TABLE II. Observations and Calculations of the Affinity Constant of Calcium-Citrate Complexes.

Solution No.	Total Ca	Total CIT	Calculated Ca^{++}	Observed Ca^{++}	Difference	CaOTC	OTC	Log K
1	2.00	1.64	1.003	1.00	−.003	1.00	.64	3.19
2	2.00	1.64	1.003	1.00	−.003	1.00	.64	3.19
3	2.00	1.64	1.003	1.03	+.027	.97	.67	3.15
4	2.00	1.64	1.003	1.00	−.003	1.00	.64	3.19
5	2.00	1.64	1.003	.99	−.013	1.01	.63	3.21

Mean K = 1.543×10^3. Log mean K = 3.19.

tions of calcium and oxytetracycline, the stoichiometry of the complex had to be determined by the spectrophotometric method(3). When 1:1 complexes were assumed, the log of the affinity constant was 3.04 and very nearly the same as –3.0 to 3.1– found spectrophotometrically. Thus while the frog heart measured calcium ions, and the spectrophotometric method measured oxytetracycline, the two methods were in remarkable agreement.

Calcium has a lower affinity for oxytetracycline than magnesium, manganese, cobalt, or any other divalent cation in blood. The amount of this substance (1.0 g day) that a man tolerates without effects produces serum concentration of approximately 10 γ per ml; approximately 75% is bound to protein and only 25% is free in solution to complex with metal ions(4). Under these conditions, calcium and oxytetracycline formed complexes chiefly in a 1:1 ratio, but in bone matrix where calcium ion concentration may be higher, the proportions might increase to 2 to 1 or as high as 3 to 1. Because of the high concentration of calcium ions, in relation to other polyvalent ions in blood, the mass law operates in favor of calcium complex formation, and this effect is localized and enhanced in calcifying tissues. The mechanism and stoichiometry of combinations of tetracycline with calcium and bone salts are under investigation and will be presented later.

Summary. Calcium complexes of oxytetracycline were analyzed by the use of the frog heart method with an improved apparatus employing a vertical shutter, photoelectric transducer, bridge circuit, and recording system. This method was readily applicable to complex biologic solutions and produced results comparable to those in the preceding article obtained by spectrophotometry on pure solutions. The log K of the 1:1 calcium oxytetracycline complex was 3.04, compared with 3.19 for the 1:1 complex of citric acid and calcium.

1. McLean, F. C., Hastings, A. B., *J. Biol. Chem.*, 1934, v107, 337.

2. Hastings, A. B., McLean, F. C., Eichelberger, L., Hall, J., Da Costa, E., *ibid.*, 1934, v107, 351.

3. Ibsen, K. H., Urist, M. R., PROC. SOC. EXP. BIOL. AND MED., 1962, v109, 797.

4. Kaplan, S. A., Yuceoglu, A. M., Strauss, J., *J. Appl. Physiol.*, 1960, v15, 106.

Received November 15, 1961. P.S.E.B.M., 1962, v109.

Impurity in RNA Preparations which Inactivates RNase Inhibitor and Increases Alkaline RNase Activity.* (27341)

HERBERT J. EICHEL

Department of Biological Chemistry, Hahnemann Medical College, Philadelphia, Pa.

Following the demonstration of both acid and alkaline RNases in rat liver homogenate (1,2), de Lamirande and Allard(3) have pointed out that different laboratories have found different ratios of alkaline to acid

* This investigation was supported by a research grant from N.I.H., U. S. Public Health Service.

Abbreviations: RNase, ribonuclease; RNA, ribonucleic acid; CMS, p-chloromercuriphenylsulfonic acid; TCA, trichloroacetic acid.

RNase activity in this tissue. They reported a ratio of 2.3 in liver(3), while Roth(1) and Maver and Greco(4) described ratios of 0.4-0.5 and 0.5, respectively. It was suggested(3) that this apparent discrepancy was due to the different assays used. For instance, Maver and Greco employed 3% (final) $HClO_4$ as a precipitating agent and RNA prepared by the method of Grinnam and Mosher(5); de Lamirande and Allard used 2% (final) $HClO_4$ and a commercial preparation of RNA; and Roth and Milstein used acid-alcohol as a precipitating agent and P^{32}-labeled RNA isolated from yeast(6). In connection with a study of the RNases present in homogenates of rat epididymal adipose tissue (7), we have found it possible to reverse the alkaline to acid RNase ratio of this tissue, as well as that of liver, with use of different commercial substrates. In addition, several substrates preclude detection of the RNase inhibitor present in the supernatant fraction obtained from homogenates of many tissues, and this appears to be related to the inactivation of the inhibitor and subsequent release of bound alkaline RNase activity.

Methods. RNA was obtained from 3 sources: Schwarz BioResearch, Inc., Pabst Laboratories, and Nutritional Biochemicals, Inc. A 5% solution of each sample was prepared, adjusted to pH 7.0, dialyzed with stirring against glass-distilled water for 24 hours at 0°, readjusted to pH 7.0, and analyzed for dry weight. The standard RNase assay was as follows: about 0.5 ml, containing 6-6.5 mg of dialyzed RNA, was added at zero time to each of 2 test tubes containing 1 ml of 0.1 M Veronal-acetate buffer, pH 6.0 (for acid enzyme) or pH 7.8 (for alkaline enzyme); 0.5 and 1 ml of 1:5 or 1:10 adipose tissue homogenate or 0.1 and 0.2 ml of 1:10 liver homogenate in water; and sufficient water to bring the final volume to 3 ml. The reaction mixture was incubated at 37° for 30-60 min. With the Schwarz and Nutritional Biochemicals preparations, the precipitant was usually 3 ml of 5% TCA + 0.5% $AlCl_3$, and the reaction mixture was allowed to stand 8 min at 25° before filtering (Whatman No. 42 paper); the results were independent of the nature of the precipitant (TCA + $AlCl_3$, acid-alcohol, or acid-alcohol + 0.5% $LaCl_3$). With the Pabst preparation, the filtrates were cloudy when TCA + $AlCl_3$ was used and, therefore, the precipitated reaction mixtures were centrifuged 10 min at 20,000 × *g*; when acid-alcohol was the precipitant, the mixtures were filtered. The clear filtrate or supernatant fluid was diluted 1:25 or 1:50 in water and the absorbancy read at 260 mμ (Beckman DU spectrophotometer). RNase inhibitor in the supernatant fraction of liver and adipose tissue was estimated similarly except that the reaction mixture contained 0.03 μg pancreatic RNase (Worthington), 1 ml of 0.1 M Veronal-acetate buffer, pH 7.8, 0.5 ml of RNA, two levels of tissue, and water to 3.0 ml. The reaction mixtures were incubated at 37° for 30-60 min, precipitated with acid-alcohol(6), and then filtered. Blanks without tissue were run in duplicate. The procedures used for maintenance of rats (220-280 g) and removal and homogenization of the epididymal fat bodies have been described (11). Liver homogenates were prepared similarly.

Results and discussion. The data in Table I (based on average values calculated from a number of experiments) show that with Schwarz RNA, ratio of alkaline to acid RNase was low in adipose tissue (0.60) and liver (0.35) but shifted to a higher value (1.6-1.8) in each tissue when Pabst substrate was used. The latter also resulted in low acid RNase (Table I) and pancreatic RNase (Table II) activities. With the Nutritional Biochemicals

TABLE I. Acid and Alkaline RNase Activities in Homogenates of Rat Tissues Using RNA Preparations from Different Sources. Figures in columns 3 and 4 represent △ absorbancy × 10^3 obtained with 1 ml of adipose tissue and 0.2 ml of liver. Readings made on 2:50 dilutions of acid-soluble filtrates. Incubation time, 30 min.

		RNase activity		
				Alkaline
Source of RNA	Tissue	Acid	Alkaline	Acid
Schwarz	Adipose	109	65	.60
	Liver	86	30	.35
Pabst	Adipose	54	95	1.8
	Liver	48	77	1.6
Nutritional Biochem.	Adipose	48	96	2.0
	Liver	94	49	.52

TABLE II. Effect of Supernatant Fraction from Homogenates of Rat Adipose Tissue and Liver on Activity of Crystalline Pancreatic RNase Using RNA Preparations from Different Sources. Figures in columns 2 and 3 represent Δ absorbancy $\times 10^3$. Supernatant fractions were prepared by centrifugation of a 1:10 water homogenate of liver and a 1:5 filtered, water homogenate of adipose tissue for 30 min. at 110,000 $\times$ *g*. Incubation time, 60 min.

Additions	RNase activity with different RNA samples		Change from theoretical (%)	
	Schwarz	Pabst	Schwarz	Pabst
.03 μg pancreatic RNase	235	122		
.3 ml adipose supernatant	26	58		
.6 ml adipose supernatant	50	119		
.03 μg pancreatic RNase + .3 ml adipose supernatant	149	186	−43	0
.03 μg pancreatic RNase + .6 ml adipose supernatant	106	258	−63	+7
.03 μg pancreatic RNase	294	142		
.1 ml liver supernatant	3	114		
.2 ml liver supernatant	11	175		
.03 μg pancreatic RNase + .1 ml liver supernatant	33	265	−89	0
.03 μg pancreatic RNase + .2 ml liver supernatant	10	342	−97	+8

RNA, the adipose tissue ratio was again shifted to a higher level but the liver was not. These results suggested the possibility that Pabst RNA contains a substance which antagonizes the action of the naturally-occurring RNase inhibitor of adipose tissue and liver, since the inhibitor is most active in the alkaline range(7,8). This idea is strongly supported by the data listed in Table II. The supernatant fractions of adipose tissue and liver readily inhibited crystalline pancreatic RNase (pH 7.8) assayed with Schwarz RNA, but did not do so when assayed with Pabst RNA; in fact, the activities of the pancreatic enzyme and those of the supernatant RNases were essentially additive. It should also be noted that RNase activities of both adipose tissue and liver supernatant were much greater when assayed with Pabst RNA than with the Schwarz preparation. This is attributed to the fact that while the supernatant fraction of each tissue contains considerable alkaline RNase activity bound to RNase inhibitor(7,8), the Pabst RNA is apparently capable of inactivating the inhibitor and releasing the bound enzyme. As might be expected from the relatively high alkaline RNase activity in adipose tissue with Nutritional Biochemicals RNA (Table I), the inhibitor cannot be detected in adipose tissue supernatant fraction with this substrate (not tabulated). The reason for the low liver ratios with this RNA is not known (Table I) but might be that the contaminating substance in the Nutritional Biochemicals preparation does not act similarly on the RNase inhibitor of adipose tissue and liver. This, in turn, would suggest a difference in some properties of the inhibitor in the two tissues. Results similar to those reported in Table I were also found for rat spleen.

When 5.0×10^{-4} M (final) CMS was added to the standard RNase assay at pH 7.8 using Schwarz RNA, the activities of adipose tissue and liver homogenates were increased 100 and 400-500%, respectively (Table III), due to inactivation of RNase inhibitor and release of bound enzyme(8); when CMS was added to the RNase assay carried out with Pabst RNA, the activities were little affected. The final level of alkaline RNase using Pabst RNA, with or without CMS, was about the same as that with Schwarz RNA with CMS. Thus, the data of Table III are consistent with the interpretation that a substance is present in Pabst RNA which simulates the action of CMS in inactivating the RNase inhibitor and releasing the bound (latent) alkaline RNase activity.

The fact that Pb^{++} is known to inactivate RNase inhibitor(9) pointed to the possibility that a metal may be the responsible agent in the Pabst preparation. That this is probably the case is demonstrated by the following observation. When Pabst RNA was dialyzed for 36 hours against 0.05 M sodium ethylenediaminetetraacetate (Versene) at pH 6, and

TABLE III. Effect of CMS on Alkaline RNase Activity of Homogenates of Rat Adipose Tissue and Liver Using RNA Preparations from Different Sources. Figures in columns 2 and 3 represent Δ absorbancy $\times 10^3$. Final CMS concentration, 5.0×10^{-4} M; incubation time, 60 min.

Additions	RNase activity with different RNA samples		Increase due to CMS (%)	
	Schwarz	Pabst	Schwarz	Pabst
.5 ml adipose homogenate	49	94		
1.0 ml adipose homogenate	86	157		
.5 ml adipose homogenate + CMS	91	91	86	0
1.0 ml adipose homogenate + CMS	181	169	115	8
.1 ml liver homogenate	18	66		
.2 ml liver homogenate	41	123		
.1 ml liver homogenate + CMS	107	81	500	23
.2 ml liver homogenate + CMS	198	160	400	30

then tested, it gave an RNase ratio of 0.62 or less in adipose tissue (Table IV A). In some instances, the alkaline RNase activities of adipose tissue or liver were reduced nearly to zero with use of Pabst RNA dialyzed against Versene. In addition, in the presence of the latter RNA preparation, strong RNase inhibitor activity was readily demonstrated in the supernatant fluid from adipose tissue (Table IV B) or liver homogenate. It will be recalled that these supernatants failed to inhibit pancreatic RNase when the substrate was Pabst RNA which had been dialyzed against water (Table II). Versene (final concentration, 3.3 mM) added directly to the assay mixture also reduced almost to zero alkaline RNase activity of liver and adipose homogenates assayed with Pabst RNA; at the same time, acid RNase activity was increased to about the level obtained with Schwarz RNA. With the latter preparation in the presence of 3.3 mM Versene, virtually no alkaline RNase activity was present but the acid RNase level was unchanged. That the Versene does not act simply by inhibiting alkaline RNase is indicated by the fact that in the presence of both CMS and Versene, unimpaired alkaline RNase activity was registered. Finally, dialysis of the Pabst preparation against glass-distilled water for 4 days or against M NaCl for one day had little effect.

The use of 2% $HClO_4$ as a precipitant, which was employed by other workers(3), did not alter the ratios obtained in Table I with either the Schwarz or Pabst preparations. However, somewhat higher RNase activities and substrate blanks were obtained with both RNA's when the precipitant was $HClO_4$ rather than TCA + $AlCl_3$.

Although this study does not appear to explain the high liver ratios of de Lamirande and Allard, since they used a Schwarz preparation, it emphasizes the role that different substrates may play in assays of intracellular RNases and RNase inhibitor.

It would seem that the alkaline RNase activity of whole homogenates of several *normal* tissues (liver, adipose), and possibly that of many others, is very low or zero due largely to interaction between the soluble RNase inhibitor and the alkaline RNase activity of the mitochondria, microsomes, and supernatant fluid. Relatively high alkaline RNase levels have been recorded for homogenates of intestinal mucosa, spleen, kidney, heart, and brain(3). These values may represent the total (or near total) alkaline RNase activity

TABLE IV. Effect of Dialyzing Pabst RNA against Versene on Activity of Alkaline RNase and RNase Inhibitor of Rat Adipose Tissue Homogenate. Pabst RNA was dialyzed at 0° for 36 hr against 0.05 M Versene and a second batch of RNA was dialyzed against glass-distilled water. Figures represent Δ absorbancy $\times 10^3$.

Additions	RNase activity
A. Effect on RNase activity at pH 7.8	
1 ml adipose homogenate, assayed with RNA dialyzed *vs* H_2O	131
1 ml adipose homogenate, assayed with RNA dialyzed *vs* Versene	64
B. Effect on RNase inhibitor activity at pH 7.8	
.03 μg pancreatic RNase, assayed with RNA dialyzed *vs* Versene	313
.03 μg pancreatic RNase + .3 ml adipose supernatant, assayed with RNA dialyzed *vs* Versene	61

of the homogenates, including that of inactive RNase bound to RNase inhibitor; thus if these homogenates were assayed under similar conditions, but with CMS added to inactivate the RNase inhibitor, there might be little further increase in RNase activity. Conversely, it is possible that the high alkaline RNase activities might be reduced to zero if different RNA preparations were utilized or if the assay mixtures contained Versene. It should be pointed out, however, that the alkaline RNase activity of the mitochondrial fractions of liver and adipose tissue and possibly of other tissues may be appreciable even when assayed with use of a pure RNA preparation —free of an inactivator of the RNase inhibitor—since these mitochondria contain only small amounts of the RNase inhibitor. That adipose tissue mitochondria are relatively free of the inhibitor is indicated by the failure of CMS to increase the alkaline RNase activity of this fraction(7).

As to the acid RNase activity, it is beyond the scope of this paper to discuss at length the effect of different assay conditions and homogenization media on the activity of the enzyme in homogenates. However, it is worth emphasizing that the values reported in Table I were obtained with water homogenates assayed at 37° for 30 min without CMS. Although it has been shown that under these conditions acid RNase activity of adipose tissue is the same whether the homogenates are prepared in water or 0.25 M sucrose(7), it is well known that the acid RNase of liver particles is fully released only by various disruptive treatments of the particles(10,12).

Immediately before this paper was submitted for publication, Shortman(13) reported observations which are in essential agreement with the conclusions reached here. He found that a preparation of RNA obtained from British Drug Houses Pty Ltd. contained a metal contaminant which inactivated RNase inhibitor of rat liver and gave rise to considerable alkaline RNase activity.

Summary. Preparations of commercial ribonucleic acid have been shown to contain a contaminating substance which interferes with assay of pancreatic ribonuclease, intracellular ribonuclease activity, and activity of the endogenous ribonuclease inhibitor. The impurity is probably a metal since its action is annulled by dialyzing the nucleic acid against Versene or by adding Versene to the assay system. The presence of the impurity results in inactivation of the endogenous ribonuclease inhibitor of the tissues examined (liver, adipose tissue, spleen) which causes the release in active form of the inactive alkaline ribonuclease of the supernatant fluid fractions and also removes the inhibition of the particulate alkaline ribonuclease. This in turn, leads to high alkaline ribonuclease activities and high alkaline/acid ribonuclease ratios in homogenates of the tissues. The results emphasize the importance of the purity of the ribonucleic acid substrate in making accurate assays, under certain conditions, for the levels of intracellular acid and alkaline ribonuclease, pancreatic ribonuclease, and ribonuclease inhibitor and may explain some previous discrepant reports of these measurements.

The skilled technical assistance of Esther K. Goldenberg and Eileen M. Figueroa is gratefully acknowledged.

1. Roth, J. S., *J. Biol. Chem.*, 1954, v208, 181.
2. de Lamirande, G., Allard, C., da Costa, H. C., Cantero, A., *Science*, 1954, v119, 351.
3. de Lamirande, G., Allard, C., *Ann. N. Y. Acad. Sci.*, 1959, v81, Art. 3, 511.
4. Maver, M. E., Greco, A. E., *J. Natl. Cancer Inst.*, 1956, v17, 503.
5. Grinnam, E. L., Mosher, W. A., *J. Biol. Chem.*, 1951, v191, 719.
6. Roth, J. S., Milstein, S. W., *ibid.*, 1952, v196, 489.
7. Eichel, H. J., Figueroa, E., Goldenberg, E. K., *Biochim. Biophys. Acta*, 1961, v51, 216.
8. Roth, J. S., *ibid.*, 1956, v21, 34.
9. ———, *J. Biol. Chem.*, 1958, v231, 1085.
10. De Duve, C., Pressman, B. G., Gianetto, R., Wattiaux, R., Applemans, F., *Biochem. J.*, 1955, v60, 604.
11. Eichel, H. J., *Biochem. Biophys. Research Communs.*, 1959, v1, 293.
12. Reid, E., Nodes, J. T., *Ann. N. Y. Acad. Sci.*, 1959, v81, Art. 3, 618.
13. Shortman, K., *Biochim. Biophys. Acta*, 1961, v51, 37.

Received November 28, 1961. P.S.E.B.M., 1962, v109.