 layers of gauze, sonicated for 25–35 sec to disrupt nuclei,[15] and centrifuged for 1 hr at 100,000 *g*. The supernatant was then recentrifuged at 100,000 *g* for 1 hr. The final supernatant was diluted tenfold with ice-cold 0·005 M EDTA and assayed for ribonuclease inhibitor by the method of Roth[11] as modified by Shortman.[14] Bovine pancreatic ribonuclease dissolved in 0·1% gelatin was used. One unit of inhibitor is defined as the amount that causes a 50 per cent inhibition of the activity of 0·005 μg of pancreatic ribonuclease under the standard assay conditions.

Ribonuclease assay. The reaction mixture, in a final volume of 2 ml, contained the following additions (in μmoles, unless otherwise stated): veronal acetate buffer, pH 7·4, 35; EDTA, pH adjusted to 7·8, 5; 0·1 ml of 1% RNA, and the tissue sample containing 50–200 μg of protein.[11] Duplicate samples were also run under identical conditions as tissue and substrate blanks. All samples were incubated at 37° for 30 min. The reaction mixture was then chilled and 2 ml of precipitating reagent (1 N HCl in 76% ethanol containing 0·5% $LaCl_3$) was added to each sample. The tubes were immediately closed by parafilm to prevent evaporation and the samples were centrifuged in the cold until the supernatant was clear. Aliquots of the supernatants were diluted with distilled water and read at 260 mμ against water. Corrections were made for substrate and tissue blanks. Under these conditions of assay, ribonuclease activity was linear for 30 min.

The activity, which is expressed as ribonuclease units, was determined by comparison with the degradation produced by standard quantities of pancreatic ribonuclease. Specific activity is defined as the activity per mg protein. Protein was estimated by the method of Lowry *et al.*,[16] with bovine serum albumin used as standard.

As a precaution against any possiole contamination of the RNA used as substrate in these studies by heavy metals, which might interfere with the activity of ribonuclease inhibitor, a 3% solution of RNA in glass-distilled water was dialyzed successively

against 0·01 M EDTA, 0·15 M NaCl, and finally against several changes of glass-distilled water in the cold (0–4°). [17]It was then diluted to a concentration of 1 per cent and stored frozen in aliquots of 1 ml.

The phosphodiesterase assay was performed with the synthetic substrate Ca bis(*p*-nitrophenyl)phosphate according to the method of Koerner and Sinsheimer[18] as modified recently by Morais and DeLamirande.[19]

RESULTS

Effect of thioacetamide on ribonuclease activity of particulate fractions. Table 1 shows the ribonuclease activity in the subcellular fractions obtained from the liver

TABLE 1. EFFECT OF THIOACETAMIDE ON THE RIBONUCLEASE ACTIVITY OF SUBCELLULAR PARTICULATE FRACTIONS*

Fraction	Sp. act. (ng ribonuclease/mg protein)			
	Control	14 hr	24 hr	48 hr
Nuclear fraction	1·0 (0·9–1·2)	2·4 (1·6–3·1)	9·0 (7·2–10·5)	7·5 (6·3–8·2)
Mitochondrial fraction	3·6 (2·4–4·2)	4·2 (3·6–4·8)	8·1 (7·1–9·5)	9·2 (8·5–10·2)
Lysosomal fraction	8·5 (6·4–10·8)	8·4 (7·2–9·6)	11·8 (9·5–13·5)	11·9 (9·5–13·7)
Microsomal fraction	1·7 (1·5–2·2)	2·2 (1·8–3·0)	5·7 (4·8–6·8)	3·6 (2·9–4·2)

* Treatment with thioacetamide and isolation of subcellular particulate fractions were done as described in 'Materials and Methods'. The data represent average values from 4 separate analyses. Variations are shown in parentheses. ng = nanogram.

of control rats and those treated with thioacetamide for 14–48 hr. As found previously, thioacetamide produces a considerable increase in the sp. act. of ribonuclease in the nuclear fraction. A two-to threefold increase was found in the sp. act. of ribonuclease in the mitochondrial[20] and microsomal fractions also. In the lysosomal fraction, there was little increase in specific activity due to thioacetamide up to 48 hr.

Fig. 1 presents the effects of thioacetamide on the distribution of ribonuclease activity in the particulate fractions. In the control liver, 60 per cent of the total ribonuclease activity was localized to the lysosomal fraction. A remarkable decrease in the percentage of cellular ribonuclease in this fraction was produced by thioacetamide. On the other hand, in the nuclear fraction the percentage of total ribonuclease increased as the injection of thioacetamide continued. In the microsomal fraction, there was an initial increase in the percentage of total ribonuclease, but later this percentage decreased. There was no significant change in the percentage of total ribonuclease in the mitochondrial fraction.

Ribonuclease activity of the particulate fractions is partly bound to membranes and can be released by freezing and thawing or by treatment with a suitable detergent.[21] When increase in activity due to freezing and thawing is taken as a measure of 'latency' of the enzyme, it will be seen from the data presented in Fig. 2 that thioacetamide decreases this latency of ribonuclease in each fraction. Since similar results were obtained when these fractions were treated with 0·1% Triton-X100 instead of with freezing and thawing, it is possible that much of the latent ribonuclease is lysosomal in origin.

Effect of thioacetamide on ribonuclease: ribonuclease inhibitor ratio. The alkaline ribonuclease activity is inhibited by a heat-labile glycoprotein, which is predominantly localized to the soluble supernatant fraction derived from rat liver[11, 14, 22] and other tissues.[23–25] The ribonuclease inhibitor of the whole cell was determined in order to

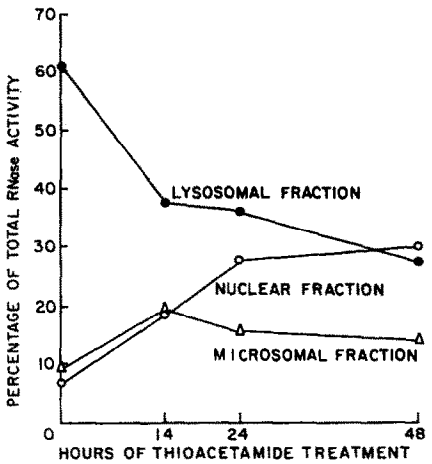

FIG. 1. Effect of thioacetamide on the distribution of ribonuclease activity in the subcellular particulate fractions. In the mitochondrial fraction (not included in the figure) the percentage of total activity varied between 23·7 (control) and 28·5 (48 hr thioacetamide).

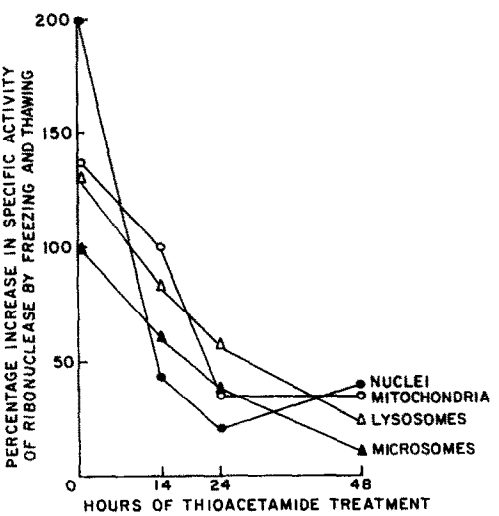

FIG. 2. Effect of thioacetamide on the 'latency' of ribonuclease in the subcellular particulate fractions.

compare the relative concentrations of inhibitor, free, and total ribonuclease. In these experiments, ribonuclease activity was also determined after freezing and thawing and after pretreatment with *N*-ethylmaleimide to determine the latent and the inhibitor-bound activities, respectively (Table 2). The samples were suspended in

TABLE 2. EFFECT OF THIOACETAMIDE ON THE TOTAL RIBONUCLEASE ACTIVITY OF THE PARTICULATE FRACTIONS AND RIBONUCLEASE INHIBITOR OF THE CELL-FREE SUPERNATANT FRACTION*

Fraction	Free activity	Inhibitor-bound activity	Latent activity	Total (particulate fractions)	Free inhibitor (cell-free supernatant)	Free inhibitor / Free ribonuclease	Free inhibitor/Total ribonuclease (particulate fractions)
Control							
Nuclear fraction	40 (36·0–48·0)	60 (52·0–70·0)	80 (72·0–96·0)	180	0		
Mitochondrial fraction	145 (96·6–169·0)	30 (21·0–41·0)	190 (132–230)	365	0	19·6	6·9
Lysosomal fraction	370 (278·7–470·3)	180 (149·9–195·1)	480 (362–600)	1030	0		
Microsomal fraction	55 (48·5–71·2)	50 (40·0–62·0)	55 (40–70)	160	0		
Total	610	320	805	1735			
Supernatant	0	650 (590–700)	0	0	2400 (2200–2560)		
Thioacetamide (24 hr)							
Nuclear fraction	460 (367·0–535·5)	240 (195·0–310·0)	93 (72·0–105.0)	793	0		
Mitochondrial fraction	345 (302·5–404·7)	35 (28·0–42·0)	120 (106·0–140·0)	500	0		
Lysosomal fraction	600 (482·6–685·8)	80 (65·0–92·0)	300 (268·0–392·0)	980	0	5·6	3·4
Microsomal fraction	262 (220·8–312·8)	128 (112·0–114·0)	42 (36·0–45·0)	432	0		
Total	1667	483	555	2705			
Supernatant	0	620 (550–690)	0	0	1870 (1690–2070)		

* Inhibitor-bound ribonuclease activity was estimated after preincubating the fractions with 0·3 mM *N*-ethylmaleimide for 10 min at 20°. Total ribonuclease activity is the sum of free, inhibitor-bound, and latent (activity released by freezing and thawing) activities. In all cases, activity is expressed as ribonuclease units (or inhibitor units) per g of liver (fresh weight). Data represent average values from 4 separate analyses. The ranges are given in parentheses. Inhibitor: ribonuclease ratios were calculated on the basis of 5 ng of ribonuclease being 50 per cent inhibited by 1 unit of inhibitor. Free inhibitor is the quantity of inhibitor capable (theoretically) of causing a 50 per cent inhibition of the free ribonuclease, e.g. for control 2400/122 = 19·6.

0·25 M sucrose and were made to 0·3 mM with respect to *N*-ethylmaleimide. Preincubation for 10 min at this concentration of *N*-ethylmaleimide was found to release maximum ribonuclease activity. However, after administration of thioacetamide for 24 hr. the free ribonuclease activity was 2–3 times that of the control. The total activity in the particulate fractions (representing the sum of free, inhibitor-bound, and latent activity) was increased by 56 per cent. The ribonuclease activity after freezing and thawing in the presence of *N*-ethylmaleimide was approximately equal to the total activity. Concomitant with this increased ribonuclease activity in the particulate fractions, there was a consistent decrease of about 20 per cent in the level of ribonuclease inhibitor in the cell-free supernatent. The inhibitor : enzyme ratio for free ribonuclease was reduced by 70 per cent in the livers treated with thioacetamide and the same ratio for total ribonuclease was decreased by approximately 50 per cent.

The high-speed supernatant fraction obtained from rat liver has been shown by Roth[11] to contain inhibitor-bound ribonuclease. This activity is quantitatively similar in control and thioacetamide-treated rat liver (Table 2). No free or latent ribonuclease activity could be detected in this fraction obtained from either normal or thioacetamide-treated rat liver.

Effect of thioacetamide on phosphodiesterase activity. The increased depolymerase activity observed in the cytoplasmic fractions due to thioacetamide is probably not associated with an increased phosphodiesterase activity since, under the conditions of ribonuclease assay (absence of a divalent cation, presence of EDTA, and the use of high molecular weight RNA as substrate which is resistant to phosphodiesterase[23]),

TABLE 3. PHOSPHODIESTERASE ACTIVITY IN THE PARTICULATE FRACTIONS*

Fraction	% Hydrolysis	
	Control	Treated with thioacetamide (24 hr)
Nuclear fraction	7·2 (8·4, 6·0)	7·4 (8·5, 6·3)
Mitochondrial fraction	7·0 (8·7, 5·3)	6·0 (5·8, 6·2)
Lysosomal fraction	8·0 (7·2, 8·8)	8·6 (8·2, 9·0)
Microsomal fraction	25·4 (24·0, 26·8)	27·0 (25·8, 28·2)

* The reaction mixture contained the following components in a total volume of 2·5 ml (in μmoles, unless otherwise stated): Tris-HCl buffer, pH 8·0, 50; Ca-bis(*p*-nitrophenyl)phosphate, 1·0; $MgCl_2$, 2·0; K_2HPO_4, 28; and the subcellular fraction containing 0·5 mg protein. The reaction mixture was incubated at 25° for 1 hr, after which 5 ml of glass-distilled water was added and then read at 440 mμ against substrate blank. The results are expressed at per cent hydrolysis; the degradation produced by 0·32 mg of commercial snake venom phosphodiesterase in 3 hr under the above conditions is taken to represent 100 per cent hydrolysis. The data are averages from 2 separate experiments. Six animals were used in each experiment, 3 as control and 3 as treated. Data of each experiment are given in parentheses.

this activity, if any, will be at its minimum. Table 3 shows that phosphodiesterase assay with *bis* (*p*-nitrophenyl)phosphate also did not reveal any remarkable increase in this activity in any of the particulate fractions after thioacetamide treatment.

DISCUSSION

One possible mechanism of action of thioacetamide on cellular metabolism emerges from the present studies. There is a decrease of latent forms of ribonuclease in the lysosomal and other cytoplasmic fractions that is accompanied by increased activity of this enzyme in other cellular fractions, except in the mitochondrial fraction. Moreover, the level of free ribonuclease inhibitor decreased. In addition, the liver cells seem to produce additional ribonuclease inasmuch as the total free and demonstrable ribonuclease in the liver cell is increased. As a result, the ratio of ribonuclease inhibitor to free ribonuclease fell precipitously and to levels that are apparently insufficient to prevent large-scale destruction of RNA of ribosomes.[6, 7] The massive increase in the biosynthesis of nucleolar RNA has been demonstrated in previous studies.[4–6] Another possibility is that the lysosomes are ruptured and the ribonuclease released is absorbed by other fractions.

The mechanism by which thioacetamide alters the ribonuclease activity is not clear at present. The possibilities that thioacetamide directly activates ribonuclease or that increased ribonuclease activity may be an accompaniment of increased nuclear volume have been ruled out in previous studies. In the present studies, thioacetamide has been found to have no demonstrable effect on the binding of pancreatic ribonuclease with ribonuclease inhibitor *in vitro*.*

The increased nuclear synthetic activity produced by thioacetamide is another example of a parallelism between increased RNA synthesis (and accumulation) and increased ribonuclease activity, which has been demonstrated in the nuclei from fetal and neontal liver,[26] regenerating rat liver,[27] and various other tissues.[28–30]. However, in these cases the RNA level increases in the cytoplasm, but it decreases in the cytoplasm of thioacetamide-treated cells. The nature of the stimulus for increased nuclear RNA synthesis is not defined at present. The mechanism or biological value of the increased nuclear ribonuclease also is not clear. These and other studies showing the increased level of ribonuclease suggest that the cell may inadvertently induce self-destruction or that ribonuclease may have intracellular roles other than hydrolysis of RNA.

* In order to investigate the effect of thioacetamide, if any, on the binding of ribonuclease to ribonuclease inhibitor, the following experiment was performed. To incubation mixtures containing pancreatic ribonuclease and the cell-free supernatont from rat liver, thioacetamide was added (to a final concentration varying from 0·6 to 6·0 mM) in different ratios to give a 50–75 per cent inhibition. The samples were assayed for ribonuclease inhibitor as described in 'Materials and Methods'. Comparable quantities of thioacetamide were added to the substrate blanks because thioacetamide has a high absorbance at 260 mμ. Under these conditions, no change in the inhibition of pancreatic ribonuclease by ribonuclease inhibitor was observed.

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