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THE CLEARANCE AND LOCALIZATION OF NUCLEIC ACIDS BY NEW ZEALAND AND NORMAL MICE

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SUMMARY

The clearance and localization of native DNA, denatured DNA, and double-stranded synthetic RNA was studied in New Zealand Black/White hybrid mice, which develop an illness closely resembling human systemic lupus erythematosus, and in normal mice. The three nucleic acids were rapidly cleared from the circulation in all strains studied. Serum nucleases did not account for this rapid clearance, indicating that the nucleic acids were taken up as macromolecules. The polymers were concentrated in the liver and spleen, suggesting uptake by the reticulo-endothelial system. Animals with circulating antibody cleared the nucleic acids even more rapidly. New Zealand mice did not differ from normal mice in their metabolism of nucleic acids.

INTRODUCTION

The fate of nucleic acids injected into the circulation is of interest for two reasons. First, nucleic acids, particularly the synthetic double-stranded ribonucleic acid, polyinosinic·polycytidylic acid (poly I·poly C), have been shown to stimulate interferon production (Field *et al.*, 1967) and to have anti-tumour effects (Levy, Law & Rabson, 1969). Poly I·poly C is currently under investigation in patients for these purposes.

Second, the glomerulonephritis of systemic lupus erythematosus (SLE) is thought to be caused by the deposition of immune complexes composed of DNA and anti-DNA antibodies (Koffler, Schur & Kunkel, 1967). The New Zealand Black/White F_1 hybrid mouse (B/W) spontaneously develops an illness very similar to human SLE (Helyer & Howie, 1963; Lambert & Dixon, 1968). Approximately 50% of the immunoglobulin eluted from such mouse kidneys has specificity for deoxyribonucleoprotein (Dixon, Oldstone & Tonietti, 1971). Like patients with SLE, B/W mice have circulating antibodies to both native and denatured DNA and to double-stranded RNA and hyper-respond to immunization with

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nucleic acids. Although these antibodies have been studied extensively (Steinberg, Baron & Talal, 1969; Tan *et al.*, 1966; Koffler *et al.*, 1969; Schur & Monroe, 1969; Schur *et al.*, 1971), little is known about the clearance and localization of nucleic acids in these and normal mice. Factors such as the rate of disappearance from the circulation and extent of degradation of nucleic acids in addition to their concentration would be important determinants of the rate of immune complex deposition. We have investigated the elimination from plasma and tissue localization of intravenously administered tritium-labelled DNA and RNA, as well as the role of serum nucleases on the degradation and clearance of these nucleic acids.

MATERIALS AND METHODS

Nucleic acids

Tritiated KB cell DNA (specific activity 90 μCi/mg) labelled at the 6-methyl group of thymidine, was obtained from Electronucleonics Co., Bethesda, Md., and used in studies of native DNA. A portion of the same preparation was denatured by heating to 100°C for 10 min before being plunged into ice (Doty *et al.*, 1960). The synthetic double-stranded RNA polyinosinic·^{3}H-polycytidylic acid was purchased from Miles Laboratories (specific activity 32 μCi/mg). Relative *S* values were determined by ultracentrifugation in 5–20% sucrose gradients made in 3·2 ml 0·2 M boric acid (pH 8·0), 0·15 M NaCl, and 5 mM sodium EDTA, and centrifuged at 35,000 rev/min in an SW-39 rotor at 8°C for 4 hr (Burgi & Hershey, 1963). T7 phage DNA (S=32) (Studier, 1965), gift from Dr Martin Gellert, served as an internal standard. The mean *S* value was 15 for the native DNA and 8 for the poly I·poly C. The same DNA and poly I·poly C preparations were used in all the clearance and localization studies, except in the comparison of poly I·poly C preparations of different molecular size. In that experiment samples with *S* values of 3 and 12 were also used. In order to make the size of the nucleic acids more comparable, the same DNA but the poly I·poly C preparation with an *S* value of 12 were used for the nuclease studies.

The native KB DNA was not precipitated by antibody specific for single-stranded DNA. After denaturation all of the DNA was precipitated by the same antibody. All the radioactivity in the poly I·poly C was precipitated by antibody specific for double-stranded RNA. Both DNA and poly I·poly C were completely precipitated by 3% trichloroacetic acid at 4°C. After the DNA was exposed to pancreatic DNAse type I and the poly I·poly C to pancreatic RNAse (both enzymes from Worthington Biochemical Corporation) no radioactivity was precipitated by 3% trichloroacetic acid.

Animals

Mice and Hartley guinea-pigs were obtained from colonies maintained at the National Institutes of Health. Female B/W F_1 hybrids were the product of NZB female by NZW male matings. DBA/2 and BALB/c mice were used as control strains. Clearance studies utilized 2-month-old NZB, NZW, B/W, DBA/2 and BALB/c females, all lacking antibodies to nucleic acid. Two-month-old B/W and DBA/2 mice were immunized with 10 μg poly I·poly C in Freund's complete adjuvant injected in all four footpads. Control animals received saline in the same amount of adjuvant.

Since antibodies to double-stranded DNA cannot be induced by immunization, 10–11-month-old B/W females with spontaneously occurring anti-DNA antibodies were used to study the elimination of DNA by immune animals.

Urine was collected by placing animals in metabolic cages for 24 hr with free access to food and water.

Nucleic acid administration

Mice were injected intravenously with 2 μg of DNA or poly I·poly C. Adult female guinea-pigs received 10 μg of nucleic acid intravenously. Serial orbital sinus punctures were performed with heparinized Pasteur pipettes. The plasma was separated by centrifugation and solubilized with NCS (purchased from Amersham-Searle). They were counted in toluene-PPO-POPOP scintillator with quench correction by external standard. In tissue localization experiments, animals were killed by cervical dislocation and perfused with iced modified Tyrode's solution through a needle inserted in the left ventricle. The organs were solubilized in NCS, decolourized with benzoyl peroxide and radioactivity determined as above.

Nuclease assays

Exonuclease activity in mouse serum was measured by incubating 40 μl fresh serum with 80 ng labelled polymer in 60 μl 25 mM Tris buffer (pH 7·4) in normal saline for 30 min at 37°C. Then 200 μl 10% trichloroacetic acid and 10 μg unlabelled nucleic acid was added, centrifuged and the supernatant assayed for radioactivity. Endonuclease activity was evaluated by incubating serum and polymer as above, then stopping the reaction with 300 μg pronase (Cal Biochemical Corporation), which hydrolysed all nucleic acid-binding proteins. The mixture was incubated at 37°C for an additional 30 min and layered on the sucrose gradient described above. Pronase alone or serum incubated with pronase had no effect on the sedimentation of either DNA or poly I·poly C.

Antibody assays

Antibodies to DNA and poly I·poly C were measured by an ammonium sulphate assay previously described (Steinberg, Pincus & Talal, 1969). In this method free radioactive nucleic acid is soluble in 35% ammonium sulphate while nucleic acid bound to antibody is precipitated.

RESULTS

Clearance from the circulation

Native and denatured DNA were rapidly cleared from the circulation of anti-DNA antibody negative B/W mice (Fig. 1a). DBA mice showed similar disappearance curves (Fig. 1b). At 1 min, the earliest bleeding, 1 ml of plasma contained 35–50% of administered radioactivity. Since the plasma volume is estimated to be 1–1·5 ml, about one-half of the DNA left the circulation prior to the first bleeding. Thereafter the rate of elimination gradually decreased and the plasma level fell to about 10% of the dose/ml at 60 min. Denatured DNA was cleared more rapidly than native in both strains.

Older B/W mice with high levels of circulating anti-DNA antibodies removed native DNA more rapidly than younger anti-DNA negative animals (Fig. 2). The difference occurred entirely in the first 4 min.

Poly I·poly C was cleared from the circulation at rates similar to that of DNA. Fig. 3 shows the results in B/W mice, immunized with either poly I·poly C or saline. Animals with

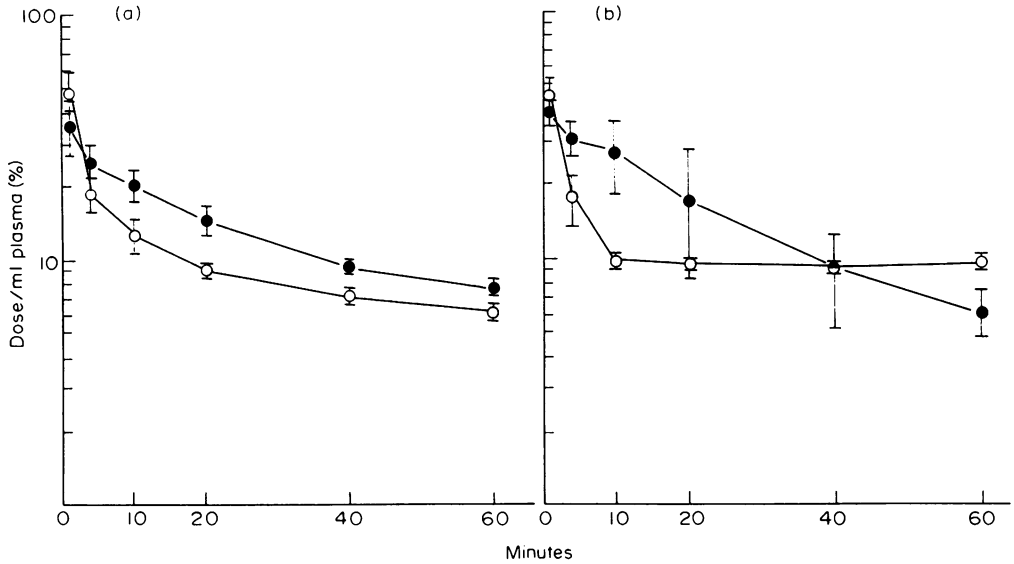

FIG. 1. Clearance of DNA from the circulation. (a) NZB/W females, (b) DBA/2 females. Each point is the mean of five mice with standard deviation shown. ●, Native; ○, denatured.

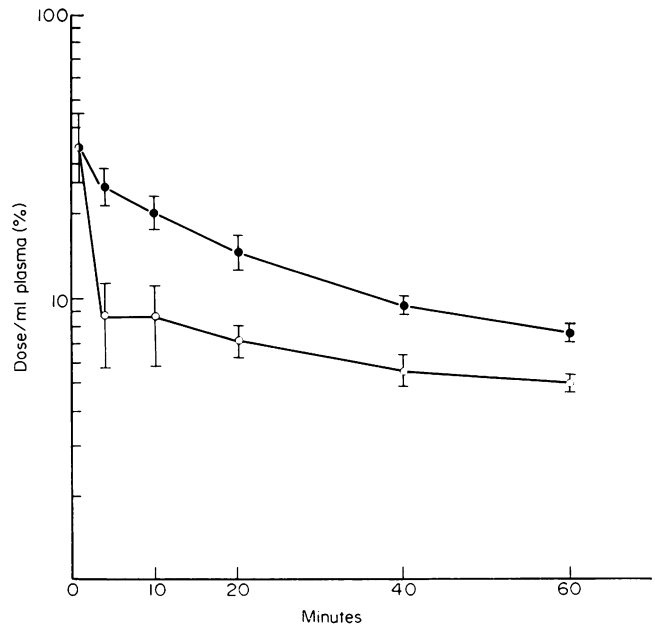

FIG. 2. Clearance of DNA from the circulation. ●, Two-month-old anti-DNA antibody negative NZB/W females. ○, Ten to eleven-month-old anti-DNA antibody positive NZB/W females. Five animals per group. Mean and standard deviation shown.

antibodies to poly I·poly C cleared the RNA more rapidly; however, unlike the elimination of DNA, this difference was observed over the entire time course. Saline immunized animals did not differ from unimmunized controls. Since the 10–30-min time period was most uniform, that interval was used to study the clearance of poly I·poly C by other mouse strains. They were found to be similar, as shown in Table 1.

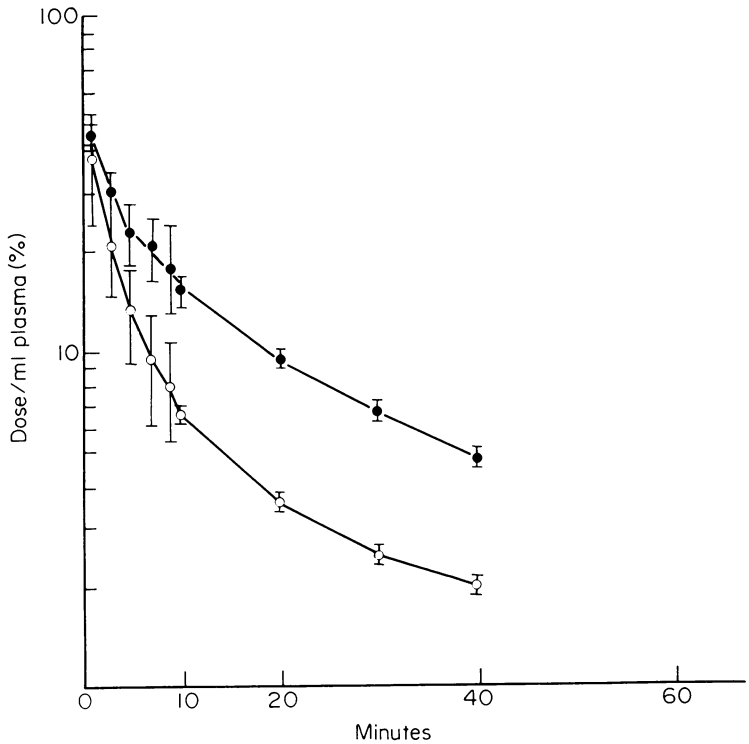

FIG. 3. Clearance of poly I·poly C from circulation of 8-week-old NZB/W mice. ●, Anti-poly I·poly C antibody negative. ○, Anti-poly I·poly C antibody positive. Mean and standard deviation for five animals.

TABLE 1. Clearance of poly I.poly C from circulation

Strain	*t*1/2, 10–30 min
NZB/W	14 min
NZW	19 min
NZB	15 min
BALB/c	15 min
DBA/2	16 min

In order to study the state of nucleic acid in the circulation, a portion of the plasma was made 35% saturated with ammonium sulphate, which precipitates nucleic acid–antibody complexes but not free nucleic acid. In animals with the specific antibody, 25–40% of the nucleic acid was precipitated. For a given antibody concentration and quantitiy of nucleic

acid, the larger the nucleic acid molecules, the greater the percentage precipitated. The ammonium sulphate precipitation study suggests that a considerable fraction of the circulating nucleic acid was of relatively large size.

The nucleic acid-antibody immune complexes were found only during the first 4 min in animals given native DNA but persisted for 30 min in poly I·poly C injected animals. This corresponds to the rapid initial clearance of DNA in immune animals (Fig. 2). No nucleic acid was precipitated in antibody-negative animals.

Guinea-pigs also cleared poly I·poly C rapidly. The half-life between 10 and 30 min was 10 min in unimmunized animals and 7 min in pigs immunized with poly I·poly C in Freund's complete adjuvant.

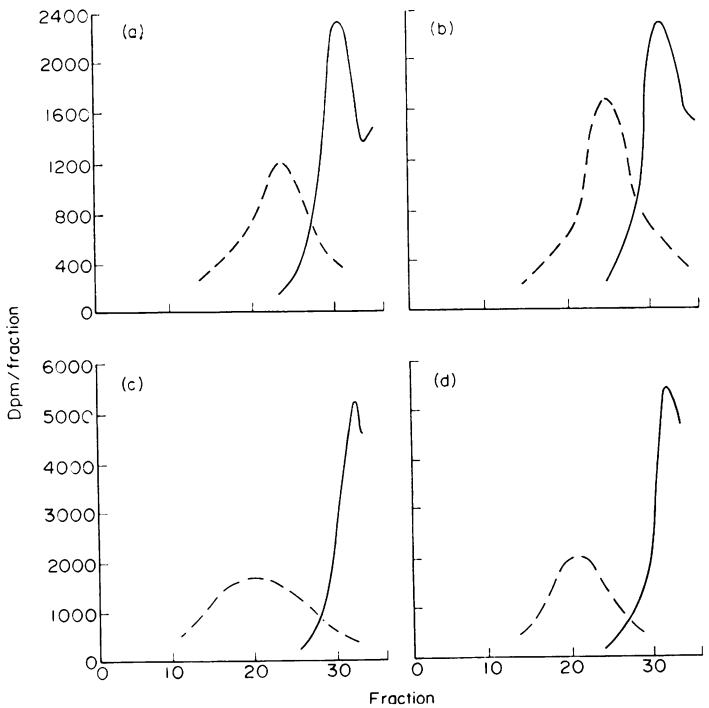

FIG. 4. Effect of serum from young, antibody-negative mice on size of RNA and DNA. Sucrose gradient fractions with top on the right. Dotted lines, no serum present. Solid lines, with serum. Conditions of gradient centrifugation described in Materials and Methods. (a) DBA–poly I·poly C. (b) NZB/W–poly I·poly C. (c) DBA–DNA. (d) NZB/W–DNA.

Role of nucleases

The rapid clearance from the circulation could have resulted from rapid breakdown of the nucleic acids to nucleotides which would be readily taken up by cells throughout the body. To test the role of serum nucleases, fresh, non-haemolysed, B/W and DBA serum pools were incubated *in vitro* with amounts of native DNA or poly I·poly C that would have been present immediately after injection in the above studies. Only 2–3% of the radioactivity was hydrolysed to trichloracetic acid soluble fragments in 30 min—several half-lives

of the material in the circulation. This suggests a negligible role of serum exonuclease activity in the rapid clearance of DNA or RNA from the circulation *in vivo*.

Significant endonuclease activity was demonstrated by the decrease in sedimentation value of both DNA and poly I·poly C after 30-min exposure to serum, as shown in Fig. 4. DNA was degraded to smaller molecules than was poly I·poly C, despite being larger initially. Both, however, were still of macromolecular size (*S* values of 2 to 4), suggesting that in the corresponding *in vivo* studies the polymers were taken up by cells as intact macromolecules.

Localization of DNA

The localization of DNA radioactivity at intervals after injection is shown in Table 2 for young, anti-DNA negative and old, anti-DNA positive, B/W mice. On the basis of per cent of dose per gram of tissue, the radioactivity was concentrated in the liver and spleen, suggesting reticulo-endothelial uptake. Kidney, thymus, heart and lung, as well as lymph node and femur (not shown), took up one-tenth to one-third of that taken up by the liver and spleen. Muscle, adrenal gland, fat, and the cellular elements of the blood each contained 1–2% of the dose per gram, comparable to the low uptake of the lung.

The localization of the radioactivity was followed with time. At 15 min after administration, 28% of the total dpm was found in the liver in young mice and 35% in old, immune mice, corresponding to the more rapid early uptake by the immune mice. By 30 min the activity in the liver was much diminished and at 24 hr it was the same as the non-concentrating organs. The splenic activity remained constant and presumably represents, in part, reutilization of the metabolized DNA through incorporation of labelled thymidine into new cellular DNA.

Under the conditions of these experiments (and the sensitivity of the method) the animals with antibodies to DNA did not deposit more radioactivity in their kidneys than non-immune animals.

Urine collected for 24 hr after injection contained 10% of the radioactivity, all soluble in 3% trichloroacetic acid. An unknown amount of volatile tritium, such as tritiated water, could have been lost through evaporation during collection. Subsequent 24-hr collections contained slowly decreasing amounts of tritium that were proportional to urine volume. The radioactivity remaining in the plasma at 24 hr was eliminated with a half-life of 4–7 days that was inversely related to daily urine volume. These data suggest that the tritium remaining in the circulation after 24 hr was in the form of water.

Localization of poly I·poly C

The localization of poly I·poly C radioactivity in immune and non-immune B/W mice was very similar to that of DNA (Table 3). The RNA was concentrated in the liver and, to a lesser extent, in the spleen and kidneys. Immune animals deposited significantly more radioactivity in the livers but not in the kidneys. It was noted that the RNA-derived radioactivity was eliminated from the liver more slowly than DNA radioactivity.

The hepatic localization of different size poly I·poly C preparations was determined. At 30 min 22% of a preparation with a mean *S* value of 3 was found in the liver, 32% for a mean *S* value of 8, and 50% for a mean *S* value of 12.

Localization of DNA and poly I·poly C in other strains of mice and in guinea-pigs

The localization of both DNA and RNA in DBA/2 and BALB/c mice was studied

TABLE 2. Organ localization of DNA in NZB/W mice*

Age	Anti-DNA antibody	Time	Liver		Kidneys		Spleen		Thymus		Heart		Lungs	
			% Dose/ gram	% Dose/ organ	% Dose/ gram	% Dose/ organ	% Dose/ gram	% Dose/ organ	% Dose/ gram	% Dose/ organ	% Dose/ gram	% Dose/ organ	% Dose/ gram	% Dose/ organ
2 mth	0	15 min	20·1	28·2	4·3	1·8	14·5	1·6	3·6	0·4	2·3	0·3	1·5	0·5
11 mth	+	15 min	13·9	35·1	2·9	1·8	8·0	3·9	1·4	0·2	1·1	0·2	1·5	0·8
2 mth	0	30 min	6·0	8·4	2·8	1·2	18·6	2·2	3·6	0·3	1·7	0·2	0·9	0·2
11 mth	+	30 min	8·6	18·5	2·9	1·8	8·3	3·9	2·1	0·4	1·2	0·2	2·1	1·0
2 mth	0	24 hr	3·7	4·4	3·0	0·8	13·1	0·9	—	—	—	—	—	—
11 mth	+	24 hr	2·4	3·0	2·4	0·4	10·2	0·9	—	—	—	—	—	—

* Standard deviation less than 15% of value.

TABLE 3. Organ localization of poly I·poly C in 8-week NZB/W mice*

Anti-Poly I·Poly C Antibody	Time	Liver		Kidneys		Spleen		Thymus		Heart		Lungs	
		% Dose/ gram	% Dose/ organ	% Dose/ gram	% Dose/ organ	% Dose/ gram	% Dose/ organ	% Dose/ gram	% Dose/ organ	% Dose/ gram	% Dose/ organ	% Dose/ gram	% Dose/ organ
0	10 min	24·2	34·1	6·1	2·2	7·6	0·7	1·6	0·2	2·9	0·4	2·3	0·7
+	10 min	33·8	47·2	5·5	2·2	11·4	1·2	1·4	0·2	2·5	0·4	1·7	0·5
0	30 min	22·8	29·4	7·5	2·6	9·3	1·1	2·6	0·2	4·3	0·5	2·6	0·6
+	30 min	23·3	32·0	8·0	2·7	10·9	1·2	2·3	0·3	4·5	0·5	2·8	0·8
0	2 hr	20·0	27·3	7·2	2·5	13·3	1·9	2·4	0·3	3·9	0·5	1·9	0·6
+	2 hr	18·6	31·7	7·6	2·6	14·1	1·8	2·3	0·2	3·8	0·6	2·4	0·8
0	24 hr	13·5	16·0	8·5	2·6	8·9	0·8	3·4	0·4	3·1	0·4	3·5	0·6
+	24 hr	14·2	16·0	7·6	2·0	12·2	1·1	3·9	0·4	3·0	0·4	3·6	0·6

* Standard deviation less than 15% of value

similarly and did not differ from the results presented for B/W mice. The Hartley strain guinea-pigs also concentrated poly I·poly C in liver and spleen. At 30 min the liver contained 37–55% of the dose and the spleen 2–5%. The immune animals deposited more in their livers but not in their kidneys.

DISCUSSION

Tsumita & Iwanaga (1963) observed that mice rapidly cleared native DNA from the circulation. This study confirms their finding and extends it to denatured DNA and poly I·poly C, and to several strains of mice. We have found that 50% or more of intravenously injected nucleic acid left the circulation within 1 min and 90% in 20 min. Differences among the three nucleic acids were small, although it was noted that denatured DNA and poly I·poly C were cleared slightly faster than native DNA.

Ledoux (1965) has demonstrated DNA of macromolecular size in the circulation up to 30 min after injection. In the present study nuclease activities were measured directly and could not explain the rapid clearance of DNA and poly I·poly C. Exonuclease activity was found to be negligible. Endonuclease activity was measured during a 30-min incubation, an interval that is several times the survival of nucleic acid in the circulation. Although present, endonucleases only reduced the size of the polymers to 2–4S. In immune animals significant amounts of the injected polymer are precipitated by the endogenous antibody indicating that the circulating nucleic acid is of relatively large size. These observations suggest that DNA and poly I·poly C are taken up by cells as intact macromolecules. This is further supported by the finding that the greater the mean *S* value of the poly I·poly C preparation the greater the uptake by the liver. Thus DNA and poly I·poly C appear to be actively removed from the circulation as macromolecules.

Both nucleic acids were localized primarily in the liver and spleen, suggestive of reticuloendothelial uptake. Some poly I·poly C, but little DNA, was found in the kidneys. This may represent relatively low molecular weight material that passed the glomeruli and was hydrolysed by the RNAse on the brush border of the proximal tubular cells (Zankowalczewska, Sierakowska & Shugar, 1966) and then reabsorbed.

Animals with the appropriate antibody showed immune elimination of both native DNA and poly I·poly C. The immune phase of elimination was more rapid with DNA, perhaps because it was ten to twenty times the size of the poly I·poly C. (Because of its rigidity a DNA molecule with the same *S* value as an RNA molecule is many times heavier.) Circulating complexes were precipitated by ammonium sulphate during the period of immune elimination.

Although immune animals deposited more DNA and poly I·poly C radioactivity in their livers, the renal deposition was the same as in non-immune mice. There are several possible reasons for the failure to observe increased renal deposition in immune animals in these acute experiments: (1) The size or antigen–antibody ratio of the complexes formed after nucleic acid injection may not have been optimal. (2) Vasoactive amines or other mediators may be necessary. (3) Partial impairment of the reticuloendothelial system may be a prerequisite. (4) Deposition of a smaller fraction of the circulating complexes than could be detected by our methods over a long period of time may be sufficient to cause the naturally occurring glomerulonephritis. These possibilities are currently being investigated.

Nucleic acid metabolism is quite similar to that of endotoxin. Endotoxin is also cleared

from the circulation in minutes and is found primarily in the liver and to a lesser extent in the spleen (Chedid *et al.*, 1966). In addition, both nucleic acid and endotoxin are immunologic adjuvants (Johnson, Gaines & Landy, 1956) and interferon inducers (Steinbring & Youngner, 1964). Perhaps these similarities are related to the fact that both are large molecules with a marked negative charge.

We have been interested in nucleic acids as antigens. They are immunogenic in Freund's complete adjuvant in several strains of mice (Steinberg, Pincus & Talal, 1971) and in guinea-pigs (van Boxel, Steinberg & Green, 1971). NZB/W F_1 hybrid mice are unique in that they spontaneously develop antibodies to both RNA and DNA and are easily immunized by RNA (given without adjuvant) and by denatured DNA. The present studies have shown that they clear nucleic acids from the circulation at the same rate as normal mice, localize them in the same organs and to the same extent, and demonstrate the same serum nuclease activity. Further, there appears not to be a sizeable endogenous pool of nucleic acids in the circulation, for such a pool should have competed for uptake with administered nucleic acid, thereby decreasing the clearance of the radioactive material. In conclusion, there is no evidence that the immunologic hyper-responsiveness to nucleic acids of New Zealand mice is mediated by abnormal clearance and degradation thereof. It is more likely related to a primary immunologic abnormality (Steinberg *et al.*, 1969; Staples & Talal, 1969).

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