TABLE I. Glucose-6-Phosphatase Activity of Tissue Homogenates Expressed as mg P Liberated per Gram of Tissue from 4.2×10^{-3}M G-6-P at pH 6.5 and 37°C for 10 Min.

Tissue	Rat*	Sheep†
Liver	2.08	1.37 ± .16 (7)
Kidney	1.47	1.60 ± .20 (7)
Intestinal wall	1.08	.13 ± .05 (5)
" mucosa	—	.21 ± .10 (3)
Salivary gland	—	.10 ± .02 (9)
Muscle	trace	trace
Brain	—	"
Rumen epithelium	—	"
Mammary gland	—	"

* Mean of 2 observations.
† Mean ± stand. error (No. of observations).

taken from sheep killed by cutting the carotid and jugular vessels. Aqueous homogenates were prepared in the cold, the supernatants were incubated with glucose-6-phosphate (G-6-P), protein was precipitated with trichloracetic acid, and phosphorus in the filtrates was estimated by the Fiske and Subbarow method (for details see 4.5).

Results are shown in Table 1. On the whole our results with rat tissues confirmed those of Hers and de Duve(4), although we found rather less glucose-6-phosphatase activity in the liver and more in kidney and intestine, but observed the same order between organs, liver having the highest activity. The glucose-6-phosphatase activity of ovine intestinal wall and mucosa was much smaller than that of kidney and liver. It was also smaller than the activity of intestinal wall tissue from the rat. The enzyme activity was detectable also in salivary gland homogenates but was negligible in the other tissues.

Discussion. The low glucose-6-phosphatase activity of sheep intestine is in accord with other types of observations (recently reviewed by Lindsay)(1), which suggest that little glucose enters the portal blood from the digestive tract of sheep. The high phosphatase activity of ovine kidney suggests that the renal contribution of glucose to the blood, which has been estimated at 4-13% of the hepatic contribution in the dog(6), may be even greater in sheep. The slight phosphatase activity in the salivary gland leaves open the possibility that a little glucose might also be added to the blood by these glands (cp. 2).

Summary. Glucose-6-phosphatase activity was estimated in various sheep tissues. It was appreciable in liver and kidney, detectable in intestine and salivary gland, but negligible in brain, muscle, stomach, rumen epithelium and mammary gland. The results suggest the relative importance of these tissues in contributing glucose to the blood in sheep. They indicate particularly the relatively small potential role of sheep intestinal wall as a source of blood glucose.

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A Biological Assay for Ribonuclease with Sub-millimicrogram Sensitivity (26151)

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Ribonuclease (RNase) activity isolated from different sources can be assayed by chemical(1,2), manometric(3), spectrophotometric(4), turbidimetric(5), and isotopic(6) methods. The substrate in these methods is usually yeast ribonucleic acid (RNA) and measurements are made of its disappearance or hydrolysis products.

Recommended enzyme levels for most of these assays range from 5 to 50 μg of enzyme in 0.1 to 1.0 ml volumes, although modifications can yield greater sensitivity particularly with the isotopic method.

The use of biologically active RNA as an ultra-sensitive substrate for determining RNase was suggested by the observation(7) that a commercial preparation of crystalline deoxyribonuclease (DNase) (Worthington Biochem. Corp.) inactivated infectious RNA derived from foot-and-mouth disease virus (FMDV). It was presumed and has now been proved(8) that such action was caused by RNase impurities in the DNase which are undetectable by conventional measurements.

This communication reports a biological assay for RNase activity capable of detecting this enzyme at concentrations as low as 0.1 mμg per ml. Infectious RNA derived from FMDV was used as the enzyme substrate and primary cell cultures of calf kidney as the RNA substrate.

Materials and methods. Infectious FMDV ribonucleic acid was isolated from purified preparations of tissue culture FMDV, type A, strain 119, by the phenol extraction procedure of Gierer and Schramm(9), as adapted to FMDV in our laboratory(7). The RNA preparations, ranging in activity from 10^3 to 10^4 plaque-forming units per ml (PFU/ml), were diluted with Hanks' balanced-salt solution containing 0.5% lactalbumin hydrolyzate. *RNA plaque assay* was conducted in 4-oz prescription bottles containing primary cell cultures of calf kidney. The cell layers were triply washed before use with Hanks' balanced-salt solution containing 0.5% bovine lactalbumin hydrolyzate to remove serum. The RNA preparations were then applied. After 15 minutes at room temperature, the cultures were overlaid with agar medium and incubated 72 hours at 37°C as described elsewhere(7). Plaques were then counted and expressed as PFU/ml. *Ribonuclease* was obtained from Worthington Corp. as a crystalline preparation.

Experimental and results. Calibration for the procedure was as follows: Using 16 x 150 mm test tubes, 0.3 ml portions of infectious RNA containing from 500 to 5000 PFU/ml were mixed by repeated pipetting with 0.3 ml portions of RNase suitably diluted in 0.02 M, pH 7.5 phosphate buffer. After 15 minutes in a water bath at 25°C, with occasional shaking, the tubes were cooled in an ice bath, and the contents assayed for RNA plaque-forming units. Table I shows typical results.

TABLE I. Calibration of RNase Biological Assay.

Dilution of RNase*	PFU/ml of enzyme treated RNA
1:2500	0
1:5000	40
1:10,000	180
1:20,000	520
Control RNA = 500 PFU/ml	

* 1 μg/ml RNase.

The results are expressed directly in terms of weight of the enzyme present, rather than as units derived from a rate determination. The end point is represented by the highest dilution of enzyme preparation which still showed activity against the infectious RNA used as substrate. As little as 0.1 mμg of RNase can be detected by this assay method. A 2-fold dilution series was used for the final determination, although 10- or 100-fold dilution series may be needed for the preliminary investigation of an unknown sample. Less than 2-fold dilutions are not practical for final determination of enzyme activity, because of the known limitations in the precision of the plaque assay for infectious RNA (7). That is, the range in number of plaques found in equal-sized samples from a single RNA population is m ± 25% when mean value, m, is about 40 plaques.

A study of the effect of time and temperature on the reaction between enzyme and substrate appears in Table II. No enzyme activity was detected at a 1:2500 dilution of 1 μg/ml RNase acting for 1 hour at 4°C, although tests had shown considerable activity with RNase at a concentration of 5 μg/ml (7). Lengthening the exposure time at 25°C produced greater sensitivity, but the 4 hours needed for maximal activity was not required for routine assays. There was no benefit in using temperatures higher than 25°C, and sensitivity even seemed to be lost.

To determine that the reaction being

TABLE II. Effect of Time and Temperature of Incubation between RNase and FMDV-RNA.

Min.	Temp. (°C)	% of control activity*
5	4	100†
15	"	100†
60	"	100†
15	25	30
30	"	35
60	"	20
120	"	15
240	"	0‡
5	37	30
5	60	65

* 1:10,000 dilution of 1 μg/ml RNase used.
† There was also 100% of control RNA activity (therefore no enzyme activity) for 1:2500 enzyme dilution.
‡ When enzyme was diluted further to 1:20,000, 20% of control RNA activity remained.

studied represented a hydrolytic breakdown of the RNA substrate and not formation of an inactive RNase-RNA complex, an RNA preparation (3,000 PFU/ml), which had been inactivated at 25°C with a 1:500 dilution of a 1 μg/ml RNase solution, was re-extracted with phenol in the usual way, and the protein-free RNA preparation then assayed. There was no infectious activity in the enzyme-treated RNA sample, while a control RNA sample re-extracted with phenol retained practically its full activity.

The RNase assay described was applied to the RNase content of sera from various sources (Table III). Except for guinea pig serum, values for the human and animal sera fall within a narrow range, 0.02-0.12 μg/ml. Since the guinea pig serum had a 20- to 30-fold greater activity, it was investigated further to determine whether its increased activity was entirely due to RNase action.

The possible presence of a low-molecular-weight RNA inhibitor or cofactor was investigated. Guinea pig serum was diluted 1:10 in 0.02 M phosphate buffer of pH 7.5 and dialyzed against 1000 volumes of the same buffer for 24 hours at 4°C; the buffer liquid was changed once during this interval. The dialyzed guinea pig serum as well as undialyzed serum were assayed against FMDV-RNA, and no significant difference was found in their RNase activity. Similarly, horse serum, used as an example of a low enzyme content serum, did not change significantly in activity upon dialysis.

Tests were made for presence of acid- or heat-labile RNA inactivators. Guinea pig serum, diluted 1:10 in phosphate buffer and adjusted to pH 3.0, was incubated 5 minutes in a boiling water bath. Such a procedure would not be expected to appreciably affect RNase itself(10). The diluted serum was then cooled quickly, readjusted to neutrality, and assayed for RNase activity. There was no change in activity compared to an untreated guinea pig serum control. A sample of phosphate-buffered crystalline RNase at the same activity level as in the guinea pig serum was subjected to the acid and heat treatments described and lost more than half of its activity. The greater stability of the serum RNase is probably due to protection offered by the other proteins present.

Discussion. It is difficult to apply any sort of kinetics to this analysis of RNase activity because concentration of the infectious RNA substrate is extremely low. Since each plaque-forming unit probably represents one molecule of RNA(7), there are less than 5,000 molecules of RNA per ml. Increasing this amount by a factor of a thousand to allow for non-infectious RNA being present still yields a substrate concentration 1,000 times lower than the 4×10^9 molecules of RNase present in a 1:10,000 dilution of a 1 μg/ml solution. Thus, there is little possibility of the reaction being zero or first order as are the usual enzyme assay reactions. Instead, this assay should be treated as a chemical assay in so far as a standard curve using known concentrations of crystalline RNase is prepared for each series of determinations on unknowns.

It has been reported that in a 0.01% salt-free, tobacco mosaic virus RNA solution, addition of RNase (molar ratio, 15%) will pro-

TABLE III. RNase Content of Several Sera.

Serum	RNase content (μg/ml)	Serum	RNase content (μg/ml)
Human	.08-.10	Mouse	.08- .10
Bovine	.08-.10	Guinea pig	2.2 -3.0
Sheep	.08-.10	Rabbit	.05- .08
Swine	.10-.12	Chicken	.06- .08
Horse	.02-.05		

duce an inactive RNA complex which can be treated with phenol to regenerate an average of 20% of the original infectivity. Kirby (13) had shown previously that phenol will inactivate RNase. The recovery of infectivity decreased both when the ratio of RNase was changed and when salts were present (12). Comparable conditions do not exist in the enzyme-substrate system used in the present report since close to physiological conditions are employed with at least a 1000-fold higher concentration of enzyme. As shown by the failure to obtain infectious RNA from RNase treated FMDV-RNA, the RNase is hydrolyzing its substrate.

This assay for RNase might also be extended to make use of any infectious RNA for which infectivity can be accurately measured. A comparable assay for DNase could also be set up by using an infectious DNA such as the one recently obtained from polyoma virus(14).

The greatest advantage of this assay is in detection of trace amounts of RNase activity not measurable by any other method. Currently available methods of assay(1-6) are most convenient to use for microgram levels of enzyme. Only under favorable conditions with extended incubation times do these methods yield greater sensitivity. The biological assay reported here using infectious RNA invariably gave a sensitivity of 0.1 mμg of RNase/ml. In the analysis of unknowns, possible interfering RNA-inactivating materials can be removed by dialysis or acid and heat treatment, since RNase itself is resistant to these treatments. As shown in the present experiments, RNase activity in the sera of several animal species and of man was not due to any acid- or heat-labile or low-molecular-weight material. Comparison of the values obtained here with those reported in the literature for rat and rabbit sera(3,11) was not feasible because the latter values are reported in terms of cu mm of carbon dioxide.

Summary. An ultra-sensitive method of assay for ribonuclease (RNase) has been devised using a biologically active ribonucleic acid (RNA) as its substrate. Infectious RNA isolated from foot-and-mouth disease virus was exposed to suitable dilutions of RNase, and the resulting loss in RNA infectivity was measured by plaque assay in calf-kidney cultures. Concentrations of enzyme as low as 0.1 mμg per ml were readily detected. The RNase level in sera from man and several animal species was determined by this method. Most sera contained 0.02 to 0.1 μg/ml of RNase, but guinea pig serum had a 20- to 30-fold higher content of this enzyme.

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