

Effects of Thioacetamide on Incorporation of Orotic Acid-2-C¹⁴ into RNA Fractions in Liver*

HELEN R. ADAMS† AND HARRIS BUSCH

(Departments of Pharmacology and Biochemistry, Baylor University College of Medicine, Houston, Texas)

SUMMARY

Thioacetamide, administered in a dose of 50/mg/kg for 9 days, produced a profound effect on the distribution and labeling of ribonucleic acid (RNA) of the rat liver. The amount of *p*-RNA (released by phenol into the aqueous phase) and *r*-RNA (which remained in the phenol), was increased two- to threefold in the nuclei. In the nucleoli, the amount of *p*-RNA was doubled, and the *r*-RNA was increased by a factor of 3.5. The amount of mitochondrial and microsomal RNA was correspondingly decreased, but the *p*-RNA of the cytoplasmic sap was increased. Thioacetamide suppressed the incorporation of orotic acid-2-C¹⁴ into the *p*- and *r*-RNA of the nuclei. As measured by specific activity, the labeling of nucleolar *p*-RNA was unchanged by thioacetamide. The labeling of the *r*-RNA of the nucleoli was suppressed by 30 per cent at early time points.

There is a marked increase in the size of the nucleoli of liver cells after administration of the carcinogen, thioacetamide, to rats (15). Comparative studies on the fate of metabolic intermediates in livers of normal and thioacetamide-treated rats may yield information on the metabolic role of the nuclear and nucleolar components, since thioacetamide may function as a blocking agent for the release of nucleolar products (1, 2, 9, 10). Recent studies in this laboratory provided evidence that, in addition to decreasing the amount of microsomal proteins, thioacetamide suppresses amino acid incorporation into microsomal proteins of normal and regenerating liver (12). On the other hand, an increased labeling was found in histones and proteins of the cytoplasmic sap when L-lysine-U-C¹⁴ was used as a tracer. Previous studies (7, 9-11) suggested that thioacetamide blocks release of RNA and/or protein from the nucleus and nucleolus that is apparently essential for formation of microsomes and their biosynthetic activity (14, 18).

The enlargement of the nucleolus of liver cells produced by thioacetamide (15) has previously

been shown to be associated with an increase in RNA content in the nucleus. Laird and others (7, 11) reported that thioacetamide produced an increase in nuclear RNA and nuclear protein and a corresponding decrease in the RNA of the cytoplasmic particles. The nuclear RNA content increased to 3.5 times the control values. Using cytochemical techniques, Kleinfeld (9, 10) obtained similar findings and found that the nucleolar volume was increased by 14 times after 10 days of thioacetamide treatment. Grant (7) found a decrease of incorporation of P³² in the nuclear ribonucleoprotein and no change in the uptake of P³² into the RNA of the other fractions of livers after treatment of rats with thioacetamide.

The present studies were designed to determine the effects of thioacetamide on RNA synthesis in the liver, by determination of the rates of incorporation of orotic acid-C¹⁴ into various subcellular fractions. The RNA was subfractionated by the modified procedure described by Sibatani and Yamana (16, 19). Direct studies on the content of nucleolar RNA were made possible by isolation of nucleoli (3, 13). The results of the present studies suggest that thioacetamide produces a dissociation of biosynthesis of RNA in the nucleolus and the chromatin and that the latter produces components which may be necessary for release of nucleolar ribonucleoproteins into the cytoplasm.

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† Predoctoral Trainee of the National Cancer Institute.

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MATERIALS AND METHODS

Albino male rats weighing approximately 200 gm. were obtained from the Holtzman Rat Company and were treated each day for 9 days with a dose of 50 mg/kg of thioacetamide. The thioacetamide was prepared as a 1 per cent solution in saline and was injected intraperitoneally. Orotic acid-2-C¹⁴ was obtained from the California Corporation for Biochemical Research and was injected intravenously in a dose of 3.3 μ c/rat. At specified times after the injection of orotic acid-2-C¹⁴ the rats were sacrificed, and the livers were excised. Two gm. of liver was homogenized in sufficient 0.25 M sucrose to make a 1:10 homogenate (w/v), and the nuclear fractions were purified as previously described (4). The cytoplasmic fractions were prepared by differential centrifugation (5); the mitochondrial and microsomal fractions were each rehomogenized in 0.25 M sucrose and re-centrifuged at 9,000 and 100,000 $\times g$, respectively.

Isolation of nucleoli.—The methods for the isolation of intact nucleoli have recently been improved (3, 13). The livers from three to four rats were removed 5 and 15 minutes after the injection of orotic acid-2-C¹⁴ (3.3 μ c/rat). They were homogenized in 0.25 M sucrose containing 0.005 M Ca⁺⁺ and centrifuged at 600 $\times g$ for 10 minutes. The precipitate was rehomogenized in 5 ml. of 0.25 M sucrose per gram wet weight of liver. Twenty ml. of the suspension were sonicated in a Raytheon sonic oscillator (10 kc.) at 1–1.1 amp. for 30–45 seconds, or until the ratio of nucleoli to nuclei was 10:1 or greater. Aliquots of the preparations were stained with Azure C for microscopic observation. The sonicate was centrifuged at 2500 $\times g$ for 15 minutes. The precipitate was resuspended in 0.88 M sucrose and centrifuged at 100 $\times g$ for 15 minutes to precipitate the unbroken nuclei. The supernatant suspension was centrifuged at 1000 $\times g$ for 15 minutes; the resulting pellet contained the nucleoli. Nucleoli were resuspended in 0.88 M sucrose, and the purification was repeated twice. The yield of nucleoli averaged 10 per cent and 16 per cent, respectively, for normal liver and livers of animals treated with thioacetamide; the yield is based upon total nucleoli in the sonicate.

Fractionation of RNA.—The RNA of the various cellular components was fractionated with phenol and buffer (6, 8, 16, 19) to give phenol-extractable RNA (*p*-RNA) and a residual RNA (*r*-RNA) which remained in the phenol phase or interphase. Six ml. of 0.2 M potassium phosphate buffer, pH 6.8, was added to the precipitates containing nuclei, mitochondria, nucleoli, and microsomes. The cytoplasmic sap was made 0.2 M by adding 2 M potassium phosphate buffer, pH 6.8.

Equal volumes of phenol saturated with 0.2 M potassium phosphate buffer pH 6.8 were added to all fractions. The fractions were shaken at room temperature for 30 minutes and centrifuged for 30 minutes at 600 $\times g$. The aqueous phase was separated, and the phenol layer was re-extracted with an equal volume of 0.2 M buffer. The RNA in the combined aqueous phases is referred to as *p*-RNA.

The aqueous phase was shaken with 2 volumes of ether, 2 times, and left in the cold 1 hour, after which sufficient 2 N HCl was then added to make a final concentration of 0.2 N HCl. This suspension was allowed to stand for 1 hour in the cold for complete precipitation of the RNA. A precipitate was obtained after the suspension was centrifuged at 600 $\times g$ for 15 minutes; it was washed with 0.2 N HCl, and 95 per cent ethanol and was then dissolved in 0.03 N NH₄OH.

The phenol phase was treated with 4 volumes of ethanol and 1 volume of ether to remove the phenol. The precipitate obtained was washed with 95 per cent ethanol, 10 per cent trichloroacetic acid (cold), 80 per cent ethanol, 95 per cent ethanol, ethanol:ether (1:1), and ether. KOH (0.5 N) was added to the precipitate (1 ml/100 mg precipitate) and the suspension was incubated 16–20 hours at 37° C. The reaction mixture was chilled ice. To precipitate the protein, DNA and K⁺, the samples were acidified with 10 N perchloric acid. For complete extraction of the nucleotides, the precipitate was washed with 0.2 N perchloric acid. The pH of the perchloric acid extracts was adjusted to 8.5 with 10 N KOH, and the samples were centrifuged at 600 $\times g$ for 15 minutes. The nucleotides in the supernatant fraction were derived from the *r*-RNA.

The samples of *p*-RNA and *r*-RNA were plated at infinite thinness, and the radioactivity was determined in a thin-window gas-flow counter. The RNA was determined by the orcinol method (17), and the specific activities were calculated. Each point on the graphs is an average of three to five experiments; in each experiment one to four animals were used; in those in which nucleoli were isolated, three to four animals were used in each experiment.

RESULTS

Quantity of RNA.—In the nuclear fractions of livers of thioacetamide-treated rats there was approximately twice the quantity of *p*-RNA and *r*-RNA as there was in the nuclear fractions of the livers of the controls (Charts 1, 2). There was also a marked decrease in amount of both *p*-RNA and *r*-RNA in the microsomal fraction. In the mitochondrial fraction there was a much smaller decrease in the amount of *p*-RNA than there was in

that of the r -RNA. In the cytoplasmic sap, there was a marked increase in the amount of the p -RNA.

Labeling of nuclear RNA.—As found previously by Sibatani and others (16, 19), the specific activity of the r -RNA was very high in the nuclear

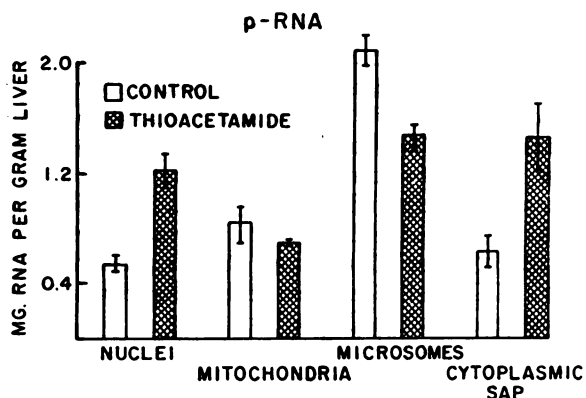


CHART 1.—Distribution of p -RNA in the various cellular components of normal rat livers and the livers of thioacetamide-treated rats. Thioacetamide was given in a dose of 50 mg/kg body weight for 9 days. The results are mg RNA/gm wet weight of liver and are averages of thirteen to fifteen experiments; the standard errors are indicated.

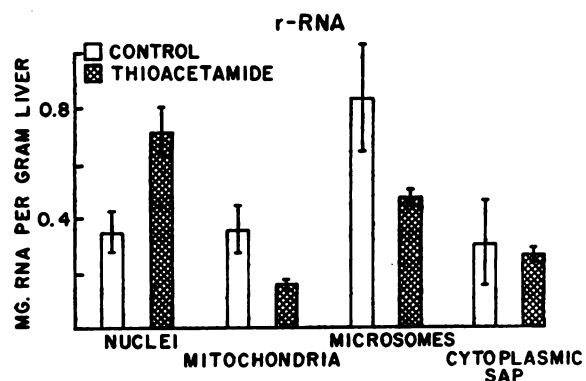


CHART 2.—Distribution of r -RNA in the various cellular components of normal rat livers and the livers of thioacetamide-treated rats. Thioacetamide was given in a dose of 50 mg/kg body weight for 9 days. The results are mg RNA/gm wet weight of liver and are averages of thirteen to fifteen experiments; the standard errors are indicated.

fractions of the normal livers by comparison with the specific activity of the p -RNA. A maximum specific activity was found at 30 minutes following the injection of the radioactive tracer (Chart 3). This corresponds to the maximum found also for the total C^{14} in the r -RNA (Chart 4). The specific activity of the p -RNA increased much more slowly. By comparison, the specific activity of the r -RNA of the nuclear fraction of the thioacetamide-treated rat was significantly lower than that

of the normal rat (Chart 5) at each point and reached a plateau at 30 minutes following the injection of the orotic acid. The total C^{14} in RNA also was lower (Chart 6) than that of the control nuclear fractions at early time points. The turnover of RNA in the nuclear fraction was more rapid in the control than in the thioacetamide-treated livers, since both the uptake into and the loss of label from the pool was rapid in the control livers by comparison with the livers of the thioacetamide-treated animals. Moreover, the lower

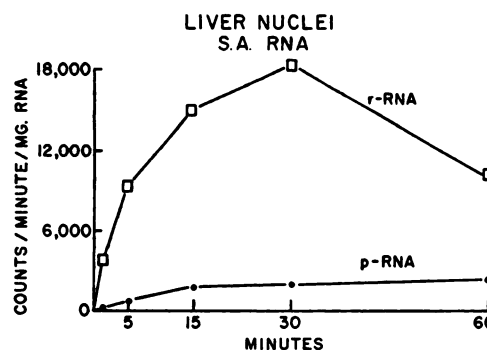


CHART 3.—Specific activities of p -RNA and r -RNA of nuclei of normal liver cells at various times after the intravenous injection of 3.3 μ c. orotic acid-2- C^{14} . At the designated time the liver was excised and carried through the procedure indicated in the section on "Materials and Methods" for isolation of RNA. The values at each point are averages of three to five experiments. The standard errors averaged 10 per cent in Charts 3-6.

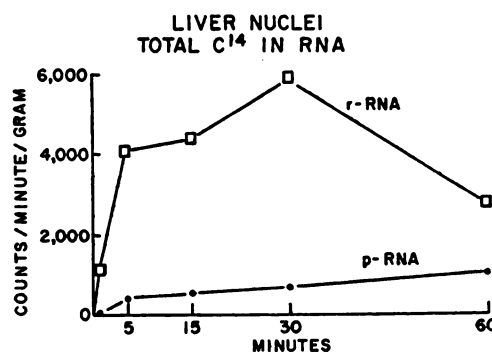


CHART 4.—Total C^{14} content per gm. wet weight of liver of the p -RNA and r -RNA of nuclei of normal liver cells at various times after the intravenous injection of 3.3 μ c. of orotic acid-2- C^{14} . At the designated time the liver was excised and carried through the procedure indicated in the section on "Materials and Methods" for isolation of RNA. The values at each point are averages of three to five experiments.

total labeling of the r -RNA in nuclear fractions of livers of thioacetamide-treated rats suggests that the biosynthesis of r -RNA is suppressed.

Cytoplasmic fractions.—A rapid increase was found in the specific activity and total C^{14} in

r-RNA in the cytoplasmic fractions of the normal livers (Chart 7). A maximum in the specific activities of the microsomal fraction and cytoplasmic sap was reached at 5 minutes following the injection of orotic acid-2-C¹⁴. In the cytoplasmic fractions of the livers of rats treated with thioacetamide (Chart 8) the specific activity of the *r*-RNA was much less, and maximal values for total C¹⁴ in *r*-RNA were found at 15 minutes. The greatest

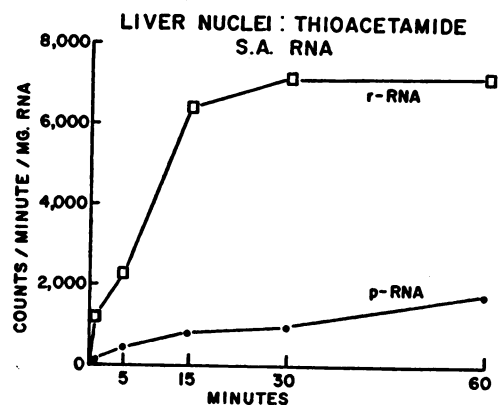


CHART 5.—Specific activities of *p*-RNA and *r*-RNA of nuclei of livers of thioacetamide-treated rats at various times after the intravenous injection of orotic acid-2-C¹⁴. Thioacetamide was given in a dose of 50 mg/kg body weight for 9 days. At the designated time the liver was excised and carried through the procedure indicated in the section on "Materials and Methods" for isolation of RNA. The values at each point are averages of three to five experiments.

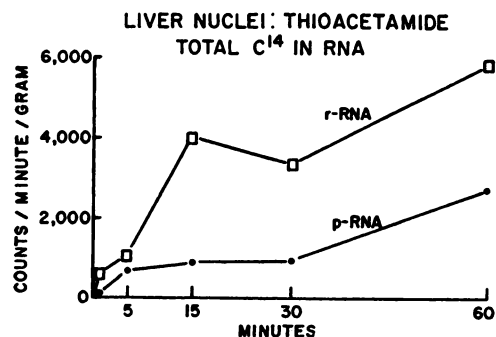


CHART 6.—Total C¹⁴ content per gm. wet weight of liver of the *p*-RNA and *r*-RNA of the nuclei of livers of thioacetamide-treated rats at various times after intravenous injection of orotic acid-2-C¹⁴. Thioacetamide was given in a dose of 50 mg/kg body weight for 9 days. At the designated time the liver was excised and carried through the procedure indicated in the section on "Materials and Methods" for isolation of RNA. The values at each point are averages of three to five experiments.

differences between the normal liver and the livers of animals treated with thioacetamide were in the total C¹⁴ and the specific activity of the *r*-RNA at 5 minutes following the injection of orotic acid-2-C¹⁴. The greater specific activity of the *r*-RNA

in the microsomal fractions of the thioacetamide-treated animals than in the controls may be related to the smaller pool size.

Along with the marked increase in the amount of *p*-RNA in the cytoplasmic sap of the livers of

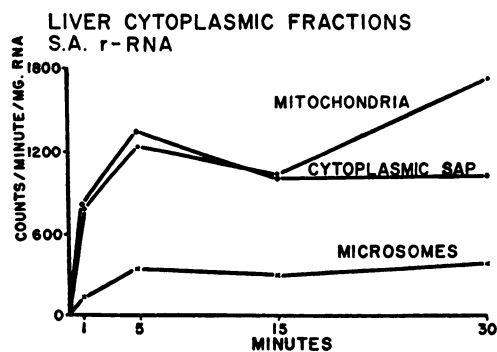


CHART 7.—Specific activities of *r*-RNA of the cytoplasmic fractions of normal liver cells at various times after intravenous injection of orotic acid-2-C¹⁴. At the designated time the liver was excised and carried through the procedure indicated in the section on "Materials and Methods" for isolation of RNA. The values at each point are averages of three to five experiments. The standard errors averaged 7 per cent in Charts 7-10.

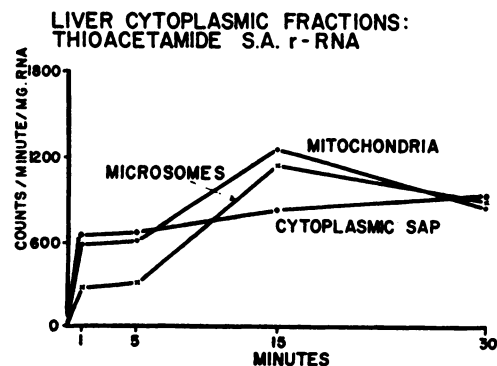


CHART 8.—Specific activities of *r*-RNA of the cytoplasmic fractions of livers of thioacetamide-treated rats at various times after the intravenous injection of orotic acid-2-C¹⁴. Thioacetamide was given in a dose of 50 mg/kg body weight for 9 days. At the designated time the liver was excised and carried through the procedure indicated in the section on "Materials and Methods" for isolation of RNA. The values at each point are averages of three to five experiments.

thioacetamide-treated rats there was a markedly lower specific activity at all time points of the *p*-RNA of the cytoplasmic sap of the thioacetamide-treated livers as compared with the control livers (Charts 9, 10). The total C¹⁴ was initially greater in both the microsomal fraction and the cytoplasmic sap in the controls than it was in the thioacetamide-treated liver. The greatest differences were noted at 5 minutes following the injection of orotic acid-2-C¹⁴. The total C¹⁴ in the microsomal fraction of the normal liver was in

excess of that of the liver of the thioacetamide-treated animals at each time point.

Nucleolar RNA.—Nucleolar preparations of high purity were obtained from livers of the thioacetamide-treated rats and from normal rats. The amount of *r*-RNA of the nucleolus of the normal

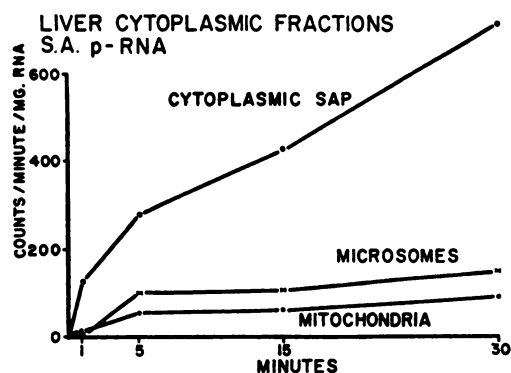


CHART 9.—Specific activities of *p*-RNA of the cytoplasmic fractions of normal liver cells at various times after intravenous injection of orotic acid-2- C^{14} . At the designated time the liver was excised and carried through the procedure indicated in the section on "Materials and Methods" for isolation of RNA. The values at each point are averages of three to five experiments.

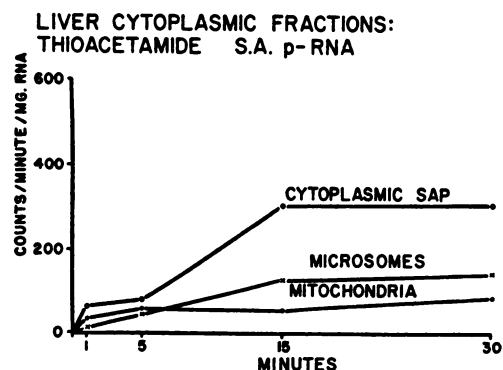


CHART 10.—Specific activities of *r*-RNA of the cytoplasmic fractions of livers of thioacetamide-treated rats at various times after intravenous injection of orotic acid-2- C^{14} . Thioacetamide was given in a dose of 50 mg/kg body weight for 9 days. At the designated time the liver was excised and carried through the procedure indicated in the section on "Materials and Methods" for isolation of RNA. The values at each point are averages of three to five experiments.

liver was slightly greater than the amount of *p*-RNA (Table 1). In the nuclear fractions of livers of the thioacetamide-treated rats, there was twice as much *r*-RNA as *p*-RNA. There was 3 times as much RNA in the nuclear fractions obtained from thioacetamide-treated rats as in the controls. The amount of *p*-RNA was doubled, and the *r*-RNA increased 3½-fold.

As measured by their specific activities at 5 and

15 minutes following intravenous injection of orotic acid-2- C^{14} , the labeling of nucleolar *r*- and *p*-RNA was essentially the same in livers of normal rats (Chart 11). The labeling of *p*-RNA in the nuclear fraction was the same in thioacetamide-treated rats as in the controls (Chart 12); however, the labeling of *r*-RNA was decreased by approximately 30 per cent. Since there was a greater amount of *r*-RNA in the nucleolar fractions of the livers of thioacetamide-treated rats, the total isotope was greater in this fraction than in the other nucleolar fractions studied.

TABLE 1

QUANTITY OF RNA IN NUCLEOLI OF NORMAL RAT LIVERS AND LIVERS OF RATS TREATED WITH THIOACETAMIDE

In the final preparations, the ratio of nucleoli to nuclear fragments was 25:1 for nucleolar preparations from normal livers and 30:1 for nucleolar preparations from livers of thioacetamide-treated rats. The values presented are averages of results of six experiments \pm standard errors of the means determined from the equation $E = \sqrt{\Sigma d^2/n(n-1)}$.

TREATMENT OF RATS	PG. RNA PER NUCLEOLUS	
	<i>p</i> -RNA	<i>r</i> -RNA
Control	0.54 \pm 0.04	0.67 \pm 0.07
Thioacetamide	0.96 \pm 0.19	2.20 \pm 0.23

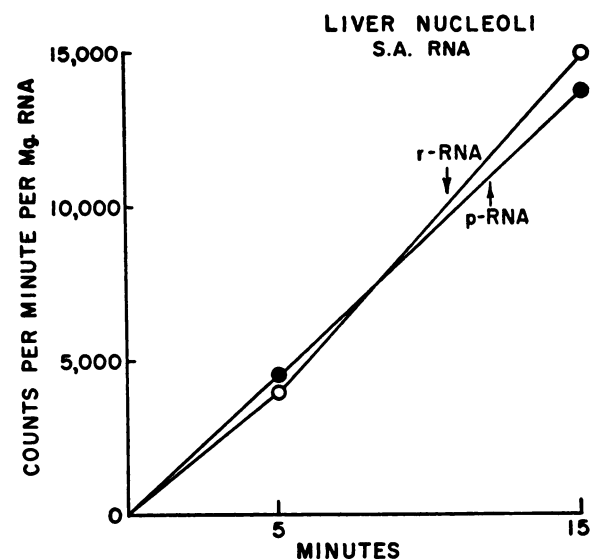


CHART 11.—Specific activities of *p*-RNA and *r*-RNA of nucleoli of normal liver cells at various times after intravenous injection of 3.3 μ c. orotic acid-2- C^{14} . At the designated time the liver was excised and carried through the procedure indicated in the section on "Materials and Methods" for isolation of RNA. The values at each point are averages of three to five experiments. The standard errors averaged 10 per cent.

DISCUSSION

Thioacetamide produces profound effects on the amounts and metabolic activities of RNA in the various cellular fractions studied. The marked increase of RNA in the nuclear fractions appears to be the result of an equal increase of both *p*-RNA and *r*-RNA. In the nucleolar fraction, however, the greatest increase was found in *r*-RNA, which was 3.5 times the normal amount. The marked decrease in RNA's in the cytoplasmic fractions suggests that these are products of biosynthetic reactions which occur in the nuclear fraction and nucleolar fraction. It is not possible, however, to

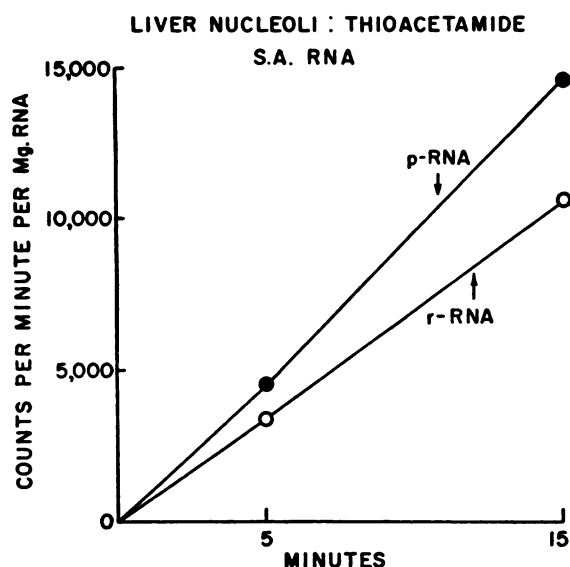


CHART 12.—Specific activities of *p*-RNA and *r*-RNA of nucleoli of livers of thioacetamide-treated rats at various times after intravenous injection of orotic acid-2-C¹⁴. Thioacetamide was given in a dose of 50 mg/kg body weight for 9 days. At the designated time the liver was excised and carried through the procedure indicated in the section of "Materials and Methods" for isolation of RNA. The values at each point are averages of three to five experiments. The standard errors averaged 10 per cent.

explain the increase in quantity of *p*-RNA in the cytoplasmic sap.

The labeling of the nuclear *r*-RNA was markedly suppressed by thioacetamide. In the control, at 5 minutes after injection of labeled orotic acid, the specific activity of nuclear *r*-RNA was twice that of the nucleolar RNA fractions. In the livers of the thioacetamide-treated animals the specific activity of nuclear *r*-RNA was less than that of the nucleolar *r*-RNA and nucleolar *p*-RNA. Similar evidence of marked suppression of labeling of nuclear *r*-RNA was found at 15 minutes after injection of labeled orotic acid into rats pretreated with thio-

acetamide. The conclusion may be drawn that thioacetamide or its products exert a relatively specific suppression of some reactions catalyzed by RNA-polymerases.

On the other hand, the labeling of the nucleolar RNA was not markedly affected by the administration of thioacetamide. At the early time points, the labeling of *p*-RNA in nucleoli of normal livers was slightly in excess of the labeling of *r*-RNA in the nucleolus, whereas at later times the labeling of the *r*-RNA became equal to that of the *p*-RNA. In the livers of rats treated with thioacetamide, however, the labeling of nucleolar *r*-RNA was less than that of the control. These data suggest that thioacetamide does not affectively block RNA biosynthesis in the nucleolus and may explain the increase in nucleolar mass under the influence of thioacetamide.

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