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Changes in Rat Liver Metallothionein and Metallothionein mRNA Induced by Isopropanol[1] (41831)

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Abstract. Administration of isopropanol (1 ml/kg body weight) via the ip route significantly depressed the serum zinc concentration within 8 hr. A maximal increase in hepatic metallothionein was observed 16 hr after isopropanol. By 48 hr after treatment metallothionein levels in liver had returned to basal levels. The extent of metallothionein induction was comparable with that observed after ip administration of zinc. Plasma glucagon concentrations were significantly elevated 4 hr after isopropanol treatment. Adrenalectomy did not prevent the isopropanol-induced changes in serum zinc or hepatic metallothionein. This suggests a nonadrenal mechanism is responsible for the observed changes. To evaluate changes in metallothionein mRNA levels in liver, *in vitro* translation with the wheat germ system was used to evaluate translational activity. Analysis of the labeled metallothionein produced *in vitro* employed both covalent chromatography as well as SDS–polyacrylamide gel electrophoresis of carboxymethylated translation products. These methods suggested the maximum metallothionein mRNA level in total RNA extract occurred about 8 hr after administration of isopropanol. Similarly, when metallothionein mRNA levels were quantitated using dot blot hybridization to [^{32}P]cDNA for mouse metallothionein I, maximum metallothionein mRNA appeared 8 hr after isopropanol administration. The overall response of these parameters in rats suggest that isopropanol administration leads to an inflammatory-like response that, with respect to zinc metabolism, has elements which are independent of the adrenal gland, but involve transcriptional regulation of the metallothionein gene in liver.

Regulation of expression of the metallothionein gene has focused on both metals and glucocorticoids as primary inducers (1–6). However, evidence is accumulating that these classical inducing agents do not account for all of the factors that may be operating to alter metallothionein tissue levels in a variety of dietary and pathophysiological situations. For example, stress and infection, as well as food restriction are all known to alter hepatic metallothionein (7–10).

Recently, we were able to demonstrate that endotoxin produces a particularly marked induction of metallothionein via an mRNA related process that occurs concomitantly with hypozincemia (11). Furthermore, we have identified that glucagon produces a synergistic action with glucocorticoids which leads to a transient increase in zinc associated with metallothionein following simultaneous administration of both hormones (12). Since glucagon levels are so frequently altered in a variety of phenomena in which trace elements seem to be altered (13, 14), it is likely that glucagon and perhaps other hormones have pronounced effects on this protein in addition to the corticosteroids which, until recently, have received most of the attention.

As described in this report, isopropanol is an extremely potent inducer of metallothionein and concomitant hypozincemia. Its mechanism of action appears to be adrenal independent. Because of its widespread availability and apparent hormonal-like mechanism of action, isopropanol may be a valuable agent with which to evaluate the influence of inflammation on hepatic zinc metabolism.

Materials and Methods. *Materials.* All chemicals and reagents were from Fisher Scientific Company (Pittsburgh, Pa.) or Sigma Chemical Company (St. Louis, Mo.). [^{3}H]-Glycine, [^{3}H]lysine, and [^{3}H]serine were purchased from Amersham (Arlington Heights, Ill.). Sephadex and Sepharose were from Pharmacia Fine Chemicals (Piscataway, N.J.). Components for the wheat germ translation

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system and nick-translation were from Bethesda Research Laboratories (Bethesda, Md.). Components for the rabbit reticulocyte lysate translation, [^{35}S]cysteine, and [α^{32}P]dCTP were from New England Nuclear (Boston, Mass.). All water was glass distilled and deionized. For mRNA work glassware was baked at 350°F for 3 hr and solutions were autoclaved when necessary.

Animals. Male rats (CD Strain, Charles River, Wilmington, Mass.) were housed in suspended stainless-steel cages and fed tap water and commercial diet (Rat Chow, Purina, St. Louis, Mo.) *ad libitum.* They were maintained on a 12-hr dark (7 PM–7 AM):12-hr light (7 AM–7 PM) cycle until ready for use. Adrenalectomies were performed by the paravertebral dorsal approach under pentobarbitol anesthesia (60 mg/kg; ip) 4 days prior to use. After surgery 0.9% NaCl was given as drinking water. Animals were injected with isopropanol (1 ml/kg, ip) or 0.9% NaCl at 8 AM. At appropriate times thereafter, the rats were killed and blood collected for serum and plasma. Livers were removed and treated as necessary for specific assays. Plasma glucagon was measured by double-antibody radioimmunoassay (Cambridge Medical Diagnostics, Billerica, Mass.) as described previously (13).

Gel filtration chromatography. Livers were excised, homogenized (1:2 w:v) in a sucrose buffer (250 m*M* sucrose, 10 m*M* Tris, 0.1% Na azide, pH 8.6), and centrifuged for 10 min at 40,000*g*. The supernatant was then centrifuged at 166,500*g* for 60 min. Aliquots (3 ml) of cytosol were applied to columns (2.5 × 60 cm) of Sephadex G-75 treated with sodium borohydride (15). Fractions (4 ml) were collected and analyzed by atomic absorption spectrophotometry. The zinc content in the metallothionein peak was calculated on a per gram liver basis.

Hg-binding assay for metallothionein. Livers (0.5 g) were homogenized in 1.15% KCl (3.5 ml). An aliquot of homogenate was incubated 15 min with a solution of Hg ($HgCl_2$ + ^{203}Hg) and then precipitated with TCA (final concentration of 2%). This is a modification of the method originally described by Zelazowski and Piotrowski (16). The sample was centrifuged and the supernatant applied to a G-75 column (0.7 × 30 cm). Fractions were collected and counted by gamma-ray spectrometry. Total Hg bound to metallothionein was calculated per gram liver.

Purification of poly(A) mRNA. Tissues (1 g) were homogenized in 30 ml 6 *M* guanidine-HCl:100 m*M* potassium acetate (pH 5) using a Polytron tissue grinder, centrifuged at 11,000*g* for 10 min, and passed through three layers of cheese cloth. Absolute ethanol was added at one-half the volume of sample. After storage at −20°C overnight the precipitated RNA was collected by centrifugation at 12,300*g* for 10 min. The RNA was precipitated twice more from guanidine–ethanol and then extracted with phenol–chloroform as described by Ouellette (17). Two volumes of 4.5 *M* Na acetate (pH 6) were added to the final aqueous phase. After an overnight precipitation at −20°C the RNA was collected as above and poly(A) mRNA was isolated by oligo-(dT) cellulose column chromatography. The mRNA was eluted and stored in sterile H_2O at −70°C until needed. We have described these methods previously (5, 18).

In vitro translation, thiol chromatography, and polyacrylamide gel electrophoresis. The wheat germ translations and assay of [^{3}H]metallothionein by covalent chromatography were as described previously (5, 18). Alternatively, translations were carried out with [^{35}S]cysteine (8 μCi) and metallothionein synthesis was measured by polyacrylamide electrophoresis. The entire translation mixture (25 μl) was centrifuged for 10 min at 13,000*g*. One-half volume of iodoacetic acid (0.3 *M* in 1 *M* Tris, pH 8.4) was added to each supernatant prior to incubation for 45 min at room temperature in the dark (19). After incubation sample buffer was added and the mixture was boiled for 5 min. Carboxymethylated samples were applied to a 20% acrylamide gel prepared according to the method of Laemmli (20). Electrophoresis was for about 2 hr at 10 W using the constant power mode. The gel was soaked in 10% TCA overnight and dried under vacuum onto Whatman 3MM filter paper. Autoradiography was carried out for at least 48 hr at −70°C with Kodak X-Omat AR X-ray film using an intensifying screen. The migration of carboxymethylated [^{35}S]metallothionein was established by comparison with coelectrophoresed carboxymethylated purified rat liver metallothionein which was visualized by Coomassie blue staining.

Hybridization of mRNA to [^{32}P]cDNA. Poly(A) mRNA was treated with glyoxal and DMSO for 1 hr at 50°C. Samples were diluted and aliquots were spotted onto a nitrocellulose filter (Schleicher & Schuell, Inc. Keene, N.H.). The filter was dried in a vacuum at 80°C for 2 hr. The filter was prehybridized overnight at 42°C in buffer containing 50% formamide, 5× SSC,[3] 40 m*M* sodium phosphate, pH 6.7, 0.05% bovine serum albumin, 0.02% polyvinyl pyrrolidone, 0.02% Ficoll, and 250 μg/100 ml calf thymus DNA. A cloned cDNA to mouse metallothionein I (21) was generously provided by Dr. R. D. Palmiter. Hybrid-select translation data demonstrated this cDNA cross-hybridizes to rat metallothionein mRNA. cDNA containing pBR322 was used to transform *E. coli* RR1 and ampicillin-resistant colonies were developed. The cDNA was nick-translated using [α^{32}P]dCTP (22) and added to the filter for 48 hr of hybridization. The filters were washed in 2× SSC (0.1% SDS) followed by two washes at 50°C in 0.1× SSC (0.1% SDS) and exposed to X-ray film as above. Following autoradiography, intensity of hybridization was measured with a densitometer at 540 nm.

Results. As shown in Fig. 1 (insert), intraperitoneal administration of isopropanol (1 ml/kg) significantly depressed the serum zinc concentration by 8 hr after the dose was administered. After 16 hr the depressive effect was less and by 24 hr postadministration the serum zinc concentration was within normal limits. No significant change was observed in the serum copper concentration or liver weight in response to isopropanol. Plasma glucagon was significantly ($P < 0.05$) altered by 4 hr after isopropanol. The percentage increase (mean ± SD) in glucagon levels in isopropanol-treated rats vs controls was 103 ± 18, 152 ± 22, and 116 ± 22 at 2, 4, and 6 hr after treatment, respectively.

Gel filtration chromatography of the hepatic cytosol fraction revealed that the maximum induction of metallothionein, based upon zinc content, occurred 16 hr after isopropanol was administered (Fig. 1). The amounts of zinc associated with metallothionein 8 and 24 hr after isopropanol were approximately half that observed after 16. By 48 hr after isopropanol was administered, metallothionein-bound zinc was at the usual basal level. When the relative abundance of metallothionein polypeptides were measured by the ^{203}Hg method, a similar enhancement due to isopropanol was observed. Specifically, the maximum metallothionein content occurred at 16 hr after isopropanol with intermediate concentrations found 8 and 24 hr after the dose. Within 48 hr after isopropanol treatment, metallothionein levels had returned to that found in controls. In contrast to the induction of hypozincemia and metallothionein by intraperitoneal isopropanol, when this compound was administered subcutaneously 16 hr before the rats were killed, the above parameters were not markedly influenced (data not shown).

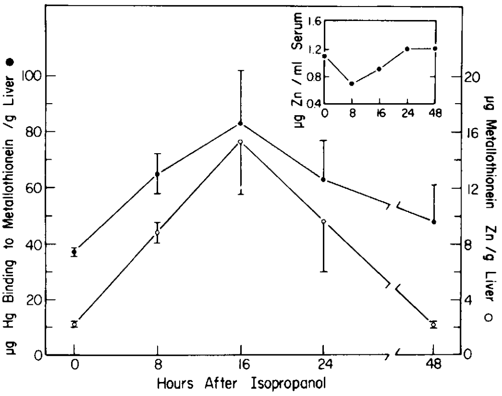

FIG. 1. Total metallothionein and metallothionein-bound Zn in liver as influenced by isopropanol given intraperitoneally. Rats were injected with isopropanol (1 ml/kg) and were killed at the indicated times. Metallothionein-bound Zn was estimated by gel filtration chromatography. Total metallothionein was measured by Hg binding. Each value represents the mean ± SD of four rats. The insert shows the serum Zn content at each time point after isopropanol administration.

The response of adrenalectomized animals to isopropanol-induced hypozincemia and hepatic metallothionein was the same as that observed in nonadrenalectomized rats, based upon zinc bound, but greater when based on Hg-binding assay (Fig. 2). When isopropanol was administered subcutaneously to adrenalectomized rats no response was observed.

In order to evaluate the role that changes in metallothionein mRNA may play in the

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[3] Abbreviations: SSC, standard saline citrate (1× SSC is 0.15 *M* NaCl/0.015 *M* trisodium citrate); SDS, sodium dodecyl sulfate; DMSO, dimethylsulfoxide.

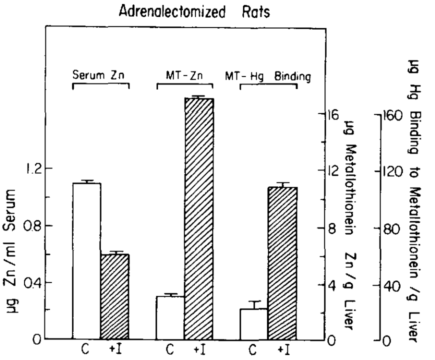

FIG. 2. Total metallothionein and metallothionein-bound Zn in liver and serum Zn content as influenced by intraperitoneal isopropanol administration to adrenalectomized rats. Four days after adrenalectomy the rats were injected with isopropanol (1 ml/kg) and were killed 16 hr later. The measurements were as described in Fig. 1.

induction of hepatic metallothionein after isopropanol, we assayed metallothionein mRNA levels by an *in vitro* translation method as well as by hybridization to a cloned cDNA for metallothionein I. When hepatic poly(A) mRNA derived from total hepatic RNA was translated in the wheat germ system, it appeared that the relative abundance of metallothionein RNA was greatest 8 hr after isopropanol was administered (Fig. 3). It is of particular interest that when translation was carried out with an equivalent amount of poly(A) mRNA from rats injected with 2 mg of zinc at a time to give maximal mRNA levels the translational activity was about the same as that observed with mRNA from rats treated 8 hr previously with isopropanol. When the ^{35}S-labeled products of the wheat germ translation system were carboxymethylated and separated by electrophoresis (SDS-20% polyacrylamide gel), the maximal translational activity was 8 hr after isopropanol treatment (Fig. 4). Note that the metallothionein mRNA level is greatest in rats injected with both zinc and isopropanol suggesting a cumulative inductive response by these two agents. Assay of metallothionein synthesized *in vitro* by either method gave comparable results. Similarly, when the poly(A) mRNA was translated with the rabbit reticulocyte system and the ^{35}S-labeled products were subjected to electrophoresis, the maximum translational activity again appeared 8 hr after isopropanol was administered (data not shown).

Total metallothionein mRNA content, rather than translational activity, was also measured. The autoradiograph shown in Fig. 5 shows the result of dot blot hybridizations obtained when equivalent amounts of poly(A) mRNA derived from total liver RNA are hybridized in the presence of a ^{32}P-nick-translated mouse metallothionein I cDNA. Increasing amounts of metallothionein RNA were observed up to a period of 8 hr after isopropanol treatment and much lesser amounts were observed at the 16- and 24-hr time points. It should be noted that in control, non-isopropanol-treated rats, the 0-hr level of metallothionein mRNA is not detectable whereas the 8-hr level was measurable. In order to obtain estimates of the relative abundance of this mRNA in liver, we related all hybridization intensities to the 24 hr level.

Discussion. The results presented in this paper clearly demonstrate that isopropanol produces an inflammatory-like response following intraperitoneal administration to rats.

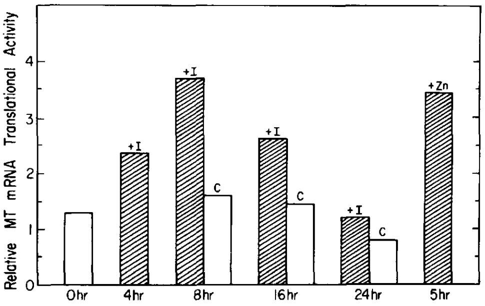

FIG. 3. Influence of isopropanol administration on the translational activity of hepatic metallothionein mRNA as measured by wheat germ translation and covalent chromatography. The rats were injected intraperitoneally with isopropanol (1 ml/kg) and were killed at the indicated times. For comparative purposes, zinc at 2 mg per animal was injected intraperitoneally to another group of rats 5 hr before they were killed. Poly(A)RNA was derived from the total liver RNA extract and translated in a wheat germ translation system using [^{3}H]glycine, [^{3}H]lysine, and [^{3}H]serine. [^{3}H]Metallothionein was isolated by activated thiol covalent chromatography. Relative translational activity for metallothionein mRNA is the percentage of ^{3}H incorporated into metallothionein compared to total ^{3}H-labeled proteins synthesized *in vitro*. The mean of separate translations using mRNA from two different rats killed at each time point are shown.

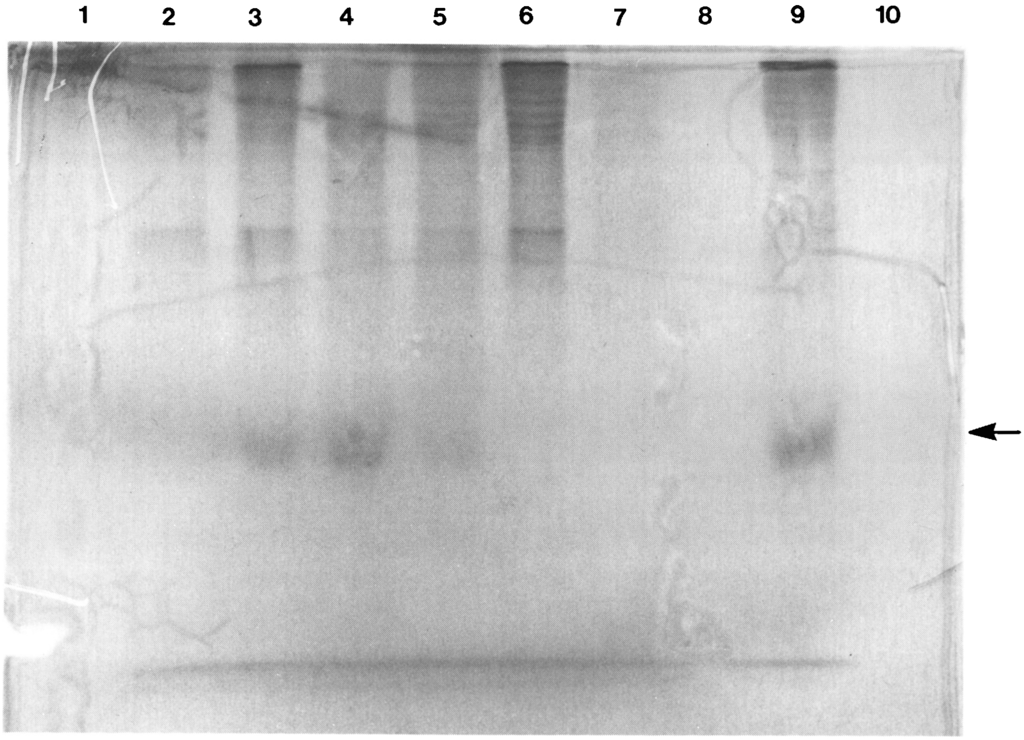

FIG. 4. Influence of isopropanol administration on the translational activity of hepatic metallothionein mRNA as measured by wheat germ translation and SDS–polyacrylamide electrophoresis. The poly(A)RNA used to program the translation system was the same as in Fig. 3 except that for comparative purposes, another mRNA sample was prepared from groups of rats given Zn (2 mg) and isopropanol, both intraperitoneally. In this series [^{35}S]cysteine was used to label proteins synthesized by translation system. The ^{35}S-labeled products were carboxymethylated and separated on a 20% polyacrylamide gel containing SDS. The gel was dried and exposed to X-ray film for 3.5 to 6 days at −70°C. Arrow on right-hand border of fluorograph denotes migration of carboxymethylated metallothionein. Lanes 1, 8 and 10 were not used. Lane 2, 0 hr isopropanol; Lane 3, 4 hr isopropanol; Lane 4, 8 hr isopropanol; Lane 5, 16 hr isopropanol; Lane 6, 24 hr isopropanol; Lane 7 is translation without mRNA and Lane 9 is 5 hr after Zn + 8 hr after isopropanol.

The response appears to be limited to alterations in zinc metabolism as no significant changes in either the serum copper concentration or intracellular distribution of copper was noted following administration of this xenobiotic. Hypozincemia produced in response to isopropanol treatment is similar to that observed in response to endotoxemia, infection and administration of leukocytic endogenous mediator (8, 11, 23).

The data in this report, however, clearly demonstrate that the intact adrenal gland is not required for either the induction of metallothionein or the hypozincemic response produced by isopropanol. In fact, in adrenalectomized rats, isopropanol was able to produce an even greater increase in hepatic metallothionein based upon Hg binding assay, but not total metallothionein bound Zn. The reason for the apparent further increase in total metallothionein protein, but not total zinc bound is not clear. In this regard, it is of particular interest because dexamethasone (a potent corticosteroid) activates the metallothionein gene and leads to increased cellular levels of metallothionein (2, 3, 6). Therefore, it is likely that another mechanism is responsible for the induction by isopropanol. For

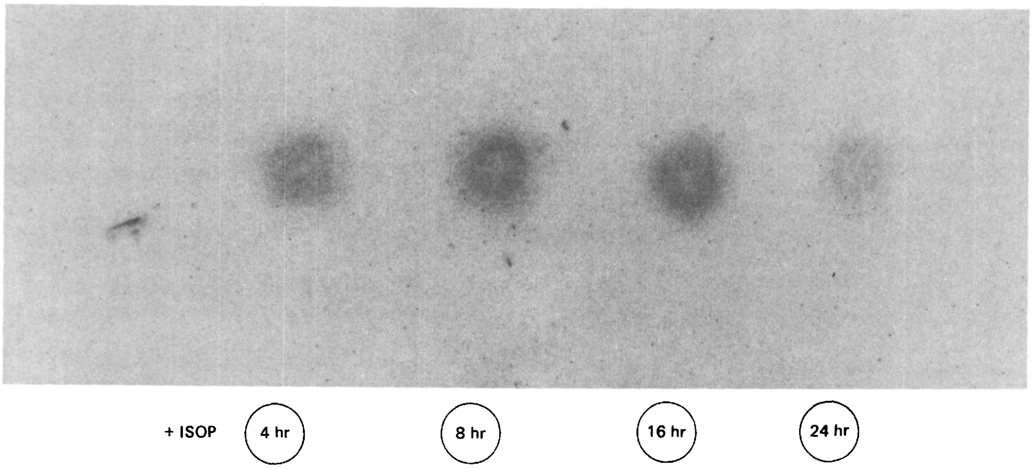

FIG. 5. Influence of isopropanol administration on hepatic metallothionein mRNA levels as measured by cDNA hybridization. Equivalent amounts of poly(A)RNA were transferred to nitrocellulose filters. Hybridization to a [^{32}P]cDNA for mouse metallothionein I was for 48 hr. Washed filters were exposed to X-ray film for a period of 48 hr at −70°C. The poly(A)RNA used was that described in Figs. 3 and 4. Relative intensity of hybridization was measured with a densitometer. Compared to the 24 hr mRNA, relative hybridization intensity for the isopropanol treated rats was 3.1 (4 hr), 6.0 (8 hr), 3.1 (16 hr), and 1.0 (24 hr). Below, template for spotting of mRNA on nitrocellulose filters.

example, we demonstrated that hepatic metallothionein-bound zinc was significantly increased in response to administration to both glucagon and dexamethasone (12). However, we observed that the independent response of hepatic metallothionein zinc to glucagon, as well as the synergistic effect of glucagon and dexamethasone were independent of actinomycin D inhibition (12). This led to the suggestion that changes in transcription were not required for this augmented response. Similarly, Sobocinski and Canterbury have suggested that inflammation which leads to metallothionein production is a response to pronounced hyperglucagonemia and concomitant transient hyperinsulinemia (24), but adrenal steroids did not appear to be involved (25).

Administration of isopropanol led to significant changes in the level of translatable metallothionein mRNA. The response to this compound was as great as we have observed using zinc and nearly as pronounced when dexamethasone is the sole inducing agent. Recently, we found that there is a significant increase in polyribosomal metallothionein mRNA levels in animals that are treated with both glucagon and glucocorticoids over levels that were obtained with either hormone alone (26). In the present work, plasma glucagon was significantly elevated prior to the maximal inductive response. This suggests that the increase in circulating glucagon may be responsible, at least in part, for the increase in metallothionein mRNA found following treatment with isopropanol. This is further supported by recent observations with streptozotocin-induced diabetic rats which showed a depressed hypozincemic response to leukocytic endogenous mediator compared to nondiabetic rats (27). Similarly, the high glucagon/insulin ratio may explain the induction of liver and kidney metallothionein observed in diabetic rats (28).

We have reported previously that endotoxin is also a particularly potent inducer of hepatic metallothionein mRNA activity in the hamster (11). It is well established that endotoxin treatment leads to elevated plasma levels of glucagon and corticosteroids (23, 24). The observation that translational activity for metallothionein mRNA and its total cellular content correspond following isopropanol treatment is a particularly interesting finding. In both cases, the mRNA population used for translation and hybridization were derived from the total cellular RNA extract. Therefore, the translational efficiency of newly transcribed metallothionein mRNA appears to be reflective of the actual total cellular content of that mRNA. The difference in levels between the 0- and 8-hr controls is of particular interest. In these experiments, the 0-hr level was obtained from rats killed at 8 AM and the 8-hr level was from rats killed at 4 PM. The differential in basal metallothionein mRNA levels suggests subtle environmental factors may influence expression of the metallothionein gene, thus leading to circadian rhymicity in the cellular levels of this mRNA. Circadian-type changes in the tissue levels of metallothionein have been reported recently (29), which supports this possibility.

In summary, the data presented in this paper suggest (i) that isopropanol produces an inflammatory-like response similar to that observed in response to leukocytic endogenous mediator, (ii) both gel filtration chromatography and ^{203}Hg-binding assays seem to give a similar estimate of the actual liver content of this protein in response to induction by isopropanol, (iii) the isopropanol induction appears to be independent of the adrenal gland, suggesting that corticosteroid hormones are not responsible for the signal required to initiate hypozincemia and differential changes in metallothionein mRNA, (iv) isopropanol administration increases plasma glucagon, (v) characterization of cell-free products from the wheat germ translation system using activated thiol Sepharose covalent chromatography and polyacrylamide gel electrophoresis, seem to give comparable estimates of newly synthesized metallothionein, and (vi) isopropanol appears to induce a rapid increase in the cellular content of metallothionein mRNA early in the induction process based upon mRNA–cDNA hybridization assay.

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