Effects of infection and endotoxin on rat hepatic RNA production and distribution

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THOMPSON, W. L., AND R. W. WANNEMACHER, JR. *Effects of infection and endotoxin on rat hepatic RNA production and distribution.* Am. J. Physiol. 238 (Gastrointest. Liver Physiol. 1): G303–G311, 1980.—Rat liver was quantitatively subfractionated into free ribosomal, bound ribosomal, nuclear, and soluble fractions to determine the effects of infection and endotoxin treatment on hepatic RNA production and distribution. A 4-h pulse label of [^{14}C]orotic acid was used to monitor newly transcribed RNA; distribution was determined by measuring RNA content at various times after infection or endotoxin treatment. A significant increase in the rate of RNA synthesis was seen by 12 h and continued through 20 h in response to *Streptococcus pneumoniae* infection. During the peak hours of the RNA response, redistribution of RNA into the bound ribosomal fraction takes place at the expense of the free ribosomes. However, in *Salmonella typhimurium* and its endotoxin, more involvement of the free ribosome fraction during the early stages of the infection was apparent. These data suggest that the hepatic RNA response takes place in two stages, an early "endotoxin" response, resulting in redistribution of cytoplasmic RNA into free ribosomes, and a later "infection" response, involving the mobilization of the bound ribosomes.

ribosomes; *Streptococcus pneumoniae*; *Salmonella typhimurium*

NUMEROUS METABOLIC ALTERATIONS OCCUR in the host in response to infection or other inflammatory substances (1, 13). One such metabolic response is an increase in the concentration of certain serum proteins during the early stages of the inflammatory response (3, 16). Several investigators have been concerned with the involvement of these "acute-phase" proteins in the host's defense mechanisms (4, 6, 8). Because the liver is responsible for the production of these proteins, it is also of interest to determine the effects of infectious organisms on hepatic RNA regulation. Therefore, this study was carried out to help understand involvement of the liver in the events taking place within the host from the time of appearance of the invading microorganisms to the appearance of increased concentrations of serum proteins. The infectious organisms were selected as the most appropriate available for the study of nonspecific aspects of host defense. *Streptococcus pneumoniae* was utilized as a model gram-positive bacterium and the MIT strain of *Salmonella typhimurium* was employed as a gram-negative infectious model in rats.

A previous report on the effects of *S. pneumoniae* infection on hepatic RNA distribution and activity described an increase in the rate of RNA transcription that was directed predominantly toward the bound ribosome fraction (10). It has been shown that an initial centrifugation step used in that study results in the loss of more than 60% of the ribosomal RNA, most of which is associated with the bound ribosomes (11). Therefore, improved techniques for quantitatively isolating undegraded rat liver subfractions (9) were used in this study that confirmed the previous observations. These techniques were further used to compare the hepatic RNA response between model gram-positive and gram-negative infections. In addition, the earlier endotoxin response to the gram-negative *S. typhimurium* was compared to the later infection-related stimulation of hepatic RNA synthesis.

MATERIALS AND METHODS

Animals. Male Fisher-Dunning rats weighing 150–200 g were supplied by Microbiological Associates (Walkersville, MD). They were maintained on a 12-h light-dark schedule (0600–1800 h), at a temperature of 25–26°C, and routinely fasted 16–20 h before killing.

Infecting microorganisms and endotoxin. Virulent *S. pneumoniae* (type I, strain A5) was used to infect rats by subcutaneous injection of 10^7 colony-forming units (CFU) contained in 0.1 ml. The virulence of this *S. pneumoniae* strain in rats has been proven by death resulting from the infection of a single microorganism. *S. typhimurium* (MIT), which was originally isolated from a naturally occurring rat infection, was inoculated intraperitoneally (ip) at a dose of 10^8 CFU contained in 0.1 ml. The same number of heat-killed *S. typhimurium* (56°C, 20 min) were given similarly. *S. typhimurium* endotoxin (type B, Difco Laboratories, Detroit, MI) was suspended in physiological saline at a concentration of 5 mg/ml and 0.1 ml was injected intraperitoneally. Control rats were given the same volume of saline by the same route of injection as the inoculum to which they were being compared. Details concerning the preparation of the inoculated microorganisms and the clinical manifestations of the infections have been published elsewhere (14, 15).

Subfraction of hepatic cells (Fig. 1). All rats were given an ip injection of 5 μCi/100 g body wt [6-^{14}C]orotic acid hydrate (40–60 mCi/mmol, New England Nuclear, Boston, MA) 4 h before killing. At the end of the experimental time period, the livers were perfused in situ with cold saline and subfractionated into nuclear, free and bound

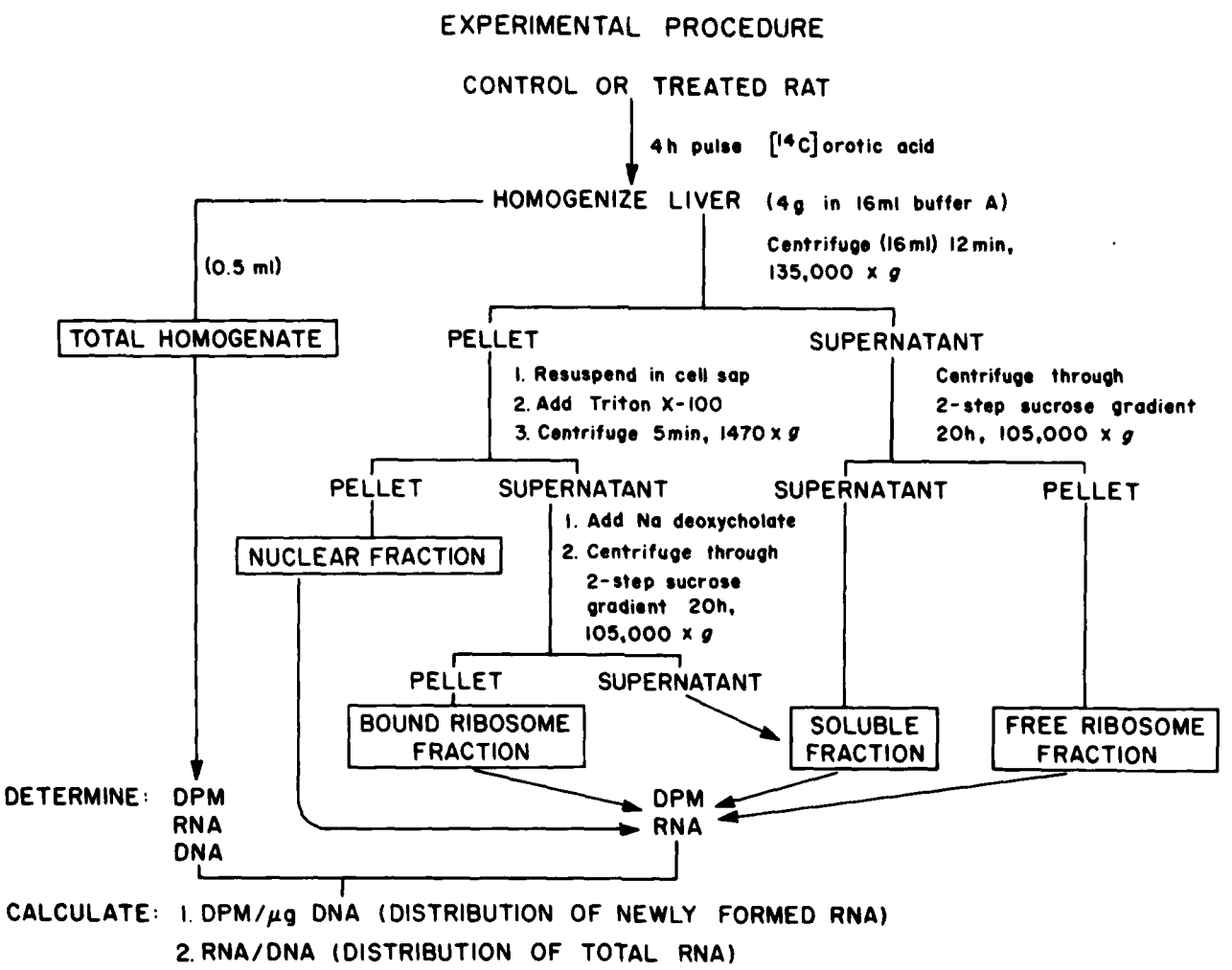

FIG. 1. Procedure for subfractionation of rat liver and determinations and calculations performed on each fraction.

ribosomal, and soluble fractions using a modification of the procedure of Ramsey and Steele (9). The livers were homogenized in 4 vol (wt/vol) of 0.25 M sucrose in buffer A (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.6, 75 mM KCl, 5 mM $MgCl_2$, 3 mM glutathione) using a Potter-Elvehjem homogenizer (A. H. Thomas, Philadelphia, PA). Aliquots of the homogenate were taken for determination of total liver RNA and radioactivity. Portions (16 ml) of the remainder were centrifuged in an SW-27.1 rotor (Beckman Instruments, Silver Spring, MD) at 135,000 *g* for 12 min at speed. Four milliliters of supernatant fluid containing the free ribosomes were layered over a two-step discontinuous gradient containing 3 ml each of 2.0 and 1.38 M sucrose in buffer A. The gradients were centrifuged for 20 h at 105,000 *g* in a 50 Ti rotor (Beckman Instruments). The resulting free ribosome pellets and supernatant fluid representing the soluble RNA fraction were collected and stored at −20°C. Each pellet from the initial centrifugation (135,000 *g*) was resuspended in 8 ml cell sap (see below), treated with 0.1 vol of 10% Triton X-100, and centrifuged at 1,470 *g* for 5 min. The resulting pellets (nuclear fraction) were collected and stored at −20°C. A 0.1 vol of 13% sodium deoxycholate was added to the supernatant fluids and 4-ml portions of this material layered over a two-step discontinuous gradient containing 3 ml each of 2.0 and 1.38 M sucrose in buffer B. These were then centrifuged at 105,000 *g* for 20 h. The resulting pellets representing the bound ribosomes were stored at −20°C. The supernatant fluid was combined with the soluble fraction from the free ribosome separation to determine the total soluble RNA content and specific activity. The RNA contributed by the addition of cell sap from untreated rats was subtracted from the total RNA found in the soluble fraction, leaving an accurate measurement of the soluble RNA content from each rat liver.

A cell-sap fraction was prepared from the livers of untreated rats. The livers were homogenized in 2 vol (wt/vol) of 0.25 M sucrose in buffer B (50 mM HEPES, pH 7.6, 250 mM KCl, 5 mM $MgCl_2$, 3 mM glutathione) and centrifuged at 105,000 *g* for 3 h, and the supernatant fluid was collected.

Determination of RNA, disintegration per minute,

and DNA content. The RNA content and radioactivity of the total homogenate and all subfractions were determined by a modification of previously described techniques (12). Aliquots of each fraction were precipitated and washed twice with cold 0.2 N perchloric acid (PCA). The precipitates were then hydrolyzed in 2 ml of 0.3 N KOH for 1 h in a 37°C water bath. The samples were reprecipitated by the addition of 0.1 ml of 60% PCA and centrifuged, and the pellets were washed twice with 0.2 N PCA. The supernatant fluid from each centrifugation that contained hydrolyzed RNA was collected. All samples were brought to a constant volume of 5 ml and aliquots were taken for measurement of radioactivity and determination of optical density at 260 nm. RNA content was determined from these values using an RNA standard curve of known concentration against absorbance at 260 nm.

After hydrolysis and extraction of RNA, the DNA content of the total homogenate and nuclear fraction was determined. The pellets were treated with 1.5 ml of 0.5 N PCA for 45 min at 95°C, centrifuged, and washed twice by suspension and recentrifugation in 0.5 N PCA. The supernatant fractions were combined and brought to 5-ml vol. The absorbance was then read at 265 and 284 nm; the difference was used to determine the DNA content according to a standard curve.

For each time period of each study, six to eight control rats were compared to an equal number of infected or endotoxin-treated rats. Each time period of each study represents a separately run experiment although all data are presented in a single value. Group mean values were compared by Student's t test and the difference between two means was considered significant at $P < 0.05$ under the null hypothesis.

RESULTS

The DNA content of total homogenate was used as a base for determination of activity and RNA concentrations on a per cell basis. This was valid due to consistent levels of DNA independent of the stage of the illness or the time of day at which the experiment was conducted. An average control value for all the samples run in this study was 2.167 ± 0.045 mg DNA/g liver as compared to 2.197 ± 0.040 mg DNA/g liver for all of the infected and endotoxin-treated samples. Also DNA values computed from nuclear fractions were within 90–100% of their complementary total homogenate DNA value.

To demonstrate the normal range of activity and distribution of RNA in the total homogenate and the four subfractions isolated, an average value was computed for the mean and standard error of the control groups from all the experiments run in this study (Table 1). The high values for disintegration per minute (dpm) per microgram DNA in the total homogenate and bound fraction were due to the large amount of RNA associated with these fractions. When their specific activities were computed on the basis of the quantity of RNA present (dpm/μg RNA), the free and bound ribosomes and soluble fraction fell in the same range of activity. The nuclear fraction was almost 10-fold higher in activity per microgram RNA, and the total homogenate an intermediate value reflecting an average of all the fractions.

TABLE 1. *Specific activity and distribution of RNA in subfractions from normal rat liver*

Fraction	dpm/μg DNA	RNA/DNA	dpm/μg RNA	% Total RNA
Total homogenate	158.2 ± 6.5	3.18 ± 0.07	49.7	
Nuclear	64.8 ± 4.3	0.27 ± 0.02	240.0	8.8
Soluble	18.7 ± 1.1	0.53 ± 0.02	35.3	17.4
Free ribosomal	22.3 ± 1.4	0.58 ± 0.02	38.4	19.0
Bound ribosomal	47.2 ± 3.4	1.67 ± 0.08	28.3	54.8

dpm/μg DNA and RNA/DNA expressed as means ± SE.

From the RNA-to-DNA ratios (and % of total RNA) of control rats, it can be seen that approximately 70% of the total hepatic RNA was associated with the ribosomes, three-fourths of which was found in the bound ribosomal fraction. The sum of the four fractions was well within the range of values for the total homogenate, indicating quantitative recovery of RNA in each of these subfractions.

To present the data in a visually comprehensive manner and avoid lengthy tables, RNA responses to the infectious organisms and endotoxin were computed as a percent of their complementary control values. The results were then plotted as time-course profiles of the hepatic RNA response to infection and endotoxin in the total homogenate and each of the four subfractions (Figs. 2–6). Significant differences between paired control and treated values in the original data are shown as asterisks above the bars. The use of a 4-h pulse of [^{14}C]orotic acid acts as a radioactive marker for newly synthesized RNA. Therefore, the left side of each figure (% of control, dpm [^{14}C]orotic acid/μg DNA) illustrates the appearance of newly formed RNA at each stage of the various studies. The concentration of both newly synthesized and preexisting RNA in each of the fractions during the course of the infection or after endotoxin administration is shown on the right side by plotting percent of control of RNA present per equivalent unit of DNA. Although small differences in the raw data existed between experiments, a consistent within-experiment ratio of activity and RNA between the five fractions was observed. Because comparisons were made between control and treated groups within experiments, the resulting time-course profiles are an accurate reflection of the host's hepatic response to the variable under study.

In evaluating the data, it should be understood that the majority of the experiments were conducted during the early stages of both infections. The onset of fever with the dosage of *S. pneumoniae* given took place around 12–16 h, with deaths beginning to occur at 48 h and extending through 72 h. With *S. typhimurium*, the rats developed fever around 8 h and began to die around 72 h postinoculation.

Figure 2 illustrates the overall hepatic RNA response to the various challenges in the total homogenate fraction. Both infectious models and the heat-killed *S. typhimurium* resulted in a significant increase in the production of RNA (dpm/μg DNA) reaching a maximum in about 14–20 h. As was seen in other fractions, the *S. typhimurium* infection caused a greater response at an

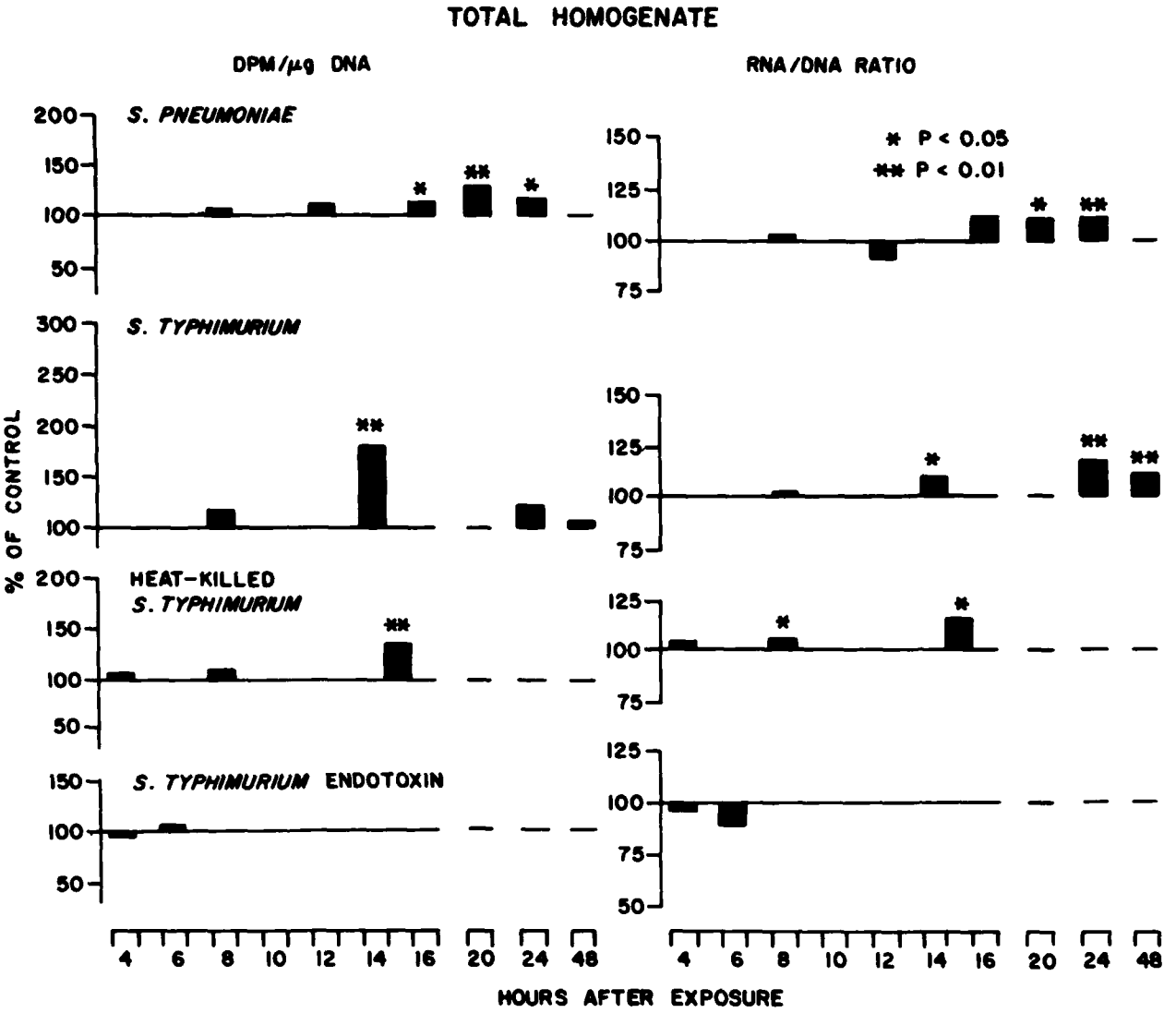

FIG. 2. Percent of control time-course profiles in total homogenate fraction in response to 4 treatments studied. Incorporation of labeled orotic acid in RNA per microgram total cellular DNA shown on left. Quantity of RNA per total cellular DNA on right. Each time period represents separate experiment containing 6–8 rats in each control and treated group.

earlier time period (80% increase over control values at 14 h) than the *S. pneumoniae* infection (30% increase at 20 h). Along with an increase in production of RNA, an overall buildup in total RNA could be seen in the later stages of both infections (right side, Fig. 2). No significant response to endotoxin was noted in the total homogenate. This indicated that any early effects due to the endotoxin content of inoculated bacteria may have been due to redistribution of existing RNA and not to changes in its rate of production.

No significant change in dpm/μg DNA was observed in the nuclear fraction in response to *S. pneumoniae* (Fig. 3), whereas the live and heat-killed *S. typhimurium* produced a significant accumulation of newly transcribed RNA in the nucleus starting around 14 h after treatment. At the same time, both infections showed accumulations of total RNA in the nuclear fraction as the infections progress. However, the nuclear response to endotoxin was characterized by a significant early decrease in total nuclear RNA in the absence of any change in RNA production. This implied a rapid movement of preexisting RNA from the nucleus to the cytoplasm in response to endotoxin.

Figures 4–6 should be viewed together because they represent the response observed in the three cytoplasmic fractions. No significant responses to *S. pneumoniae* were noted in the free ribosomal fraction with respect to the appearance of newly formed RNA, but greater than a twofold increase over control concentrations was seen at 14 h with the *S. typhimurium*. The most significant increases in dpm/μg DNA in response to both infections was seen in the bound ribosomal fraction; the soluble fraction showed a similar but slightly reduced response.

A general decrease in the amount of RNA associated with the free ribosomes in response to *S. pneumoniae* infection (right side, Fig. 4) is noted with a highly sig-

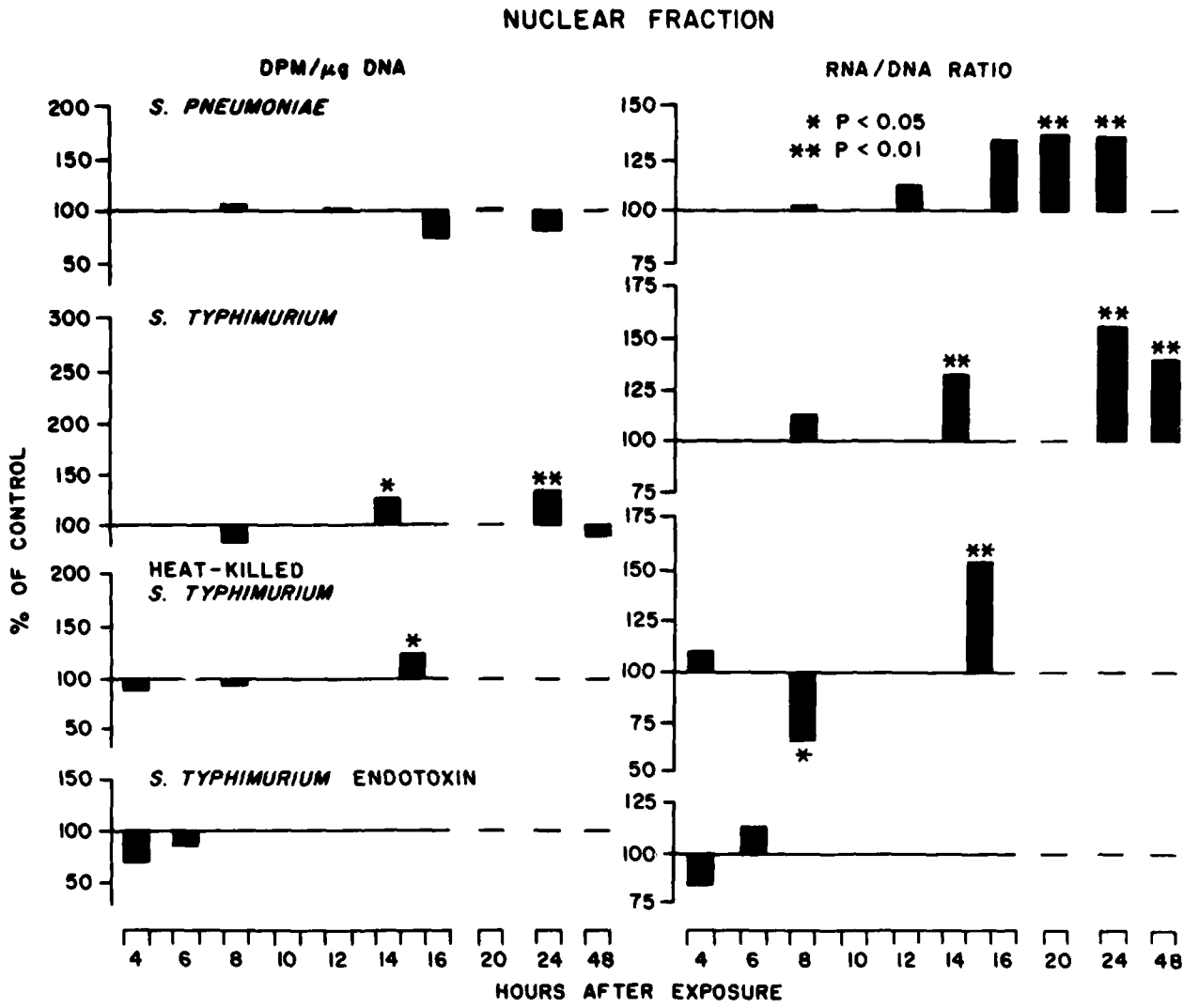

FIG. 3. Percent of control time-course profiles in nuclear fraction in response to 4 treatments studied. Incorporation of labeled orotic acid into RNA per microgram total cellular DNA shown on left. Quantity of RNA per total cellular DNA on right. Each time period represents separate experiment containing 6–8 rats in each control and treated group.

nificant increase at 16 h in the concentration of RNA in the bound ribosomes (Fig. 5). There was no significant change in the RNA content of the soluble fraction (Fig. 6). The *S. typhimurium* infection had no effect on free ribosomal RNA distribution and only a late-stage increase was observed in bound ribosomal RNA. The effects of endotoxin on hepatic RNA distribution demonstrated a very early increase (4 h) in RNA associated with the free ribosomes in response to both heat-killed *S. typhimurium* and its endotoxin. At the same time, an opposite effect was observed in the bound ribosomal RNA by decreasing to 70% of control values at 6 h. The soluble fraction also showed an early increase in RNA in response to the endotoxin (Fig. 6).

DISCUSSION

Liver cells may be classified into two groups: parenchymal cells or hepatocytes and sinusoidal cells, which are comprised almost entirely of Kupffer and endothelial cells (5). Although the sinusoidal cells represent almost one-third of the total liver cells, they occupy only 3% of the total liver volume and contain an even smaller percentage of the total liver RNA (5, 7). Due to the predominance of lysosomes in the Kupffer cells, it has been implied that they function mainly in the clearance of foreign material. Therefore, no attempt was made to separate these cell types, because the contribution to liver RNA activity by the sinusoidal cells is probably negligible in comparison to hepatocytes.

An effective procedure for the quantitative isolation of rat liver subfractions has been recently developed (9) and employed in this study to compare the hepatic RNA response to *S. pneumoniae* and *S. typhimurium* infections and to the endotoxin contained in gram-negative bacteria. Results from this study and previous observations (10) provide evidence that *S. pneumoniae* infection stimulates a gradual increase in the transcription of RNA

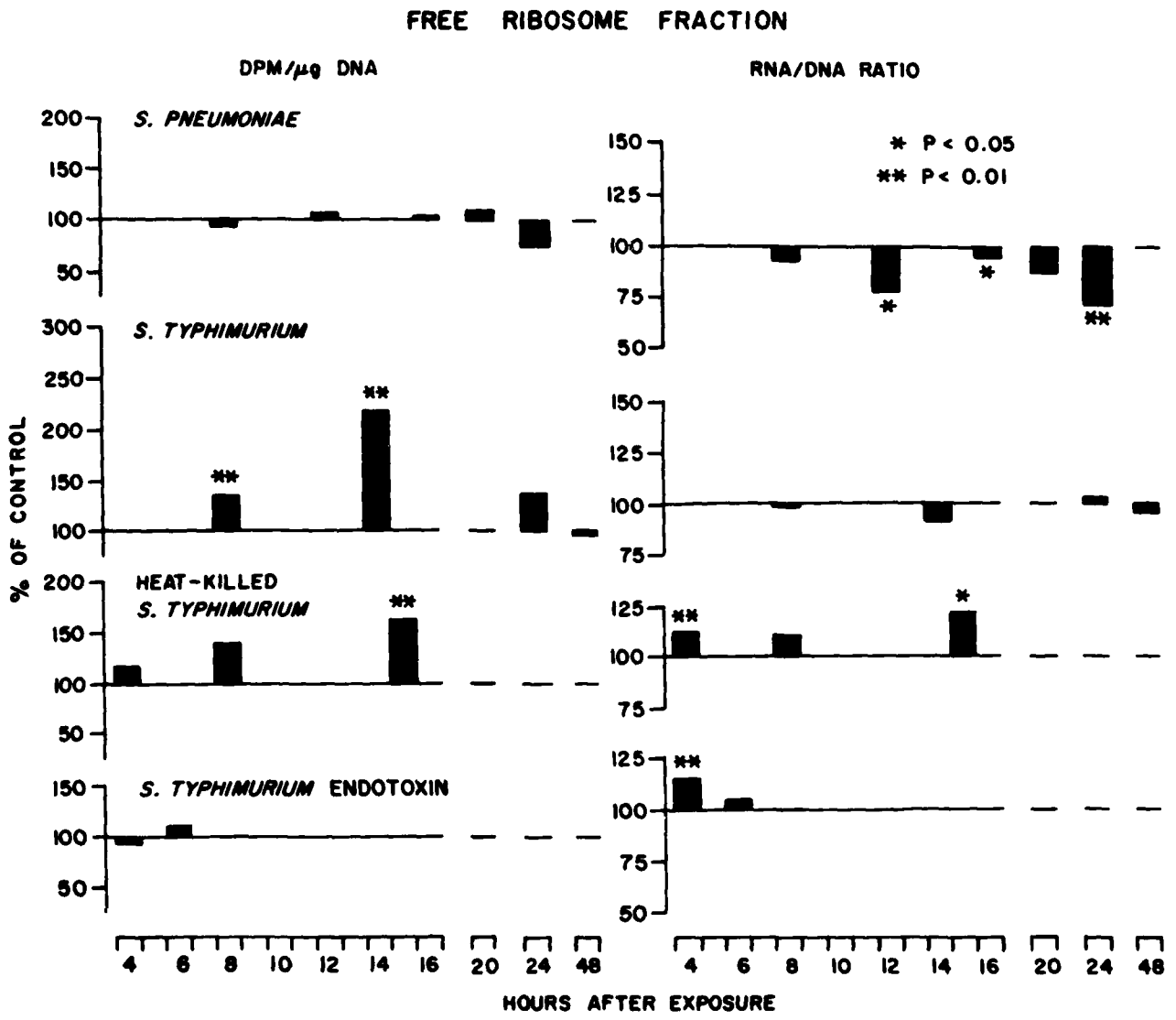

FIG. 4. Percent of control time-course profiles in free ribosomal fraction in response to 4 treatments studied. Incorporation of labeled orotic acid into RNA per microgram total cellular DNA shown on left. Quantity of RNA per total cellular DNA on right. Each time period represents separate experiment containing 6–8 rats in each control and treated group.

reaching a peak response around 16–20 h after inoculation of the liver bacteria. Because there was no significant change in RNA activity associated with the nuclear fraction, the newly formed RNA appears to be processed as quickly as it is made. A redistribution of cytoplasmic RNA from the free to the bound ribosomal fraction appears to take place at the peak of the RNA response. This fits well with the proposed function of the bound ribosomes in the production of extracellular proteins, because an increase in certain plasma proteins takes place during the same stage of the pneumococcal infection (8).

A similar response in hepatic RNA production was observed with the *S. typhimurium* infection, reaching a peak response around 14 h after inoculation of live bacteria. A notable exception was the response seen in the nuclear fraction. A buildup of newly formed RNA during the peak response along with an overall buildup of RNA during the entire course of the infection may be due to differences in the infectious model. The early and rapid enhancement of transcriptional rates in response to *S. typhimurium* infection might result in the production of RNA at a rate greater than it can be transported from the nucleus to the cytoplasm. Also, because endotoxin has been shown to enter parenchymal cells and become associated with both cytoplasmic and nuclear fractions (17), this toxic component of the bacteria may cause some impairment in transport of RNA from the nucleus to the cytoplasm. Although no significant depression in RNA associated with the free ribosomes was noted with *Salmonella* infection, the RNA content of the bound ribosomal fraction increased during the later stages of

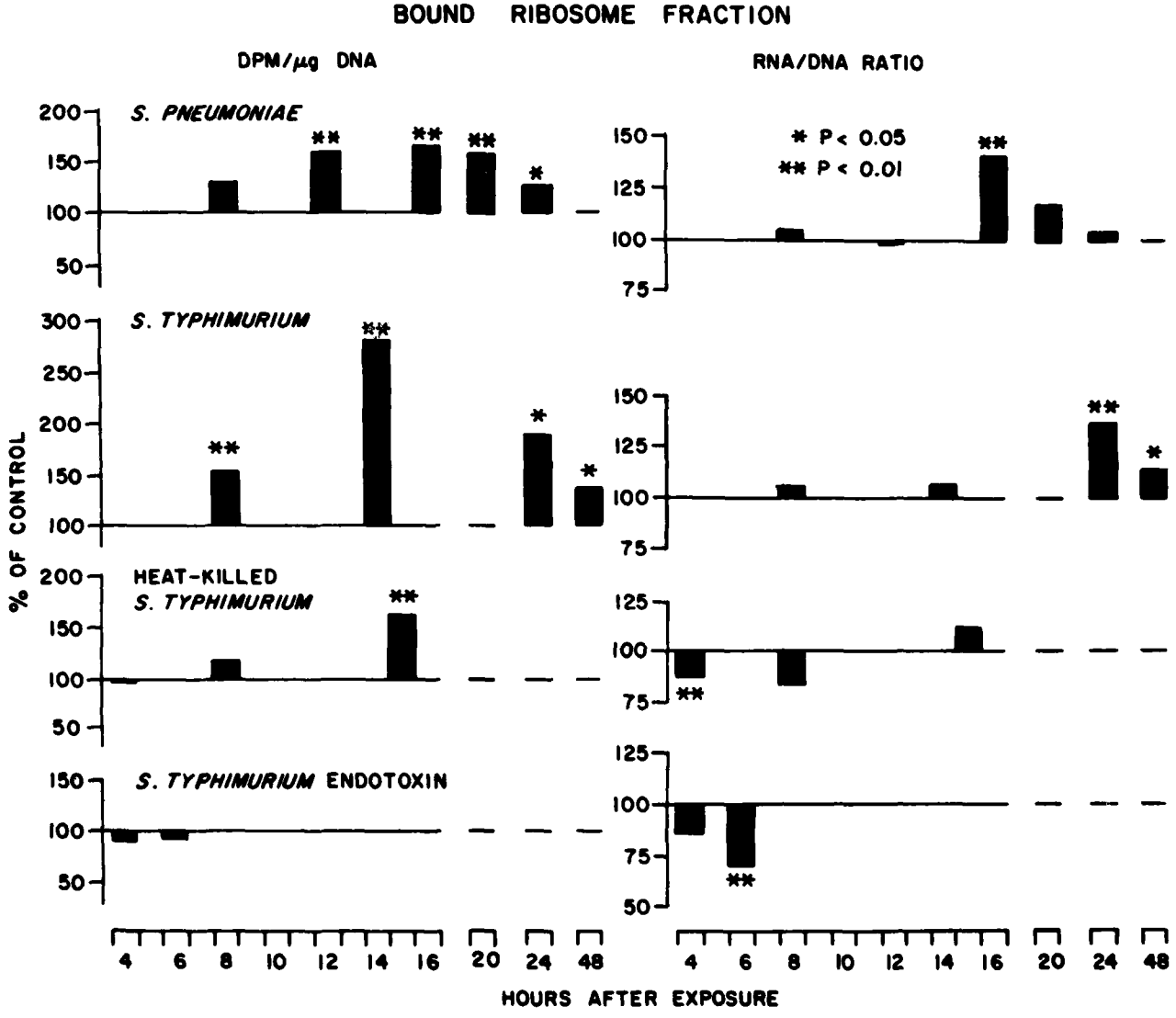

FIG. 5. Percent of control time-course profiles in bound ribosomal fraction in response to 4 treatments studied. Incorporation of labeled orotic acid into RNA per microgram total cellular DNA shown on left. Quantity of RNA per total cellular DNA on right. Each time period represents a separate experiment containing 6–8 rats in each control and treated group.

the infection along with an earlier increase in bound ribosomal radioactivity. This indicates once again a preferential movement of newly formed RNA to the bound ribosomes during the peak and later stages of the infectious response. The RNA response to both infectious models in the soluble fraction was what would be expected from a fraction containing support materials for the translation process or RNA in a transition state between the free or bound ribosomes. Significant increases in soluble RNA activity reflect the response seen in the bound ribosomes in the *S. pneumoniae* infection and in both free and bound ribosomes with the *S. typhimurium* infection.

The different pattern observed during the early stages of *S. typhimurium* infection appears to be due to endotoxin effects. A similar response was seen with both heat-killed *S. typhimurium* and the purified *S. typhimurium* endotoxin, although a lower concentration of endotoxin in the heat-killed preparation in comparison to the purified endotoxin resulted in a delayed or reduced effect in some of the fractions. This endotoxin response was associated with an earlier increase in RNA in the free ribosome fraction along with a decrease in the RNA content of the bound ribosome fraction. This was an opposite effect to that seen during the later "infectious" stage. The early decrease in RNA observed in the nuclear fraction in response to endotoxin treatment implies that at least a part of the RNA being redistributed in the cytoplasm was supplied by the rapid movement of preexisting RNA from the nucleus to the cytoplasm. The soluble fraction response again fits in well with this proposed theory. The early increase in RNA at 4 h,

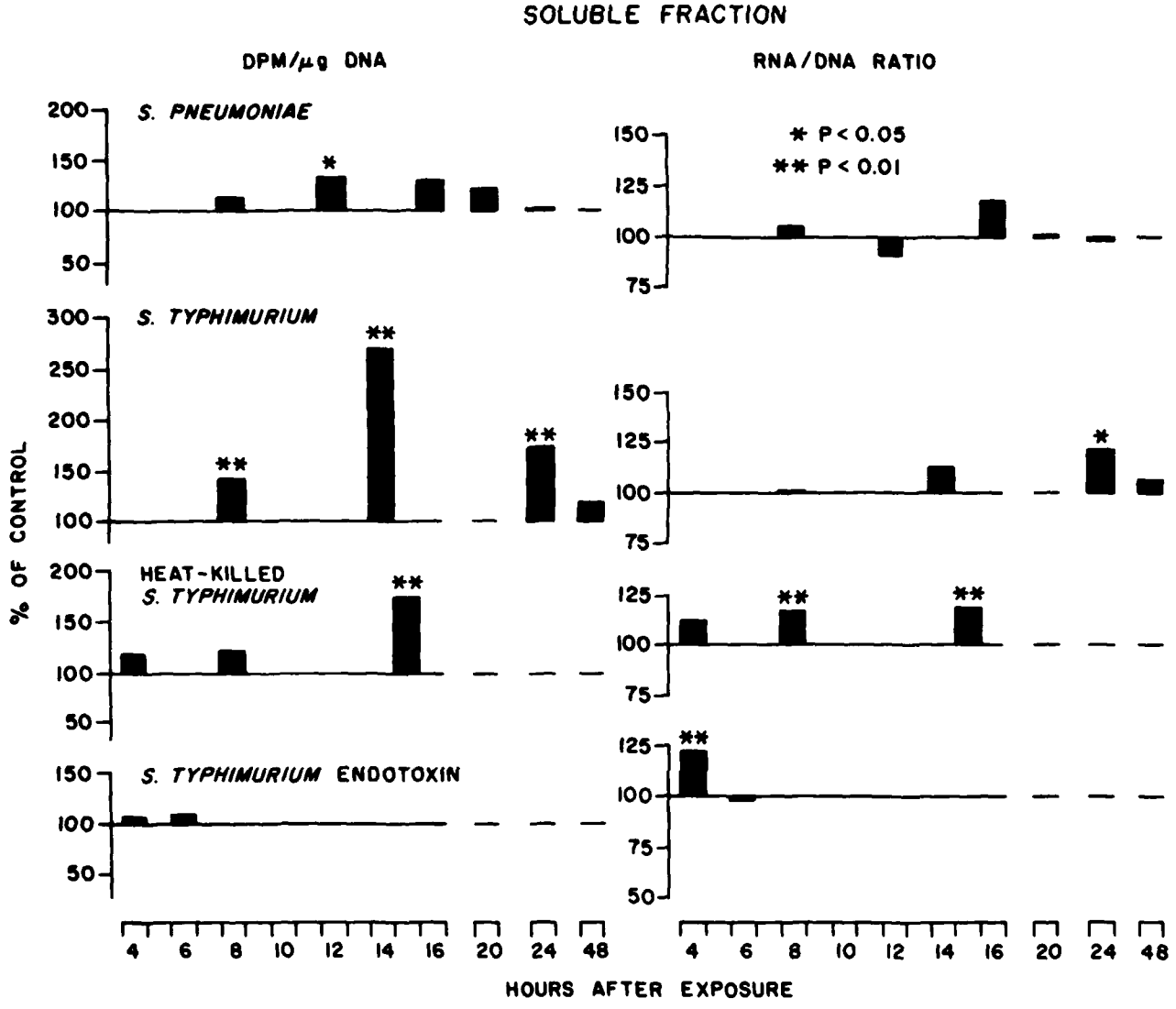

FIG. 6. Percent of control time-course profiles in soluble fraction in response to 4 treatments studied. Incorporation of labeled orotic acid into RNA per microgram total cellular DNA shown on left. Quantity of RNA per total cellular DNA on right. Each time period represents separate experiment containing 6–8 rats in each control and treated group.

probably due to release of RNA from the nucleus, is in this case in support of the free ribosomal response. These data along with information available in the literature on the mechanism of endotoxemia (17) provide strong support for a two-stage response in hepatic RNA regulation to an infection by endotoxin-containing bacteria.

The first stage, due to the early entry of endotoxin into the hepatic cells, causes a rapid movement of existing RNA from the nucleus to the cytoplasm and the mobilization of the cytoplasmic intracellular machinery for its defense. Many possibilities exist for regulatory mechanism(s) involved in the redistribution or mobilization of cytoplasmic RNA. This response may be due to a direct effect of the endotoxin, stimulation of soluble cytoplasmic regulatory factors, the presence of rapidly transcribed mRNA produced in response to the presence of endotoxin, or any of the above combinations. Future studies on translational rates of free and bound ribosomes and soluble cytoplasmic fractions from control and endotoxin-treated hepatic cells are required to elucidate the mechanism of action of endotoxin on hepatic RNA metabolism.

The second stage, which was similar in both infectious models studied, involves increased rates of RNA transcription and a subsequently greater involvement of the bound ribosomal fraction presumably for the increased production of specific acute-phase serum proteins. This latter stage appears to be a nonspecific response to inflammatory stimuli.

In conducting the research described in this report, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals*, as

promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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