artery may be an inadequate means of inducing renal hypoxia, or that insufficient quantities of erythropoietin are engendered by unilateral renal hypoxia over a 24 hour period. A moderately severe reduction in renal artery pressure may, by an interpretation of the "cell separation theory," cause an increase in the cell percentage of the normally cell-poor fraction of blood circulating through the peritubular epithelium, and the tissue would therefore receive an adequate supply of oxygen. A more severe and uncompensated restriction in arterial flow might result in failure of tubular function and inability to secret erythropoietin. However, there is evidence (unpublished data) that the juxaglomerular apparatus secreted renin in a group of rats having chronically and severely ligated kidneys. An extended rise in blood pressure was noted in these animals. Since ischemia was seen in even the series of moderately restricted kidneys, the tubular epithelium may very likely have been hypoxic.

Summary. Plasma taken from rats having moderately constricted or severely constricted right renal arteries failed on 2 occasions to induce a significant increase in the 18 hour RBC uptake of Fe^{59} when compared with similarly treated controls that received plasma from unoperated animals. Microcapillary hematocrit values were also the same for the experimental and control assay groups.

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Effect of Ribonucleic Acid Extracts upon Viability of Skin Homografts in the Rat.* † (26993)

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The phenomenon of "actively acquired tolerance" has been described as an induced state in which tissue homografts become immunologically acceptable to hosts which normally would react against them(1). In these now classic experiments, tolerance of skin grafts was produced by exposure of the hosts to foreign homologous tissue cells sufficiently early in foetal life. Further studies have demonstrated the induction of actively acquired tolerance of homologous tissues in newborn mice(2) and rats(3). An extensive series of investigations by the Minnesota group has provided ample evidence that in-

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† Abbreviations: EDTA, ethylenediaminetetraacetate; Tris, tris (hydroxymethyl) aminomethane; ATP, adenosine triphosphate.

duction of tolerance may also be observed in more mature animals(4-6).

In the above reports tolerance was produced by administration of viable cells or through parabiotic union. Attempts to produce tolerance with cellular extracts have generally proven unsuccessful(7-9). Accordingly, the recent study of Ashley *et al.* (10) in which tolerance of skin grafts was induced in neonatal rats by administration of ribonucleic acid (RNA) extracts was of considerable interest. In the present study, we have attempted with various modifications to reproduce these results with olders rats.

Methods and Materials. Animals and diets. Male, weanling rats of the Long-Evans strain and male rats of the Holtzman strain, weighing 180-200 g, purchased from commercial sources, were housed in individual cages. The Long-Evans animals, serving as skin recipients, were fed a purified, basal diet and daily vitamin pills described previously(11). Holtzman animals, serving as skin and RNA donors, were fed a commercial stock ration (Purina chow). All diets were consumed *ad libitum*. Solutions of deoxypyridoxine (25 μg/ml) and pyridoxine (1 mg/ml) were prepared in isotonic sodium chloride and adjusted to pH 7.0 for intraperitoneal injection.

Grafting procedure. Pieces of skin of full thickness, measuring 2.5 $\times$ 3.5 cm, were excised from the anterior abdominal wall of the donor animal and applied to the host area as described previously(12). In the present experiments, the recipient bed was prepared by removing a similar-size piece of skin from the mid-dorsum as described by Hansen and Hubay(13). Pressure dressings were applied for one week subsequent to grafting. A protective dressing consisting of a Telfa pad held in position by narrow strips of adhesive was maintained during the remainder of the experiment. The criteria utilized to judge survival of a skin graft have been presented elsewhere(12,14).

Preparation of RNA extracts. Approximately 85% of the spleen was removed from prospective donor rats, frozen on solid carbon dioxide, and stored at -5°C until extraction one week later. RNA was extracted from the various cellular fractions described below by the phenol method of Gierer and Schramm(15) and residual ether removed from the final extracts by bubbling with nitrogen. All extracts were adjusted to pH 7.0 prior to injection.

1) *Spleen homogenate.* A 10% (w/v) homogenate of spleen in a solution of 0.01 M sodium citrate and 0.14 M sodium chloride of pH 7.0 was prepared from minced spleen with a hand homogenizer.

2) *Cytoplasmic Fraction.* This fraction was prepared by the method of Colter and Brown (16).

3) *Mitochondrial, Microsomal, and Soluble Fractions.* These fractions were obtained by differential centrifugation at 0°C of a splenic homogenate prepared as described under Section 1 above. The method utilized in the present study incorporated various features of procedures employed for isolation of liver subcellular fractions(17-19) and was conducted as follows. The supernatant resulting from centrifugation of the splenic homogenate at 2000 $\times$ g for 10 minutes was centrifuged at 6590 $\times$ g for 20 minutes to yield a mitochondrial precipitate and a supernatant fraction. Centrifugation of the latter at 105,000 $\times$ g for 80 minutes precipitated a microsomal pellet. The remaining supernatant constituted the soluble fraction.

Analytical procedures. 1) RNA was determined by the orcinol reaction(20) and protein by the method of Lowry *et al.*(21). 2) For electron microscopy, the microsomal pellet was fixed in buffered osmium tetroxide, dehydrated and embedded in Vestopol W. Sections displaying gold interference colors were examined in a Philips 200 electron microscope. 3) *Cholinesterase assay.* Cholinesterase activities of a splenic homogenate, mitochondrial and microsomal fractions of spleen were determined by a null point potentiometric titration procedure conducted with a pH-stat.‡ The splenic homogenate was prepared by homogenizing one gram of spleen in 10 ml of a solution of 0.01 M sodium citrate and 0.14 M sodium chloride of pH 7.0. Total mitochondrial and microsomal pellets prepared as described above by differential centrifugation of the homogenate were homogenized in 10 ml of a pH 7.4 buffer

‡ TTTI b Titrator Radiometer, Cophenhagen.

TABLE I. Analytical Data of Various Splenic Fractions.[1]

Fraction	Mg RNA/ 100 mg spleen[2]	Mg RNA/mg fraction protein	Specific cholinesterase activity[5]
Original Homogenate	.55	.033	.017
Cytoplasmic[3]	.38	.058	
Microsomal[4]	.17	.17	.22
Mitochondrial[4]	.05	.02	.038
Soluble[4]	.35	.05	

[1] RNA was determined in extracts prepared from the various fractions by the phenol method.
[2] Fresh weight.
[3] Isolated by method of Colter and Brown(16).
[4] Isolated by differential centrifugation of original homogenate.
[5] Expressed as μmoles of substrate hydrolyzed per min. per mg of protein.

consisting of 0.1 M tris, 0.001 M EDTA and 0.001 M ATP. The final reaction volume (4.0 ml) consisted of 0.2 ml of the mitochondrial or microsomal homogenate, 0.2 ml of 0.25 M $MgCl_2$, 0.4 ml of 0.03 M acetylcholine iodide and 3.2 ml of ion-exchanged distilled water. An aliquot (0.2 ml) of the original homogenate was assayed in an identical fashion with the exception that 0.2 ml of water was replaced by an equal volume of the tris buffer. Titrations were performed with 0.30 N NaOH at 30°C and pH 7.4.

Experiments and results. Analytical data obtained for the various splenic fractions are presented in Table I. Determinations of both cholinesterase activity and RNA content proved useful in assessing purity of the mitochondrial and microsomal fractions. The validity of the cholinesterase measurements as a microsomal marker derives from observations that this enzyme is concentrated in the microsomal fraction of brain(22,23) and muscle.§ With the assumption that mitochondria are free of RNA and cholinesterase, one can calculate that this fraction prepared by our procedure is contaminated with either 11% (RNA data) or 17% (cholinesterase data) of microsomes. Thin sections of the microsomal pellet examined in the electron microscope consisted of granules approximately 150Å in diameter and some rough-surfaced membranes characteristic of a microsomal fraction. No mitochondria were observed in sections of the microsomal pellet.

§ Unpublished studies of Drs. J. Smith and R. Basford of these laboratories have demonstrated that rat brain and muscle mitochondria contain 5% of microsomes as determined by cholinesterase assay.

Fig. I depicts growth responses of Long-Evans recipient rats. Following a 2-week period during which weanling rats consumed a diet furnishing 10 μg of pyridoxine daily, the animals were fed a pyridoxine-free diet and given daily intraperitoneal injections of 25 μg of the pyridoxine antagonist, deoxypyridoxine, per 100 g of body weight for 3 weeks. During this time, growth ceased and skin symptoms characteristic of pyridoxine deficiency appeared. After 2 weeks of pyridoxine deprivation, each experimental animal received a single, intraperitoneal injection of RNA extracted from the total cellular fraction (Table II) derived from the spleen of its prospective skin donor. Each RNA extract was obtained from a separate spleen. One week following RNA administration, deoxypyridoxine treatment was discontinued and the animals transferred to a dietary regimen furnishing 50 μg of pyridoxine daily and given 3 daily intraperitoneal injections of one mg of pyridoxine per 100 g of body weight. After one week of pyridoxine therapy which produced immediate growth responses and gradual alleviation of the skin symptoms, the Long-Evans animals received skin homografts from Holtzman rats. Thus, one Holtzman rat donated both skin and RNA extract to a single Long-Evans recipient. For the remainder of the experiment,

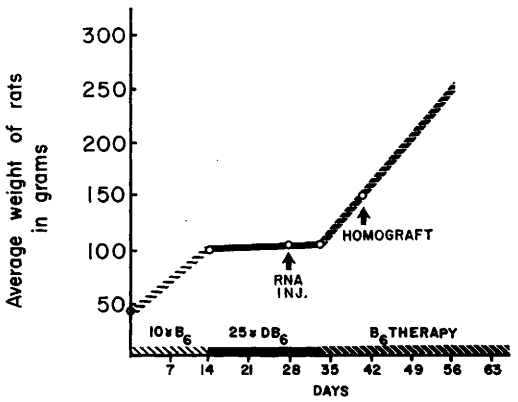

FIG. 1. Growth responses of Long-Evans recipient rats. DB_6 refers to deoxypyridoxine.

TABLE II. Effect of RNA on Viability of Skin Homografts.[1]

Source of splenic RNA	No. of recipients	No. of exp.	RNA (μg)/100 g body wt[2]	% homograft survival (weeks after grafting) 2	3	4	6	8	12
(Controls)	175	17	0	0					
Original Homogenate	40	4	1500-1800	30	0				
Cytoplasmic Fraction	20	2	1170-1500	30	0				
Soluble "	20	2	1250-1580	0					
Microsomal "	10		455-590	100	60	20	0		
	8		327-490	100	100	87	37	0	
	10		750-792	50	0				
	10		490-580	100	100	100	80	40	10[3]
	10		400-450	100	100	70	20	10	10[3]
	10		490-580	100	100	60	30	0	
	12		490-580	100	100	100	25	8	8[3]
	10		490-580	100	100	100	50	10	10[3]
	15[3]		490-580	100	100	—			

[1] Results of individual experiments with microsomal RNA are presented. Composite data are given for those groups receiving RNA derived from other fractions and for control groups.
[2] Range of values is presented.
[3] Animals still under observation.

the recipient animals received the purified diet furnishing 50 μg of pyridoxine daily. Except for omission of RNA, treatment of the control Long-Evans recipients was identical with that described above. Equal numbers of control and experimental recipients were utilized in every experiment.

The effect of RNA extracts upon the viability of skin homografts is shown in Table II. In controls, initial signs of rejection were evident 10 days after grafting and in 175 animals the skin homografts were completely rejected within 2 weeks. In no case did a control homograft survive beyond this period. Thirty per cent of the skin homografts in animals treated wih RNA extracted from the original homogenate and the cytoplasmic fraction were still viable 2 weeks after grafting. However, all skin homografts in these groups, as well as in the one receiving RNA derived from the soluble fraction, were rejected within 3 weeks after homografting. In 8 separate experiments, however, 57 of 80 skin homografts in rats receiving RNA extracts from the microsomal fraction were in excellent condition 4 weeks after grafting. All grafts in the last experiment, still in progress, are surviving at 3 weeks. Unfortunately, the inordinate degree of graft mutilation beyond the 4-week period makes it difficult to assess the effects of RNA upon further prolongation of the skin homografts. In some experiments as high as 70% of the grafts surviving at 4 weeks were lost in this manner. We are at a loss to account for this high degree of graft mutilation which has not been observed over a 4-year period during which homotransplantation experiments have been conducted in our laboratory. Our extensive experience with skin homografts(12, 14) would lead us to expect a high survival rate in grafts which are in such excellent condition at 4 weeks. The percentage survival figures (Table II) are based upon the number of original grafts in each experiment, and, accordingly, present a conservative estimate of the RNA effect.

Discussion. Data presented here have indicated that the viability of skin homografts can be enhanced if the recipient is pre-treated with microsomal RNA extracts while in a state of pyridoxine deficiency. This beneficial effect of RNA agrees with the findings of Ashley *et al.*(10) in neonatal rats. The inefficiency of large doses of RNA derived from the original splenic homogenate or the cytoplasmic fraction has no ready interpretation. The presence of molecular species of RNA in these fractions which are antagonistic to microsomal RNA may afford a pos-

sible explanation. There is also some basis for the belief that the dosage of RNA may be a critical factor. This possibility gains credence from the observation that, in one experiment (Table II), higher doses of microsomal RNA were ineffective. This question can only be resolved by further experimentation with varying doses of microsomal RNA administered singly or in combination with RNA derived from other fractions. Clarification of the specificity of RNA will derive from studies in which RNA of the extracts is selectively removed by the action of ribonuclease.

The role of microsomal ribonucleoproteins in protein synthesis has been documented (24) and a variety of experiments have indicated that administration of RNA or ribonucleoprotein may lead to *in vivo* protein synthesis(25-28). It may, therefore, be presumed that administration of microsomal RNA in the present study leads to the formation of donor antigens in the host. For induction of actively acquired tolerance, antigenic stimuli are generally applied to the host while it is in a state of immunological unresponsiveness. For this purpose, various experimentors have utilized the foetal or newborn animal(1-3) as well as older recipients treated with cortisone(29), x-irradiation(30) or 6-mercaptopurine(31). We administered RNA during a period of pyridoxine deficiency since previous studies have shown that this deficiency can produce a desired state of immunological inertness(32). The experimental approach of the present study was, therefore, designed to simulate the more classical methodology utilized for induction of acquired tolerance. On the basis of his nucleation theory of antibody production, Goldstein(33) has suggested a mechanism for production of acquired tolerance by the use of antimetabolites. Our findings could be interpreted within the framework of this rationale.

Summary. Administration of an RNA extract prepared from the microsomal fraction of splenic cells increased the viability of skin homografts. RNA derived from a normal, adult rat which later served as the skin donor was administered to a pyridoxine-deficient rat of another strain. Skin homografting was performed after the RNA-treated recipient had received intensive therapy with pyridoxine.

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