INTRACELLULAR LOCALIZATION OF ENZYMES IN SPLEEN

II. Some Properties and the Distribution of Ribonuclease in Rat Spleen

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ABSTRACT

Some properties of rat spleen ribonuclease have been studied, and the intracellular distribution of the enzyme and ribonucleic acid have been presented. Spleen ribonuclease exhibits maximal activity at pH 5.8, and although there is some evidence for the presence of an enzyme with an optimum at pH 7.0, it is not conclusive. The enzyme is concentrated primarily in the mitochondrial fraction, but significant quantities occur in the supernatant fluid. The latter contains ribonuclease inhibitor similar to that found in liver. The effects of whole body x-irradiation, magnesium ion, substrate concentration, type of buffer, presence of *p*-chloromercuriphenylsulfonic acid, deoxycholate, and Triton X-100 on ribonuclease activity are examined.

In a previous paper from this laboratory (paper I of this series (1)), it was shown that the technique of separating liver homogenates into four subcellular fractions by differential centrifugation in sucrose could be adapted successfully to rat and guinea pig spleen homogenates, as judged by the localization of three respiratory enzymes—DPNH and succinic cytochrome *c* reductases and cytochrome *c* oxidase—and deoxyribonucleic acid (DNA). As a follow-up study, it was considered of interest to determine in spleen the intracellular distribution of the hydrolytic enzyme, ribonuclease (RNase), and to compare the results with those for rat liver RNase and the oxidative spleen enzymes. The properties of rat liver RNase have been studied extensively by several investigators (2–7), and the presence of at least two ribonucleic acid (RNA)-degrading enzymes, one optimally active at pH 5.8 (acid RNase) and the other optimally active at pH 7.8 (alkaline RNase), is well substantiated. In addition, the occurrence in the supernatant fraction of a RNase inhibitor and latent RNase activity has been reported (8–10). The distribution of RNases in rat liver has been determined (3, 4, 11), and the greater part of both acid and alkaline enzyme activity has been found to separate with the mitochondrial fraction. An alkaline RNase[1] appears actually to be associated with the mitochondria, while the acid RNase, as well as other acid hydrolases, is said to reside in lysosomes, particles which are morphologically distinct from mitochondria (12). Evidence for the presence of two acid RNases in rat liver has been reported (13). Fewer studies of spleen RNases have been made, the earliest being those of Maver and Greco, who described some properties of a calf spleen RNase (14, 15). This enzyme was also studied by Kaplan and Heppel (16), who reported that it closely resembled crystalline

[1] There is increasing evidence for the association of an alkaline RNase with ribosomes of rat liver (41, 42). Whether this is the same as, or similar to, the mitochondrial alkaline enzyme is not yet clear.

pancreatic RNase in its specificities and properties. Hilmoe and Heppel (17) suggested the presence of three RNases in calf spleen, but at least one was a non-specific phosphodiesterase. De Lamirande and Allard (6) have investigated some of the characteristics of RNase activity in rat spleen and reported an alkaline to acid RNase ratio of 4.3/1 in this tissue. The intracellular distribution of RNase activity in the spleens of control and x-irradiated rats, both fasted for 16 to 64 hours, has been described (18).

Since it could not be assumed that the spleen and liver RNases have the same properties, and since, to our knowledge, no detailed study of rat spleen RNase has appeared, it was essential first to survey the properties of this enzyme in spleen. This report describes these properties, some factors affecting spleen RNase activity, and the intracellular localization of the enzyme.

METHODS

Preparation and Fractionation of Spleen Homogenates

Homogenization of the spleens and separation of the four subcellular fractions were carried out according to the procedures previously described (1). Male Wistar rats (Charles River Labs, Brookline, Massachusetts), weighing 200 to 400 gm and fed Purina fox chow checkers, were used, except where indicated otherwise.

Ribonuclease Assay

One milliliter of a 1 per cent solution of commercial RNA (Schwarz BioResearch, Inc., Mount Vernon, New York), adjusted to pH 7.0 with dilute NaOH, was added at zero time to each of two test tubes containing 1 ml of buffer (as indicated below), 0.05 to 0.1 ml of a 10 per cent tissue homogenate or derived fraction, and sufficient water to bring the final volume to 3 ml. The reaction mixture was incubated at 37°C for 30 or 60 minutes. Three milliliters of 5 per cent trichloroacetic acid (TCA) containing 0.5 per cent aluminum chloride was then added. The mixture was allowed to stand for 5 minutes and was then filtered through circles of Whatman No. 42 filter paper 9 cm in diameter. One milliliter of the clear filtrate was diluted to 50 ml with water and the absorbancy read at 260 mμ in the Beckman DU spectrophotometer. A blank, without enzyme, and tissue blanks, without RNA, were set up with each experiment, as well as a standard containing 0.015 μg of crystalline pancreatic RNase. The absorbancy increment of the standard was 0.100 to 0.110 under the above conditions, and the activity was linear from 0.001 to 0.05 μg of pancreatic RNase. The range of linearity for tissue homogenates was considerably less. The standard deviation of the method was ± 5 per cent on duplicate determinations of the same sample. Modifications in the standard assay procedure are given in connection with individual experiments. Nitrogen determinations were carried out by a micro-Kjeldahl procedure.

RESULTS

Distribution of RNA in Subcellular Fractions Isolated from Rat Spleen

Before studying the properties and distribution of RNase in the four subcellular fractions, it was considered important to characterize further the fraction which was separated at 110,800 *g* as being primarily microsomal. Previously, it was demonstrated by Eichel (1) that this fraction contained 40 per cent of the DPNH cytochrome *c* reductase activity, minor amounts of cytochrome *c* oxidase and succinic cytochrome *c* reductase activity, and no DNA, properties which are similar to those of rat liver microsomes. Ninety-one per cent of the DNA was recovered in a nuclear fraction (1) isolated by the sucrose-$CaCl_2$ "layering" technique (19). One of the most striking properties of microsomes is a high concentration of RNA; at least 50 per cent of the RNA of whole rat or mouse liver is present in this fraction, and microsomes comprise the only liver fraction in which RNA is concentrated in terms of total N (20). Although two other laboratories have reported that spleen microsomes are also rich in RNA, the data were only fragmentary and obtained with the mouse (see below).

Rats (180 to 340 gm) bred in the Wistar Institute were sacrificed by ether anesthesia. After excision, the spleens were washed free of adhering blood and homogenized in cold 0.25 M sucrose with a Ten Broeck tissue homogenizer. Two spleens were pooled in each of four experiments and single spleens were analyzed in two others. RNA was extracted from the homogenates and all fractions according to Schneider (21) and estimated by the orcinol test (22). In Table I are presented the results of the distribution of RNA in the four fractions isolated from rat spleen homogenates. In order to express the RNA concentration in terms of N, analyses of the latter component were performed on the same preparation. For comparison, the RNA distribution found in rat liver by Schneider (23) is also given in

Table I. It can be seen that 41.0 per cent of the total spleen RNA was recovered in the microsomes, only 9.5 per cent in the mitochondria, and 20.4 per cent in the supernatant fluid. These figures are in excellent agreement with those obtained in liver. The presence of more than twice as much RNA in the nuclear fraction of spleen as in the corresponding fraction of liver parallels previous findings in the nuclear fractions of these two tissues with respect to N content and DPNH cytochrome *c* reductase activity (1). Total reductase and total N recovered in spleen nuclei were greater than those found in liver nuclei by a factor of at least 2. It is probable, as has been contended elsewhere in regard to the localization of respiratory enzymes in spleen (1), that a large part of the RNA recovered in spleen nuclear fractions is due to contamination with unbroken cells and possibly adsorbed microsomes. This idea is supported by the results in Table I, which show that the spleen microsomal fraction is the only one in which RNA is concentrated in terms of total N, the average RNA/N ratio being 692/1, as compared with 278/1 for the whole homogenate. The RNA/N ratios of spleen are generally in excellent agreement with those of liver. The content of RNA in whole spleen in terms of micrograms per milligram wet tissue averaged 7.56 for six different preparations involving ten spleens (not tabulated); this compares very well with an average of 7.70 recalculated from the RNA-P data listed by Leslie (24). Maxwell and Ashwell (25), in an analysis of fractions isolated from a single normal mouse spleen homogenate in 0.44 M sucrose, also reported a high RNA level in the microsomes. Finally, the work of Petermann *et al.* (26) on the distribution of RNA in four normal mouse spleen homogenates is somewhat difficult to interpret because of (*a*) the low centrifugal force used for sedimentation of the microsomal fraction and (*b*) the low recoveries of RNA in several instances. However, their RNA/N ratios do indicate that RNA is concentrated in the microsomes.

TABLE I

Comparison between Data of Schneider and This Report on the Distribution of RNA in Subcellular Fractions of Liver and Spleen, Respectively

	Schneider, rat liver‡		This report, rat spleen§	
Fraction*	% of total homogenate RNA	μg RNA per mg N	% of total homogenate RNA	μg RNA per mg N
H	(100)	290	(100)	278 (296–254)
N	13.8	294	30.0 (35.5–25.0)	228 (288–185)
M	7.2	79	9.5 (14.1–6.4)	252 (348–196)
P	52.6	798	41.0 (45.6–30.0)	692 (756–560)
S	23.2	160	20.4 (29.9–16.5)	152 (217–125)
Recovery	96.8		100.9 (109.8–93.7)	

* H, homogenate; N, nuclei; M, mitochondria; P, microsomes; S, supernatant fluid.

‡ The figures represent the average values of four experiments in which Sprague-Dawley rats were used. Extraction and determination of RNA were carried out as indicated for spleen in the text. Original data of Schneider (23) were recalculated from RNA-P by assuming an average P content of 9.4 per cent in RNA.

§ The figures represent the average values and ranges of six experiments.

Effect of pH on RNase Activity of Rat Spleen Homogenate and Fractions

The homogenate and each of the separated fractions were assayed over a range of pH values with different buffers to obtain an indication of the presence of one or more RNases and to determine the optimum pH for enzyme activity. Fig. 1 A, B, and C illustrates the effect of pH on the RNase activity of normal spleen homogenate using either 0.02 M veronal-acetate (VA) (27), 0.067 M acetate-borate-cacodylate (ABC) (28), or a combination of 0.033 M tris(hydroxymethyl)-aminomethane (Tris) and 0.033 M succinate buffers, respectively. With both VA and ABC buffers, two peaks of activity were observed, one occurring at pH 5.8 and the other at pH 7.0 to 7.3, suggesting the possible presence of an acid

and an alkaline RNase. The enzyme activity in the alkaline range is optimal at a pH considerably lower than the pH optimum of liver alkaline RNase, a fact already reported for calf spleen alkaline RNase (16). It is of interest to note that with the succinate and Tris buffers, only one peak of activity was found (pH 5.8) with spleen homogenate, but Tris may exert an inhibiting effect on the alkaline activity. With separated spleen microsomes, using ABC buffer, a single sharp peak of activity at pH 6.5 was obtained (Fig. 1 *D*).

Fig. 2 *A* and *B* shows the effect of VA and ABC buffers on the RNase activity of spleen mitochondria. A single broad pH optimum was observed ranging from 5.7 to 7.4 in the case of VA buffer, and 5.3 to 6.3 with ABC buffer. This is in contrast to rat liver mitochondria, which exhibit distinct peaks of activity, one at pH 5.8 to 6.0 and the other at pH 7.8 (2). Supernatant fraction showed a sharp activity peak at pH 5.3 with ABC buffer (Fig. 2 *C*) and pH 5.7 with VA buffer (Fig. 2 *D*), indicating that the active enzyme in this fraction is of the nature of an acid RNase.

The pH-activity curve of the nuclear fraction of

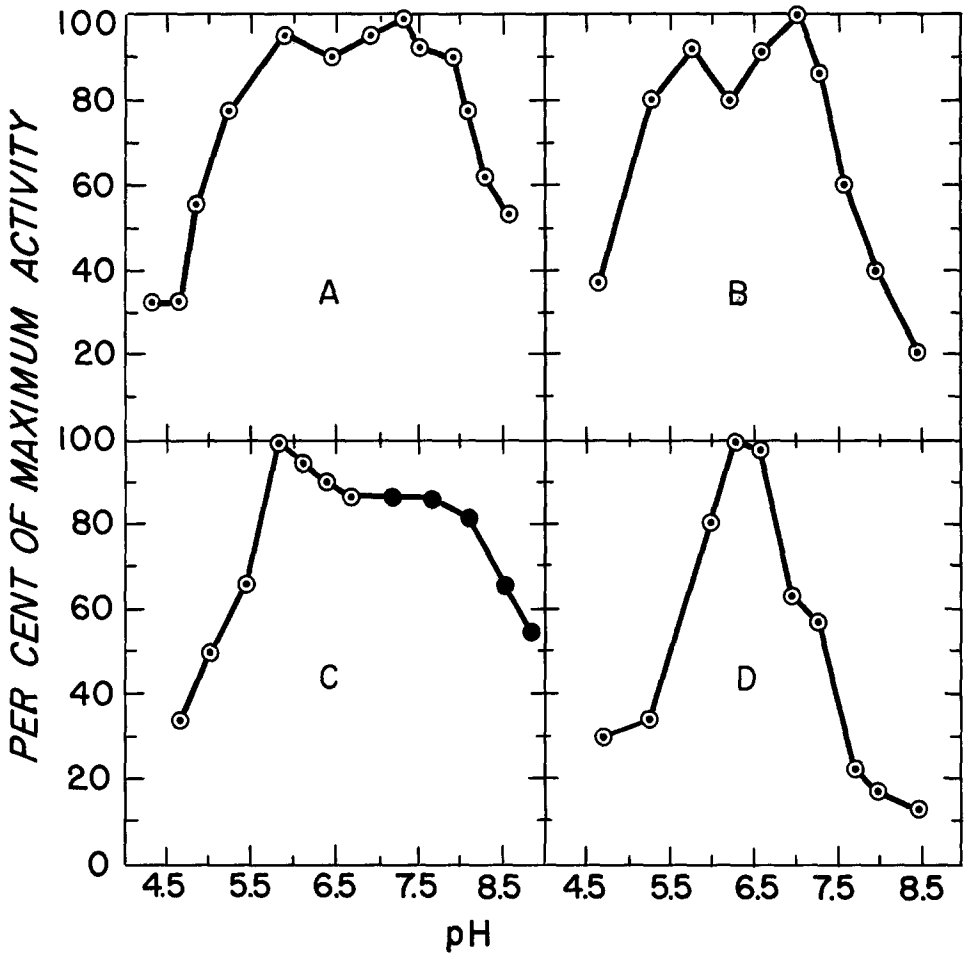

FIGURE 1

The effect of pH on RNase activity of rat spleen homogenate and microsomes. *A*, homogenate, veronal-acetate buffer; *B*, homogenate, ABC buffer; *C*, homogenate, Tris and succinate buffers (○——○ succinate buffer, ●——● Tris buffer); *D*, microsomes, ABC buffer.

spleen is illustrated in Fig. 3 *A* using ABC buffer. Two peaks of activity were obtained. This fraction is quite impure (although twice washed) and probably contains many whole and fragmented cells as well as a considerable number of mitochondria. The mitochondrial contamination has been shown to be markedly diminished (1), as judged by cytochrome *c* oxidase and DPNH cytochrome *c* reductase assays, by isolating nuclei from homogenates using the sucrose-$CaCl_2$ "layering" technique (19). With such a "purified" nuclear preparation, the pH 7.0 peak was much reduced (Fig. 3 *B*), indicating that a considerable amount of the pH 7.0 enzyme was removed with the contaminating material. When VA buffer was used, this contaminating peak was almost absent (Fig. 3 *C*). Only one major peak, at pH 6.3, is visible; however, the presence of a small amount of contaminating pH 7.0 enzyme is suggested by the slight bulge in the curve at this point.

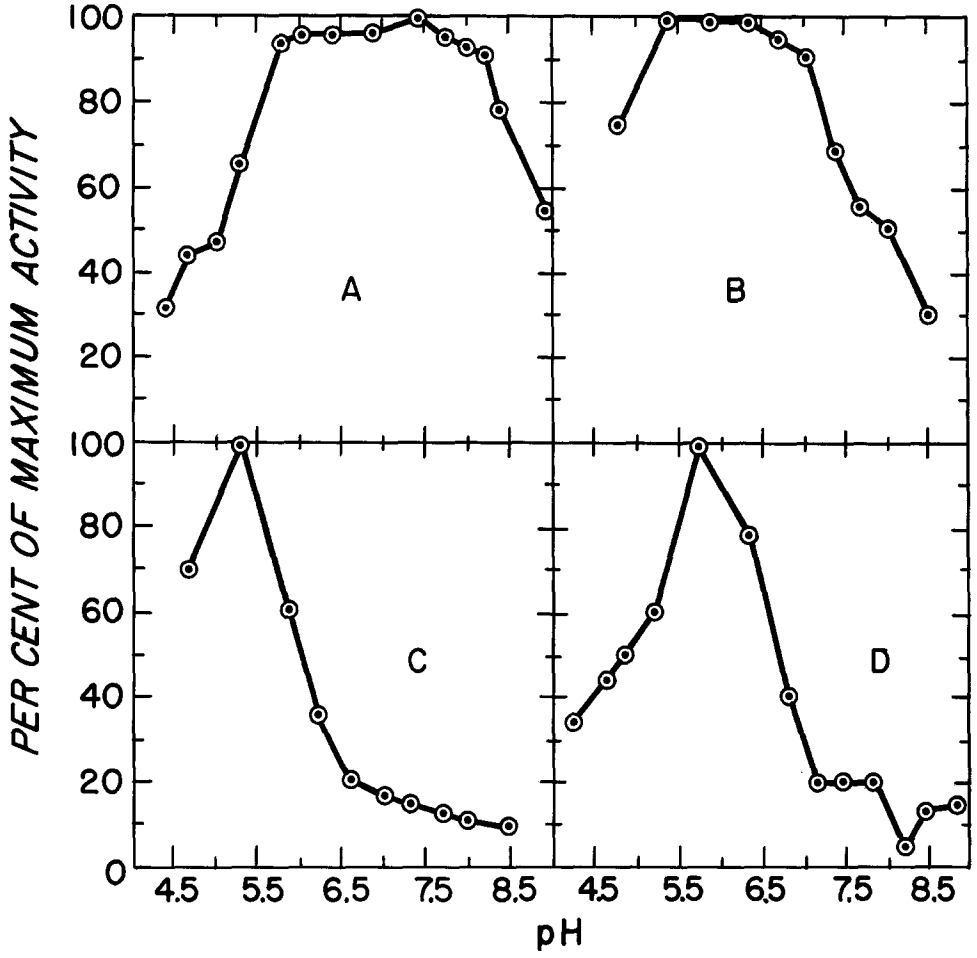

FIGURE 2

The effect of pH on RNase activity of rat spleen mitochondria and supernatant fraction. *A*, mitochondria, veronal-acetate buffer; *B*, mitochondria, ABC buffer; *C*, supernatant fraction, ABC buffer; *D*, supernatant fraction, veronal-acetate buffer.

To determine whether a spleen RNase inhibitor might be repressing activity at alkaline pH values, RNase assays were carried out on spleen homogenate and supernatant fraction in the presence of *p*-chloromercuriphenylsulfonic acid (CMS) over a range of pH's using ABC buffer. In all, four experiments were run and similar results were obtained. A typical one is illustrated in Fig. 4 *A*,

which shows that 4 × 10^{-4} M CMS increased RNase activity in spleen homogenate from pH 4.5 to 8.5, but the per cent increase rose from 24 at the lowest pH to 258 at the highest pH, indicating clearly the increased effectiveness of the inhibitor in the alkaline pH range. The shape of the curve in Fig. 4 *A* does not confirm the presence of an alkaline RNase, since the RNase inhibitor may simply be repressing the activity of a single enzyme more effectively in the alkaline pH range. In separate experiments, it was determined that CMS, in a final concentration of 1.6 to 4 × 10^{-4} M, gave the maximum increase in the RNase activity of 0.1 to 0.2 ml of a 10 per cent spleen homogenate.

The RNase activity of spleen supernatant fraction, with and without the addition of CMS, is illustrated in Fig. 4 *B*. This curve gives better evidence for the existence of an alkaline RNase in the supernatant fraction, for in this case the outlines of a second peak are evident at pH 7.0. The increase in RNase activity in the supernatant fraction upon addition of CMS is more striking than that observed in whole homogenate and

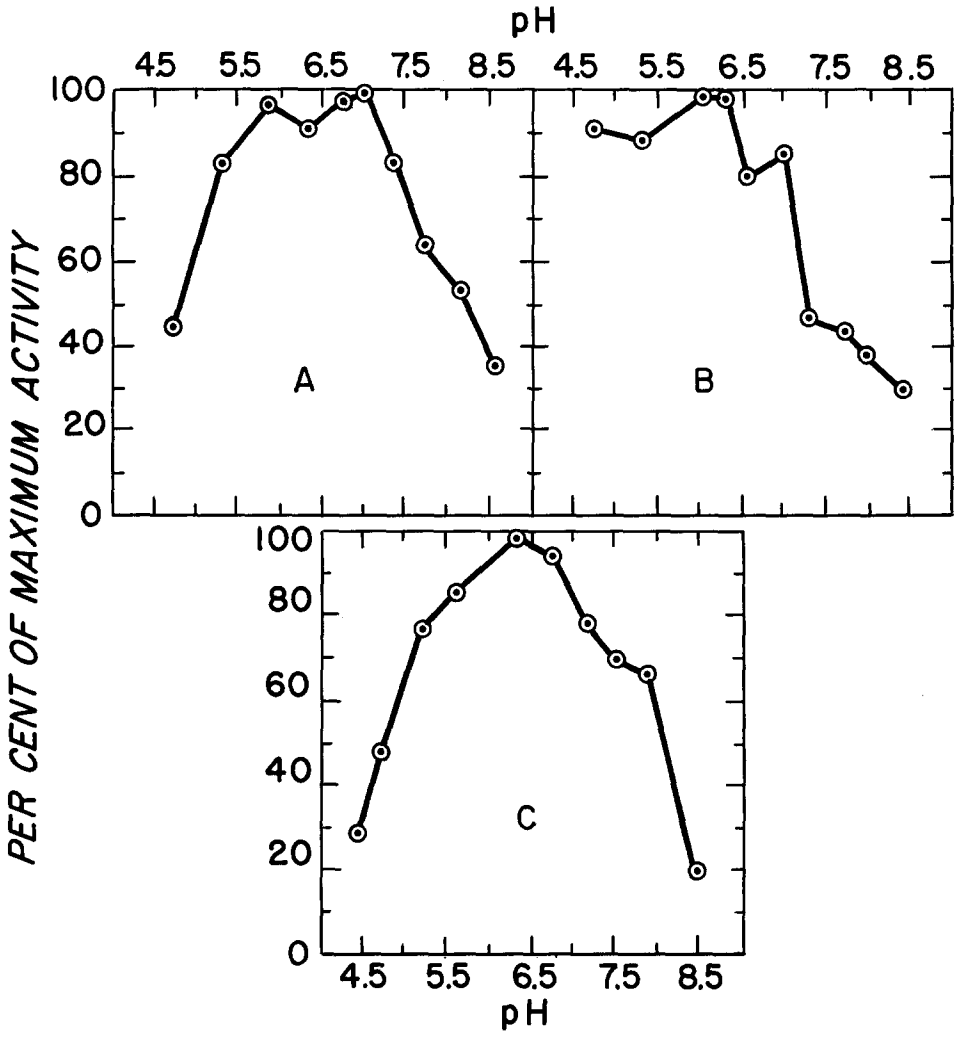

FIGURE 3

The effect of pH on RNase activity of the nuclear fraction of rat spleen. *A*, nuclei, ABC buffer; *B*, "purified" nuclei, ABC buffer; *C*, "purified" nuclei, veronal-acetate buffer.

also occurs over the entire pH range measured, although it is greatest above pH 6.0. The increase is especially dramatic from pH 7.0 to 8.5, since, without CMS, spleen supernatant fraction usually exhibits little or no activity at these pH values.

It is of interest to note that if the supernatant fraction is heated at 80°C (pH 6.5 to 6.7) for 5 minutes, its RNase activity is found to be 95 to 100 per cent destroyed when assayed over the entire pH range in the absence or presence of CMS. Thus, the spleen enzyme(s) appears to be different from latent rat liver RNase, which was only slightly affected by heating the supernatant fraction at 75°C at pH 5.0 (10). Acid RNases are heat-labile, in general. Further studies on the effect of heating on rat spleen RNase activity are described below.

The above observations, considered in their entirety, do not present a good case for the existence of an alkaline RNase in rat spleen. It is possible that the activity peak observed with whole homogenate at pH 7.0 to 7.3 may be explained in other ways. For example, it is well known that the relationships among RNases and RNase inhibitor are complicated (8, 9) and that the optimum pH of crystalline pancreatic RNase

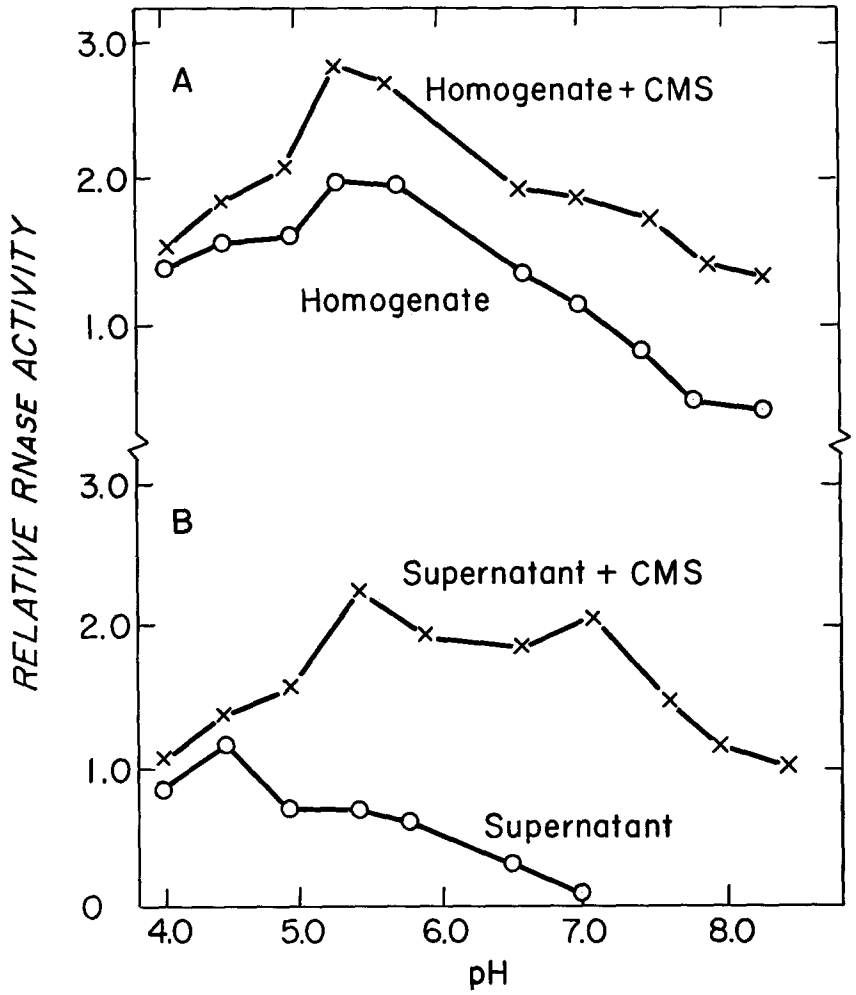

FIGURE 4

The effect of *p*-chloromercuriphenylsulfonic acid (CMS) on RNase activity. *A*, rat spleen homogenate; *B*, rat spleen supernatant fraction.

may vary widely depending on the buffer and, particularly, on changes in ionic strength and protein concentration (29, 30).

As mentioned in the introductory paragraph above, de Lamirande and Allard (6) reported an alkaline/acid RNase ratio of 4.3/1 in rat spleen homogenate, whereas in this paper the ratio is close to unity (Fig. 1 A or 1 B). These workers also found in liver a higher alkaline/acid RNase ratio than that reported by Roth (2) and by Maver and Greco (14). De Lamirande and Allard (6) suggested that the apparent discrepancies in the case of liver RNase ratios may be explained by the fact that different precipitants and substrates were used by different workers. To check this, spleen homogenate was assayed with three different buffers at pH 5.8 or 7.8, using both 5 per cent TCA plus 0.5 per cent aluminum chloride (the precipitant used in this report) and 2 per cent $HClO_4$ (the reagent used by de Lamirande and Allard) and centrifuging the precipitated reaction mixtures in the cold instead of filtering. The results with use of both precipitants were essentially the same. Furthermore, a wide variation in the conditions of a large number of experiments failed to give ratios greater than 1/1 for activity measured at pH 7.8 and 5.8. When RNase was assayed with 0.1 per cent RNA (final) in the presence of 0.01 M Mg^{++} at pH 5.8 and with very dilute buffers—conditions used by de Lamirande *et al.* (4)—the ratio of alkaline to acid RNase activity was not significantly affected. When the effect of 0.01 M Mg^{++} on the RNase activity of spleen homogenate was determined over a range of pH values with ABC buffer, it was found to increase activity 30 to 40 per cent from pH 4.5 to 5.3, while from pH 5.8 to 7.0 it decreased the activity by a similar amount. This action of Mg^{++} would, of course, tend to decrease the ratio of alkaline to acid RNase. It should be noted, too, that the data of Maver and Greco (14) for bovine spleen RNase also indicate little alkaline RNase activity. In connection with the discrepancy in the spleen and liver ratios, it may be of interest to note some recent results obtained in this laboratory using two different RNA samples (31). With dialyzed Schwarz RNA, alkaline/acid RNase ratios of about 0.3/1 and 0.5 to 1/1 were found in homogenates of rat liver and adipose tissue, respectively; with RNA purchased from Pabst Laboratories, and later dialyzed, the ratio was about 1.8/1 in both tissues. Further, it was demonstrated that the Pabst RNA preparation contains a substance (metal) which inactivates the RNase inhibitor found in the supernatant fraction of liver and adipose tissue (31). It would appear that a high alkaline RNase level results from the destruction of RNase inhibitor and the subsequent release of bound alkaline RNase activity.

Effect of Different Buffers on RNase Activity of Rat Spleen Homogenates and Fractions

Since the highest RNase activities in most of the separated fractions were generally observed at or near pH 5.8, this pH was employed for the studies described in this section. The effect of various buffers was determined by preparing homogenates and subcellular fractions and assaying for RNase activity in the presence of the buffers at pH 5.8. RNase activity of the homogenate and fractions was, in most instances, linear for periods up to 1 hour. Examination of the data given in Table II indicates that there is considerable variation in activity depending on the buffer used. This has been noted for rat liver RNase (3), as well. It is also of interest to note that although methylarsenate gives consistently the highest RNase activities with crystalline pancreatic RNase (30) and some intracellular RNases (14, 30), it gives poor results with spleen mitochondria. Since the use of ABC or VA buffer generally resulted in the highest RNase activities, these buffers were used in most of the experiments summarized below.

Stability of RNase Activity of Rat Spleen Homogenate to Storage (0°C) and Heating

During a study of the effect of x-irradiation on the distribution of rat spleen RNase, it had been noted in two experiments that the RNase activity in control homogenates increased to the extent of 40 to 45 per cent on standing for 6 hours at 0°C, but there was little increase in the first 2 hours. To study this phenomenon more carefully, four spleens were homogenized individually, and the RNase activity of each was assayed immediately and 1, 2, 4, and 24 hours later. Although some changes occurred, no important trends were noted and the activity at 4 and 24 hours was approximately the same as that measured initially. In addition, two experiments were carried out in which rat spleen homogenate and each subcellular

fraction were assayed for RNase activity at 0, 2, 4, 6, and 24 hours after preparation. In one experiment, homogenate activity increased 30 per cent at the end of 6 hours but no significant changes were observed in any of the fractions. In the second experiment, no changes were noted in the homogenate or any of the fractions. It appears that the increase in RNase activity that has been observed in whole homogenate is a variable phenomenon. In making assays over a long time interval, it would be advisable, therefore, to redetermine the activity of the control samples at the same time as the experimentals. Destruction of the RNase inhibitor in the homogenate might be expected to result in an increase in measured RNase activity.

The effect on RNase activity resulting from the heating of homogenate and mitochondria at various temperatures for 5 minutes is shown in Fig. 5; the activities are calculated as percentages of the activities of samples heated at 37°C. In this experiment, the homogenate and mitochondria were adjusted to pH 7.8 with dilute NaOH before heating, but similar curves were obtained when the preparations were adjusted to pH 5.8 before heating. It can be seen that the enzyme is almost completely inactivated at 80°C. This is in contrast to the results obtained with a partially purified alkaline RNase of rat liver mitochondria (3), which is stable at least to 70°C under these conditions. Calf spleen alkaline RNase has been reported to be heat-labile (32).

Effect of Triton X-100 and Sodium Deoxycholate on RNase Activity of Rat Spleen Homogenate and Mitochondria

When 0.1 ml of a 10 per cent rat spleen homogenate was treated with concentrations of Triton X-100 ranging from 0.03 to 0.3 per cent, no significant change in RNase activity was noted. When similar concentrations of sodium deoxycholate were utilized, there was a large decline in RNase activity; complete inhibition was observed above a concentration of 0.25 per cent. With mitochondria, Triton X-100 had no effect at the lowest concentration but led to increased RNase activity (20 to 25 per cent) at the highest concentration used. On the other hand, mitochondrial RNase activity was completely inhibited by concentrations of deoxycholate greater than 0.067 per cent. The small increase in activity observed with Triton-treated mitochondria may be due to a true activation of enzyme activity rather than to a rupturing of lysosomes by the Triton, since the assay procedure (30 or 60 minutes at 37°C in

TABLE II

Effect of Various Buffers on the Ribonuclease Activity of Homogenates and Subcellular Fractions of Rat Spleen

Each value is the average of two experiments and represents the relative activity. RNase activity was assayed by measuring acid-soluble P^{32} split from uniformly labeled P^{32} yeast RNA as described previously (43). The composition of the reaction mixture for this assay was identical with that of the standard RNase assay given above under Methods, with the exception that a labeled RNA was employed. All buffers were pH 5.8 and were present in the following final concentrations: VA, 0.02 M; ABC, 0.067 M; succinate, 0.033 M; methylarsenate, 0.033 M.

Fraction	Series 1*			Series 2‡		
	VA	ABC	Succinate	VA	ABC	Methyl-arsenate
Homogenate	857	1112	784	660	930	600
Nuclei	817	574	510			
Mitochondria	1680	1604	1915	1860	1140	540
Microsomes	176	236	75			
Supernatant	1325	745	912			

* Incubated 30 minutes at 37°C.

‡ Incubated 60 minutes at 37°C.

water) probably results in complete disintegration of lysosomes and thus allows a full expression of the RNase activity (12).

Effect of RNA Concentration on RNase Activity of Rat Spleen Homogenate and Mitochondria

In general, no significant differences in RNase activity were observed with final concentrations of RNA from 0.07 to 0.33 per cent. The proportionality of RNase activity with increasing amounts of spleen homogenate and mitochondria at different RNA concentrations was also examined, since, in some cases, poor proportionality was observed previously at higher enzyme concentrations when 0.33 per cent RNA was employed (18). Usually, good proportionality was obtained with 0.17, 0.33, or 0.66 per cent RNA when the amount of 10 per cent homogenate was varied between 0.025 and 0.1 ml and incubated 1 hour; however, the highest RNA concentration resulted in somewhat greater activity. Mitochondria, under similar conditions, gave less satisfactory proportionality, but, again, 0.66 per cent RNA caused somewhat higher activities. The optimum conditions for assay appear to be 0.02 M VA buffer, pH 5.8; 0.66 per cent RNA (although 0.66 per cent gave higher activities, 0.33 per cent was used in this report since many experiments had already been run at this concentration); 0.025 to 0.1 ml of 1:10 spleen homogenate in sucrose; and 1 hour incubation at 37°C.

Effect of X-Radiation on the pH-Activity Curve of RNase of Rat Spleen Homogenate

Modification of the pH-activity relationship of acid deoxyribonuclease of spleen has been reported in rats given 850 r of total-body x-irradiation (33). Besides the normal deoxyribonuclease optimum at pH 5.15, there appeared a second peak at pH 4.8. Although this qualitative change is believed to be an indirect, and not a specific, effect of the ionizing radiations (34), it was considered of interest to determine whether irradiation has a similar effect on spleen RNase activity. Rats were given 700 r of whole-body x-irradiation, and 16 hours later the spleens were removed, homogenized, and assayed for RNase activity. Controls were unirradiated rats treated similarly. With ABC buffer, an increase in RNase activity was noted at pH values below 6.0 (although the maximum increase was somewhat less than that previously reported (18)), but no change in the pH optimum was noted. With VA buffer, a shift of approximately one-half unit to a more acid pH optimum was observed. It should be pointed out that the controls here may exhibit some variability in pH-activity curves, and thus a

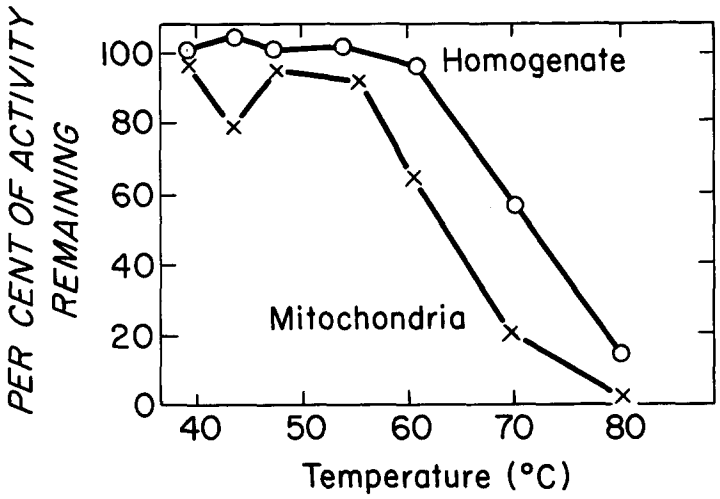

FIGURE 5

Effect on RNase activity of heating rat spleen homogenate and mitochondria at various temperatures for 5 minutes.

large series of animals would have to be used to obtain statistically significant results.

RNase Inhibitor in Rat Spleen

Some indication as to the amount of RNase inhibitor present in rat spleen supernatant fraction was obtained by adding increasing quantities of crystalline pancreatic RNase to 0.5 ml of rat spleen supernatant fraction. Under these conditions, 100 per cent inhibition of 0.06 μg of crystalline pancreatic RNase, 91 per cent inhibition of 0.1 μg, and 67 per cent inhibition of 0.2 μg was obtained. The amount of inhibitor present in spleen supernatant fraction is, therefore, of the same order as that present in rat liver supernatant fraction (8).

Distribution of RNase Activity in Subcellular Fractions Isolated from Rat Spleen

The distribution of RNase activity in subcellular fractions of rat spleen has been reported in normal rats fasted 16 to 64 hours (18). Since fasting is known to have a profound effect on enzyme activities (35), it was deemed advisable to repeat these studies on normal, fed animals. The results obtained are given in Table III, which gives the averages and ranges for five complete fractionation experiments. The per cent recovery of total RNase activity in each fraction was obtained in the following way: A value of 100 per cent was assigned to the sum of the recoveries of the four fractions, and the total RNase activity of each fraction was expressed as a percentage of this. The distribution of nitrogen in each fraction was determined, but is not given since it was almost identical with the control values previously reported (18). It can be seen that about half of the total RNase activity is recovered in the mitochondrial fraction and that the enzyme is concentrated to a considerable extent in this fraction when its specific activity is compared to that of the homogenate. Comparison of these results with those previously obtained for the fasted animals (18) indicates that fed rats have more RNase activity in the mitochondrial fraction and less in the microsomal and supernatant fractions. The RNase specific activity of the spleen homogenate of the fed animals was lower than that of the fasted rats, and this was reflected in a lower specific activity in the microsomal and supernatant fractions; the nuclear and mitochondrial specific activities in fed animals were not significantly different from those in the fasted rats.

The intracellular localization of RNase activity in spleens from normal rats, as illustrated in Table III, is similar to that observed in liver. In

TABLE III

Specific and Total Activities of Ribonuclease in Subcellular Fractions Prepared from Rat Spleen Homogenates

The numbers represent the average values and ranges of five experiments. In three experiments, 0.067 M ABC buffer, pH 5.8, was used; in one experiment, 0.02 M VA buffer, pH 5.72, was used; and in one experiment, 0.033 M methylarsenate buffer, pH 5.8, was used. One unit = Δ absorbancy of 0.001 at 260 mμ in 30 minutes measured under the conditions given above under Methods.

Fraction	RNase activity	
	Specific (units × 10^{-3} per mg N)	Total %
Homogenate	0.15 (0.07–0.21)	(100)
Nuclei	0.15 (0.07–0.16)	16.8 (12.9–19.9)
Mitochondria	1.13 (0.71–1.70)	47.4 (35.3–64.0)
Microsomes	0.32 (0.17–0.41)	12.4 (4.8–17.7)
Supernatant	0.22 (0.11–0.30)	23.4 (11.9–40.1)

this tissue, de Lamirande and coworkers have reported (4) that the nuclear, mitochondrial, microsomal, and supernatant fractions contain 14, 47, 19, and 17 per cent alkaline RNase and 11, 54, 16, and 13 per cent acid RNase, respectively.

In the experiments described in Table III, the over-all recoveries of total RNase activity averaged 182 per cent and ranged from 125 to 200 per cent. In our laboratory, such high recoveries have been a general finding in RNase distribution studies in both liver and spleen and are attributed to the presence of the RNase inhibitor in the supernatant fraction. Distribution studies are complicated by this inhibitor, and by the fact that a variable amount of lysosomal RNase activity may be released in the course of separating and assaying the cell fractions. The recoveries near 100 per cent reported by de Lamirande *et al.* for liver may possibly be attributed to use of a substrate that destroys RNase inhibitor.

It was of interest to determine the effect of CMS on the intracellular distribution of spleen RNase; CMS releases latent RNase activity (presumably bound by RNase inhibitor) in rat liver and spleen (see Fig. 4). Enzyme activity was assayed in normal rat spleen homogenate and fractions in the presence and absence of 4×10^{-4} M CMS. A third assay, without CMS, was carried out at the same time on homogenate and fractions to which 0.25 M sucrose instead of water had been added, the increased sucrose concentration favoring maintenance of the integrity of lysosomal particles. A procedure somewhat different from that described above under Methods was used in two experiments of this type. Two rat spleens were pooled and the fractions prepared as before. To 125 ml Erlenmeyer flasks containing 10 ml of 0.06 M VA buffer, pH 5.8, was added either 1 ml of homogenate, 5 ml of nuclei, 5 ml of mitochondria, 5 ml of microsomes, or 5 ml of supernatant fraction, and water or sucrose to make 20 ml, and CMS where desired. At zero time, 10 ml of 1 per cent RNA was added, and 3 ml aliquots were withdrawn from each flask at 10, 30, 45, and 60 minutes and added to 3 ml of precipitating agent. The RNase activities at each time interval were plotted for the homogenate and fractions, and a straight line was drawn through the points. The slope of the line was taken as the correct activity and the distribution calculated from this. In one experiment, the reaction rates of the homogenate and fractions were linear for 60 minutes, but in the second experiment the rates of some of the fractions deviated considerably from linearity. With this method, however, it is possible to obtain a more accurate assessment of enzyme activity, since even if two points are out of line the results will not be seriously affected; when a single time interval is used in the assay, such an error cannot be detected or corrected. The distribution of RNase activity in rat spleen fractions treated with either CMS or sucrose was not found to be significantly different from that of the controls. However, total activities of the sucrose-treated fractions were lower, and of the CMS-treated fractions higher, than those of the controls. The distribution of RNase activity in the control fractions was slightly different from that reported in Table III, less activity being observed in the mitochondria and more in the supernatant fraction. Whether this was due to the different experimental method or to some other factor is not known.

DISCUSSION

Ribonucleases have been divided into three groups based on the pH of optimal activity (7, 36)—neutral, acid, and alkaline—and rat spleen RNase appears to fall into the acid group. It is possible that a second RNase, with optimal activity at about pH 7.0, is present, but the evidence for this is not yet clear-cut. Studies on the purification of rat spleen RNase by column chromatography may give an unequivocal demonstration of more than one enzyme. Such studies, as well as investigations of the specificity of the spleen enzyme(s), are in progress and will be reported at a later date.

De Duve and his collaborators, as a result of some preliminary enzyme studies, have indicated that lysosomes may be present in spleen (37), and electron microscopical studies have pointed to a high content of "lysosome-like" bodies in this organ (38). The spleen is also rich in the acid hydrolases which characterize liver lysosomes, but several of these—acid phosphatase (37, 39), β-glucuronidase (39), cathepsin (37), and phosphoprotein phosphatase (39)—have been shown to occur to a large extent in the supernatant fraction, suggesting that lysosomes of spleen may be more sensitive to the preparative manipulations than those of liver. This study has shown that the distribution in spleen of another acid hydrolase which characterizes liver lysosomes, acid RNase,

is similar to that in liver; this study taken together with the distribution studies on acid phosphatase and β-glucuronidase suggests that if lysosomes occur in spleen, either they have a heterogeneous composition or the linkage of the various hydrolases to the particles may differ, resulting in the release of enzymes to varying degrees during fractionation. Although RNase is normally concentrated to the greatest extent in the mitochondrial fraction of spleen, all the enzyme may be lysosomal. Since it is difficult to separate mitochondria completely from lysosomes, the intriguing question whether there is any true mitochondrial RNase activity in spleen cannot be answered at present.

After the work described here was completed, a paper by Belousova (40) reporting the intracellular distribution of RNase in fractions of rat spleen came to our attention. Using the method of Schneider and Hogeboom (11) for RNase assay, and buffered (pH 7.4) 0.25 M sucrose homogenates with 0.018 M $CaCl_2$, Belousova found 34, 40, 9, and 18 per cent of the activity in the nuclear, mitochondrial, microsomal, and supernatant fractions, respectively. In addition, 115 per cent of the original homogenate activity was recovered among the four fractions.

Finally, it is of interest to compare the RNase distribution with the results previously reported on the localization of some oxidative enzymes in spleen (1). Cytochrome *c* oxidase and succinic cytochrome *c* reductase were localized almost exclusively in the mitochondria, while DPNH cytochrome *c* reductase was split between the mitochondria and microsomes. None of these enzymes appeared in the supernatant fraction.

Since the spleen is a heterogeneous organ with regard to cell type, the results presented here give a picture only of the average behavior of RNases of the different cells present. It is possible that the properties and distribution of RNases might vary considerably among individual cell types.

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