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THE NUCLEIC ACID OF A MURINE LEUKEMIA VIRUS

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Of the several viruses which are known by now to cause leukemia in mice and related animals,[1] the one which was isolated by Rauscher[2] is especially suitable for study because its biological effects are extremely rapid. Upon inoculation of this virus into mice, a palpable enlargement of the spleen occurs in about 15 days. Those mice which survive the spleen enlargement die with lymphocytic leukemia in 4–6 months.[2] Other sensitive and rapid assay methods[3–5] may also be employed. Further interest in this virus derives from the fact that it may have antigens similar to those which occur in some human leukemias.[6]

While the biological properties of this and of other murine leukemia viruses have been extensively investigated,[1] as yet there is not much known about their chemical nature, partly because of the difficulty in obtaining these viruses in sufficient quantity. Some information has been deduced from studies by electron microscopy and by density gradient centrifugation. The results of the first technique suggested[7, 8] that the Rauscher virus, in common with the other murine leukemia viruses,[11] probably contains an outer membrane derived from the lipid-rich plasma membrane of the virus-producing cells. The ether sensitivity and the low buoyant density observed in density gradient centrifugation[9, 10] support this suggestion. Recently, somewhat larger amounts of the Rauscher virus became available.

This communication reports the results of some preliminary chemical studies on its nucleic acid.

The nucleic acid of other membrane-enclosed oncogenic viruses, such as the Rous sarcoma virus,[12] the avian myeloblastosis virus,[13] and the mouse mammary tumor virus,[14] was found to be RNA, present in small quantities (~1%). When recently a structurally quite different, lipid-free, and symmetrical plant tumor virus, the so-called "wound tumor virus," was found to contain double-stranded RNA,[15] similar to a reovirus,[16] a suggestion was made[15] that RNA in the double-helix structure might be generally associated with the cancer-causing ability of an RNA virus. We found that the Rauscher murine leukemia virus also contains RNA, but the RNA appears to occur substantially in a single-stranded state.

Materials and Methods.—Preparation of the virus: Concentrated stocks of the Rauscher murine leukemia virus were prepared from the plasma of viremic mice by differential centrifugation similar to that described previously.[2] The preparations were carried out at Charles Pfizer and Company, Inc., Maywood, N. J., under the supervision of Dr. F. J. Rauscher of the National Cancer Institute. The virus concentrates, kindly supplied by Dr. Rauscher, were "tenfold concentrates" in 0.05 *M* sodium citrate-citric acid buffer, pH 6.8, and were preserved at −70°C in sealed glass vials. The virus is known to maintain its biological activity under these conditions for a considerable length of time.[2] Infectivity (ED50) with such virus preparations was demonstrated by the spleen enlargement assay after a 10^5 to 10^6 dilution or more.[2]

Purification of the virus: It has been observed in our laboratory[9, 20] that after brief centrifugation in a shallow potassium citrate density gradient, all the biologically active Rauscher virus can be collected in a sharp zone at a buoyant density of about 1.16 gm/ml. Electron microscopy showed that essentially all the material in this zone consisted of intact or slightly degraded virus-like ("c-type") particles, similar in all of the murine leukemia viruses.[17] In this laboratory, during the last three years, a large number of virus preparations were purified in potassium citrate and other density gradients, and studied by various chemical and biological methods to develop optimum methods of purification. Taking advantage of these studies, about ten large virus preparations were purified by density gradient centrifugation in potassium citrate; the 1.16 gm/ml density zone was collected. This zone was extracted for nucleic acid, using the cold phenol method with slight variations. A representative example of purification and extraction is given below. All work was carried out in the cold (~ 2°C), and with minimum delays and storage periods.

Concentration of the virus: Four ml of concentrated potassium citrate solution with a density of 1.4 gm/ml, brought to pH 7 with small amounts of citric acid, was put into each of two Spinco SW25.1 centrifuge tubes, to act as a "cushion."[18] These solutions were then carefully overlaid with 25 ml virus suspension. After centrifugation at 22,500 rpm (av. 51,500 *g*) for 90 min, a thick zone settled on the top of the salt solution. The zones from each tube were combined and their density was determined. An appropriate volume of 0.05 *M* Na citrate-citric acid buffer, pH 7, was added to adjust the density to 1.13 gm/ml. This suspension was then centrifuged in the SW39 tube at 36,000 rpm (av. 107,100 *g*) for 3 hr and 20 min. The supernatant was carefully siphoned off and discarded. The pellets readily resuspended by gentle stirring in a total of 1.5 ml citrate buffer.

Density gradient centrifugation: The concentrated virus solution was divided into three 0.5-ml aliquots which were layered on the top of preformed K citrate-citric acid gradients (density 1.05–1.40 gm/ml) in Spinco SW39 centrifuge tubes and centrifuged at 36,000 rpm (av. 107,000 *g*) for 2 hr and 45 min. The main zone, which appeared as a thin layer at 1.16 gm/ml density and contained all of the biologically active virus,[9] was separated by careful unloading from the top. The virus band was diluted with 20 vol of 0.05 *M* citrate buffer, and sedimented at 36,000 rpm (av. 86,000 *g*) for 1 hr in a Spinco no. 40 rotor.

Extraction of the nucleic acid: The resulting virus pellet was overlaid with 1 ml of 0.02 *M* phosphate buffer, pH 7, containing 10^{-3} *M* Mg^{++}. (In some of the extraction experiments at this point, an anionic RNase inhibitor, polyglucose sulfate,[19] was added at 50 μg/ml concentration.) One milliliter of 0.2% solution of sodium lauryl sulfate (SLS) in the same phosphate-Mg buffer

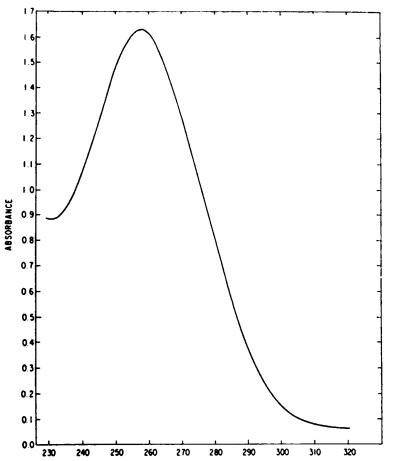

FIG. 1.—Ultraviolet spectrum of the nucleic acid extract of the virus. The virus purification and extraction conditions were given under *Materials and Methods*.

was added, and the pellet was dispersed by gentle stirring with a glass rod for 15 min. Two milliliters of freshly distilled, water-saturated (at 2°C) phenol was added, and the tube was very vigorously shaken for 15 min. The suspension was centrifuged for 15 min at 27,000 *g* to allow the phases to separate. The clear aqueous phase was carefully pipetted off from a thick interphase and then re-extracted similarly with an equal volume of phenol. After one more phenol extraction there was no visible interphase. The final aqueous phase was freed of phenol by extracting eight times with anhydrous ether, and freed of the ether by bubbling with nitrogen. The extract (2 ml) was then dialyzed for 24 hr against three changes of 50 ml of 0.1 standard saline-citrate (SSC = 0.15 *M* NaCl + 0.015 *M* Na citrate).

Measurements: Sedimentation velocity experiments on the nucleic acid extracts were made in 0.2 *M* NaCl by following the sedimenting boundary at 265 mμ in the Spinco model E analytical ultracentrifuge equipped with a monochromator attachment. Reported *S* values are uncorrected, and were calculated from the 50% point of the generally diffuse boundaries.

Optical density measurements were carried out in a Cary 15 or in a Zeiss PMQII spectrophotometer. Thermal denaturation was followed by absorbancy measurements at 260 mμ in a Beckman DU spectrophotometer, equipped with thermospacers. Ground-glass-stoppered cuvettes were sealed with a silicone rubber compound (GE RTV60) before the measurements. Temperatures in the cell carriage were measured in a mock-up cell equipped with a calibrated copper constantan thermocouple. Solvent spectra were also taken in the heating compartment, and all values were recorded as differences in absorbancy. Hyperchromicity ($A_t/A_{25}°$), the ratio of the absorbancy at 260 mμ at a given temperature to the original absorbancy of the untreated sample at 25°C (about 0.5 OD), was plotted against temperature.

Ribose-equivalent estimation was made by a modified[21] orcinol method[22] using xylose as standard.

RNase and DNase assays[23] were carried out in 0.02 *M* phosphate buffer containing 0.01 *M* Mg^{++}. Lysozyme (50 μg/ml) was added as a polycation to counteract[24] the enzyme-blocking effect of the polyglucose sulfate, when the latter substance was employed prior to the extraction. Both the beef pancreatic RNase and the DNase employed were Worthington preparations and they were present at 20 μg/ml concentration. Absorbancy changes were measured at 260 mμ and at 300 mμ at 30°C.

Alkaline (0.3 *M* KOH, 18 hr, 37°C) or acid conditions (1 *N* HCl, 1 hr, 100°C) were used to hydrolyze the nucleic acid preparations. For separation of the mononucleotides, paper chromatographic[25] and ion-exchange resin[26] techniques were employed. The four ribonucleotides were detected visually under the UV light as single spots on the chromatographic paper in positions corresponding to those of the mononucleotides of a purified yeast RNA after similar hydrolysis, or of authentic mononucleotides.

For comparative purposes other RNA preparations were used. Unfractionated yeast sRNA was a gift of Dr. G. L. Cantoni.[27] "Cytoplasmic" RNA[28] was essentially an extract from the cytoplasmic fraction of rat liver homogenates, prepared in this laboratory by Dr. C. E. Smith by a modified procedure of Dingman and Sporn.[29]

Results.—(a) *Characterization of the nucleic acid extract:* The nucleic acid extract of the virus gave an ultraviolet spectrum characteristic of nucleic acid with a 260-mμ absorbance maximum. The absorbancy ratio of 280/260 mμ was 0.494, and of 230/260 mμ was 0.529, indicating satisfactory purity from proteins (see Fig. 1).

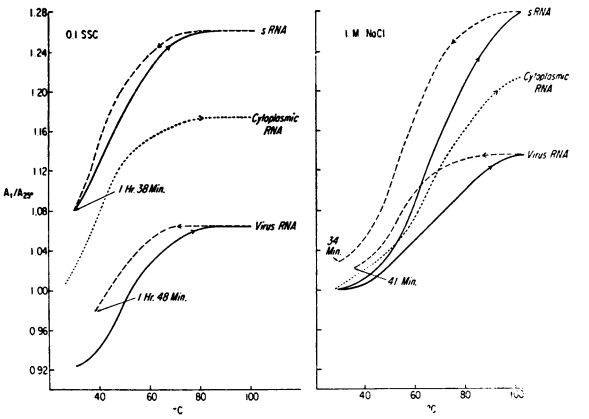

FIG. 2.—Hyperchromicity at 260 mμ ($A_t/A_{25°}$) upon heating of the RNA extract of the virus, of a sRNA, and of a cytoplasmic RNA preparation (*solid lines*) in 0.1 SSC, and in 1 *M* NaCl. Values are relative to the room temperature absorbance of the particular RNA preparation in 1 *M* NaCl. Cooling curves (*broken lines*) show the rapid "annealing" in the case of the sRNA, and the somewhat slower loss of hyperchromicity of the virus extract. The time values with arrows indicate the absorbance after the cooling has been started from 100°.

Similar nucleic acid absorption curves were obtained by similar methods from about ten other virus stock solutions. In each case potassium citrate density gradient centrifugation was employed to purify the virus, and the main zone at 1.16 gm/ml density was extracted. There was no significant amount of extractable 260 mμ-absorbing material outside of this main zone. Slight modifications in the virus concentration steps prior to density gradient centrifugation, digestion of the purified virus with beef pancreatic RNase, or incorporation of RNase inhibitors during the extraction steps did not significantly alter the shape of the UV curve, or the recoverable optical density at 260 mμ.

That the nucleic acid extract was mainly RNA was shown by agreement (within 10%) of RNA estimation by the orcinol method, by absorbancy at 260 mμ, assuming 45 μg/OD, and by enzymatic experiments. Addition of DNase caused no observable change in absorbancy at 260 or 300 mμ, while addition of RNase caused a rapid increase at 260 mμ and a decrease at 300 mμ, complete within 15 min. Also, substantially complete hydrolysis of the nucleic acid extract was obtained after 18 hr in 0.3 *N* KOH at room temperature, as detected by the hyperchromic shift. Paper chromatographic separation of concentrated, acid-hydrolyzed nucleic acid extracts showed the presence of only the four common ribonucleotides. There was no other material detectible by the methods employed in the hydrolyzates of about 100 μg RNA.

The average sedimentation coefficient of the nucleic acid extract was 3.9 *S*. Similar extraction experiments on other virus preparations yielded similar low sedimentation coefficients (4–8*S*) for the major portion of the optical density material present. However, in one extraction experiment a diffuse boundary was present, indicating about 50 per cent of 48*S* sedimenting material. This variability emphasizes the sensitivity of the RNA to hydrolytic breakdown, and the necessity for further studies on the conditions of extraction, so that the extracted RNA would be more representa-

tive of the RNA molecule as it occurs in the virus. This problem will be discussed further.

(*b*) *Experiments pertaining to the secondary structure of the virus RNA:* We report the extraction and the properties of this particular 3.9*S* preparation because it was our largest, providing sufficient material for several experiments on the hyperchromic shifts at elevated temperatures. However, it should be pointed out that similar hyperchromic behavior was observed on other virus extracts. For comparative purposes we include the yeast sRNA (4*S*) and the "cytoplasmic" rat liver RNA preparation.

To compare the relative amount of organized (secondary) structure in the various RNA preparations, the changes in absorbancy at 260 mμ upon heating and cooling are presented (Fig. 2). The optical density of each type of RNA at 25° in 1 *M* NaCl was used as the denominator in calculating the ratio $A_t/A_{25°}$. This allows a suitable comparison of the various RNA samples for the effects of heat and of salt concentration on the secondary structure, as manifested by the absorbancy changes.

It can be seen from Figure 2 that in 1 *M* NaCl the hyperchromic shift of the sRNA is both sharper and greater (~28%) than that of the cytoplasmic RNA (~22%), which in turn is greater than that of the virus RNA (~13%). This indicates that the sRNA suffers the greatest disruption of organized structure upon heating, cytoplasmic RNA less, and the virus RNA, the least. Upon cooling, both the sRNA and the virus RNA rapidly recovered the lower values of absorbancy.

The differences between the various RNA preparations are further evident (Fig. 2) from experiments in 0.1 SSC. In the case of the sRNA at room temperature almost one third of the hyperchromic shift occurred merely upon lowering the ionic strength. This is presumably due to greater electrostatic repulsion between the phosphate groups leading to partial disruption of the secondary structure. In this low-ionic-strength medium the transition upon heating was sharper; it showed a considerably lower median transition temperature (T_m = 50°), and reached the full hyperchromicity at lower temperature (80°) than in 1 *M* NaCl (T_m = 68°, max hyperchromicity at 100°C). In the cytoplasmic RNA there was no significant evidence of loss of organized structure at room temperature at lower ionic strength. However, the transition upon heating also occurred at lower temperatures.

In contrast, the virus RNA not only did not show evidence of loss of organized structure in low ionic strength, but in fact had a considerably lower optical density in 0.1 SSC (actual value 0.606) than in 1 *M* NaCl (0.656) and this difference remained even at 100° (0.698 and 0.744, respectively). The hypochromic shift upon lowering the ionic strength is shown by the lower starting point of the virus RNA curve. The total attainable hyperchromic shift upon heating is equal at both ionic strengths, in contrast to the other two RNA preparations. However, the hyperchromic shifts upon heating occurred at lower temperature ($T_m \sim 50°$), and total hyperchromicity was obtained at 80°, similar to the two other RNA preparations.

The "annealing" of sRNA in 0.1 SSC was very rapid and complete, but distinctly less so in the case of the virus RNA (compare the cooling lines in the left half of Fig. 2).

We also observed a decline in absorbancy between repeated heating and cooling cycles in the case of the virus RNA, when we stored a previously heat-treated sample at refrigerator temperatures (~2°C) in 0.1 SSC. The greatest decline observed in

the absorbancy amounted to 10 per cent in 48 hr. The total hyperchromicity attainable upon reheating, however, remained about the same, and so did the ability to regain the lower absorbancy upon slow cooling. No such loss in absorbancy was observed upon similar refrigeration of a previously heated virus RNA in higher salt concentrations (1 *M* or in 5 *M* NaCl). The ability to regain nearly the original absorbancy upon slow cooling was also retained in 1 *M* or 5 *M* NaCl solutions after several prior heating cycles. By way of comparison, yeast sRNA annealed rapidly and completely in 0.1 SSC, but not so completely in 1 *M* NaCl after the second heating cycle; it showed no loss in absorbancy after storing at 2°C, but perhaps a slight decrease (3% loss) in total attainable hyperchromicity.

When the virus extract in 1 *M* NaCl was heated for 1 hr at 100°C and then rapidly cooled ("quenched") in ice, the absorbancy at 260 mμ, the range of the "melting" region, and the total attainable hyperchromicity remained similar to those of the unheated sample.

Discussion.—The limited amounts of virus samples which were available from time to time for chemical studies on the virus nucleic acid allowed only a few key experiments on each sample. As mentioned above, we extracted numerous other density gradient purified virus preparations, and obtained nucleic acid extracts each with one or more properties entirely similar to those detailed above: thus, they absorbed in the ultraviolet characteristic to nucleic acid, showed similar "melting" behavior, were hydrolyzed slowly by 0.3 *N* KOH and rapidly by RNase, and yielded only the four common ribonucleotides as detected by paper chromatography. None of the samples available, however, was sufficient, for example, to report a fair estimation of base ratios.

The low sedimentation coefficients and the diffuse boundaries generally obtained indicate that it was difficult to maintain the molecular integrity of the RNA and extensive degradation of the viral genome probably occurred during extraction. Therefore, it should be emphasized that the data presented in Figure 2 and the conclusions we may reach from these data necessarily pertain only to the RNA extracts investigated to date, and the pertinence of these results to the state of the RNA in the virus must await development of extraction techniques which may better preserve the integrity of the RNA molecule. As more virus samples become available, we are planning to evaluate more thoroughly the effectiveness of various RNase inhibitors to prevent degradation.

With the above reservations, one can, however, say that the higher total hyperchromicity upon heating of the yeast sRNA and of a rat liver cytoplasmic RNA preparation (especially in 1 *M* NaCl) as compared to the virus RNA, and the more gradual melting transition of the virus RNA preparation, all indicate that the latter RNA extract contains less organized secondary structure disruptible by heating than either of the two other RNA samples. Thus, these melting experiments are only suggestive, that the RNA may be single-stranded in the virus. This is in line with the apparent fragility of the molecule during extraction, and with its rapid susceptibility to RNase digestion.

The RNA preparations obtained by phenol extraction at room temperature from wound tumor virus[15] and also from reoviruses[16] showed sharp melting profiles with almost full hyperchromic changes occurring within 5°, similar to the melting profile of a DNA. Our results on the Rauscher virus RNA extract are obviously quite

different. A broad "melting" transition curve very similar to that of the Rauscher virus RNA extract was observed on the RNA of the mouse mammary tumor virus, another membrane enclosed oncogenic virus, indicating a single-stranded RNA structure.[14]

The observed decrease in the absorbancy upon low-temperature storage of the RNA extract from the Rauscher virus after heating in 0.1 SSC is indeed puzzling. Since no such loss was observed in the case of sRNA, cytoplasmic RNA, or of the virus RNA at higher salt concentration, it is not likely that this lowering of absorbancy was due to some trivial cause (such as breakdown or sedimentation or bacterial contamination). Apparently, it represents an increased ability of a heated virus RNA to anneal into a more ordered structure under specific conditions. It is also interesting that the virus RNA, in contrast to the other two RNA preparations, has a lower absorbancy at the lower ionic strength.

At this time the limited availability of this murine leukemia virus did not allow more accurate chemical measurements. Further investigations of this and other RNA tumor viruses are planned as methods of production, assay, and purification improve.

Summary.—The Rauscher murine leukemia virus contains RNA. It is difficult to maintain the molecular integrity of this RNA during extraction. On the RNA extracts obtained up to date, melting experiments suggest a single-stranded (random-coil) configuration.

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EXCITATION OF INTERNALLY PERFUSED SQUID GIANT AXONS IN SODIUM-FREE MEDIA

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Many excitable tissues are known to maintain their ability to develop action potentials in Na-free media. For example, plant cells such as *Nitella* produce electrical responses in media containing only salts of K and divalent ions.[1] Crustacean muscle fibers produce large action potentials in media containing only $BaCl_2$ or $CaCl_2$.[2, 3] Electrical activity is reported to persist in frog heart muscle[4, 5] and various smooth muscles[5, 6] in the absence of Na-ion in the external medium. Dorsal root ganglia of the frog also remain excitable in the absence of external sodium.[7, 8] In large myelinated fibers of the frog, Lorente de Nó and his associates[9, 10] have demonstrated that when neural conduction is initially blocked by Na-ion deprivation, conduction may subsequently be restored by external application of a variety of nitrogenous compounds, including hydrazine, hydroxylamine, and guanidine. Similar observations have been made in frog nerve-muscle preparations[11] and isolated single nerve fibers.[12]

The fact that Na-ions may not always be required in the excitation process for squid giant axons has been reported in a recent abstract from this laboratory,[13] but it is generally believed that squid axons rapidly lose excitability in the total absence of Na or Li salts in the external medium. The present article is designed to demonstrate that under intracellular perfusion with favorable salt solutions, squid giant axons maintain distinct excitability in Na-free media.

Some of the conditions under which excitation may take place in squid giant axons without external Na-ion have been briefly described in a recent article,[14] in which an extensive comparison of the relative "favorability" of various cations and anions introduced into the axon interior was made by the technique of intracellular perfusion. Among the anions examined, the following order was found:

$$\ldots < \text{glutamate, aspartate} < \text{phosphate} < F,$$

where fluoride ion was the most favorable. The cations studied formed the following sequence: