ginal protein. Further studies on the protein moiety, and its possible role in endotoxin tolerance, are in progress. That the *in vivo* biological properties of endotoxins may be mediated by distinctive antigenic properties of the intact macromolecule(19) must be considered.

Summary. The influence of endotoxins of varying nitrogen content on water intake and numbers of hemolysin-forming spleen cells in mice and on delayed skin reactivity in rabbits has been investigated. Pretreatment with an aqueous-ether endotoxin of *S. enteritidis* results in heightened reactivity to a subsequent test dose of the aqueous-ether or protein-free (Ribi) endotoxin, whereas pretreatment with the protein-free endotoxin fails to modify subsequent reactivity to either endotoxin. Similarly, pretreatment with the Boivin endotoxin of *S. abortus equi* increases reactivity on subsequent test with the Boivin or Westphal endotoxin, whereas pretreatment with the partially deproteinized Westphal endotoxin does not enhance reactivity to subsequent test with either endotoxin. The implications of these findings for the hypothesis that adult host reactivity to endotoxins is contributed to by an acquired delayed hypersensitivity are discussed.

1. Bacterial Endotoxins, M. Landy, W. Braun, ed., Rutgers Univ. Press, 1964.

2. Molecular Biology of Gram-Negative Bacterial Lipopolysaccharides, A. Nowotny, ed., Ann. N. Y. Acad. Sci., 1966, v133.

3. Westphal, O., in Polysaccharides in Biology, 2nd Conf., Joshiah Macy, Jr., Foundation, 1957, p115.

4. Ribi, E., Anacker, R. L., Fukushi, K., Haskins, W. T., Landy, M., Milner, K. C., in Bacterial Endotoxins, M. Landy, W. Braun, ed., Rutgers Univ. Press, 1964, p16.

5. Atkins, E., Physiol. Rev., 1960, v40, 580.

6. Zweifach, B. W., Janoff, A., Ann. Rev. Med., 1965, v16, 201.

7. Ribi, E., Haskins, W. T., Landy, M., Milner, K. C., J. Exp. Med., 1961, v114, 647.

8. Schaedler, R. W., Dubos, R. J., ibid., 1961, v113, 559.

9. Watson, D. W., Kim, Y. B., in Bacterial Endotoxins, M. Landy, W. Braun, ed., Rutgers Univ. Press, 1964, p552.

10. Stetson, C. A., Jr., Bact. Rev., 1961, v25, 457.

11. ———, in Bacterial Endotoxins, M. Landy, W. Braun, ed., Rutgers Univ. Press, 1964, p658.

12. Uhr, J. W., Physiol. Rev., 1966, v46, 359.

13. Holborow, E. J., Loewi, G., Brit. Med. Bull., 1967, v23, 72.

14. Freedman, H. H., Fox, A. E., Schwartz, B. S., Proc. Soc. Exp. Biol. & Med., 1967, v125, 583.

15. Freedman, H. H., Nakano, M., Braun, W., ibid., 1966, v121, 1228.

16. Dubos, R. J., Schaedler, R. W., J. Exp. Med., 1961, v113, 921.

17. Fox, A. E., Schwartz, B. S., Freedman, H. H., J. Reticuloendothelial Soc., 1966, v3, 48.

18. ———, Bact. Proc., 1967, M116.

19. Freedman, H. H., Sultzer, B. M., in Bacterial Endotoxins, M. Landy, W. Braun, ed., Rutgers Univ. Press, 1964, p537.

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Some Factors Affecting the Interferon-Induced Antiviral State. (32347)

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The interferon system may be composed of 2 distinct cellular proteins. The first is the interferon protein which is induced by viral infection(1) and which has been interpreted to react with cells to induce a second substance—the proposed intracellular, antiviral protein(2). The present communication will consider some of the factors which influence the interferon-induced, second component of the interferon system—the antiviral protein.

Materials and methods. Interferon was produced in the serum of NIH strain mice by intravenous injection of Newcastle disease virus, as previously described(3). It was

desirable to measure interferon-induced antiviral activity at a time as close to the time of virus challenge as possible. For this purpose antiviral activity was determined by inhibition of yield of vesicular stomatitis virus, Indiana strain (VSV), in a single growth cycle rather than the plaque reduction assay because the latter requires multiple virus growth cycles over several days for interferon assay. It is likely that this method determines antiviral activity close to the time of VSV infection since the antiviral effect, induced by interferon can inhibit early steps in viral replication(4). The unit of interferon is defined as the highest dilution of the sample which decreases the yield of VSV by 0.5 $\log_{10}$ in a 1-step growth cycle. For the present assay of mouse interferon and for determination of the level of antiviral activity, primary mouse embryo cells (ME) were cultured in loosely covered 16 $\times$ 150 mm tubes in 1.0 ml of medium in a vertical position in a CO_2 incubator at 37°C until a confluent and non-dividing cell population developed. Thereafter cultures were maintained at 33°C in Eagle's medium containing 10% skim milk for 3-5 days. Experimental procedures utilized 3 cultures per point, Eagle's medium containing 2% fetal bovine serum and a temperature of 37°C. Dilutions of interferon were applied, and at the intervals described for each experiment the cultures were washed 3 times and challenged with 0.2 ml of a VSV preparation containing an input of at least 20 plaque-forming units (pfu) of VSV per cell. After one hour at 37°C the cultures were rinsed 4 times, refed with maintenance medium and reincubated for 14 to 24 hours. Culture fluids were then harvested and assayed for virus content by plaque assay on mouse L cell cultures. Control experiments demonstrated that VSV yield was maximum at 12 hours, and virus titers remained constant through 30 hours. Fluids from the 3 replicate cultures were pooled before assay. Inhibition of 0.5 $\log_{10}$ yield of virus was determined to be significant. The NIH reference mouse interferon titered 500 units/ml in this assay system.

Rates of cellular protein or RNA synthesis were determined as follows. ^{14}C phenylalanine in phenylalanine-free medium or ^{3}H uridine (New England Nuclear Corp.) were added to triplicate cultures of cells, to a concentration of 0.1 μc/ml. The cells were incubated at 37°C for 45 minutes, the medium poured off, the cells washed 3 times with phosphate buffered saline, and scraped into 1 ml of 10% trichloracetic acid. The cells were washed twice with 10% TCA, and dissolved in 1 ml of 1 N NaOH. 0.4 ml was acidified, filtered onto a Schleicher and Schuell membrane filter #B6 and counted in a Packard Tricarb scintillation counter. Protein contents were determined on 0.2 ml of the NaOH solution by the method of Lowry *et al*(5).

Results. Kinetics of induction of intracellular antiviral activity. The time course of development of antiviral activity by cultured mouse embryo cells exposed to varying amounts of interferon is shown in Fig. 1. The level of antiviral activity is expressed as $\log_{10}$ inhibition of VSV yield. It may be seen that the time of onset of antiviral activity is directly correlated with units of interferon applied. The rate of increase of antiviral activity at each dose level began to diminish at 7 hours. These findings are similar to those reported for mouse L cells exposed to interferon(6). After 7 hours the level of antiviral activity at each dose level tended to remain at a fixed level which was related to the dose of interferon. Previous studies have shown that in this experimental system, the extracellular concentration of interferon does not detectably decrease during the development and maintenance of the antiviral state (see ref. in 7-9). However, it should be noted that the amount of interferon has been observed to decrease under the experimental conditions of certain laboratories(10-13).

Effect of dose of interferon on antiviral activity. As may be seen in Fig. 1 the final level of resistance to virus was directly related to the dose of interferon up to 16 units of interferon per ml. The apparent lack of increased inhibition of VSV by doses greater than about 16 units per ml was shown to be due to unremoved challenge virus. With in-

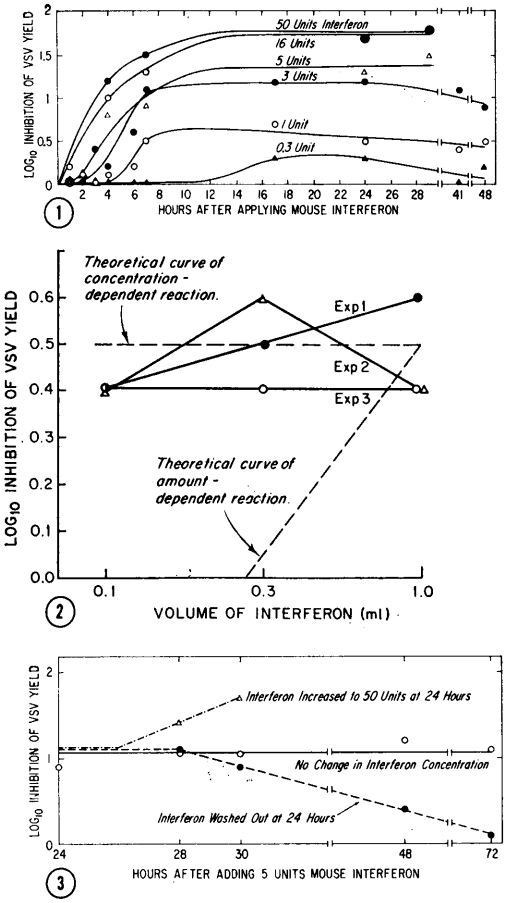

FIG. 1. Time course of development of resistance to multiplication of vesicular stomatitis virus, by cultures of mouse embryo cells treated with varying amounts of interferon.

FIG. 2. Relationship of concentration of interferon and total amount of interferon to degree of antiviral activity.

FIG. 3. Effect of changing concentration of interferon on preexisting level of resistance to vesicular stomatitis virus by mouse embryo cultures pretreated with interferon.

creased washing to remove more of the unadsorbed virus inoculum, inhibition of VSV increased through at least 100 units/ml of interferon. These findings indicate that the degree of resistance to viruses by ME was directly proportional to the dose of interferon.

To help determine whether resistance was dependent on the concentration or total amount of interferon, cultures were exposed to different volumes of interferon (0.1 ml-1.0 ml) at a fixed concentration of 1 unit/ml. As may be seen in Fig. 2 the degree of antiviral activity was dependent on the concentration applied and was largely independent of the total amount of interferon. Similar concentration dependency has been observed independently by Youngner and Hallum(14). However, the finding that the degree of antiviral activity is independent of the amount of interferon has not been observed under all experimental conditions(15,16).

Effect of changing concentration of interferon in the extracellular fluid on established antiviral activity. Experiments were done to determine whether the continued presence of a uniform concentration of interferon in the fluid was required for maintenance of established antiviral activity.

ME were treated with 5 units/ml of interferon (enough to inhibit VSV yield 1.2 $\log_{10}$) for 24 hours at 37°C so that their antiviral activity had reached a steady level. A group of cultures was then washed 5 times with Earle's balanced salt solution to remove interferon and refed Eagle's medium containing 2% fetal bovine serum (maintenance medium). Fig. 3 shows that following removal of extracellular interferon there was no detectable loss of antiviral activity during the first 7 hours of continued incubation at 37°C in the absence of interferon. Thereafter the level of antiviral activity decreased but at a slower rate than the initial rise of antiviral activity (Fig. 1). This rate of decline is comparable with previous reports of decline of antiviral activity following removal of interferon(6,17,18).

In the same experiment another group of cultures were also pretreated with 5 units of interferon for 24 hours. The interferon was then replaced by a 10-fold higher concentration of interferon (50 units/ml), and the level of antiviral activity was determined 4 and 6 hours later. It may be seen that the degree of antiviral activity increased at 4 and 6 hours (Fig. 3). The degree of activity at 6 hours was equal to that of cultures which had not been pretreated with interferon but which were exposed to 50 units/ml of interferon for 6 hours.

The findings demonstrate that prolonged maintenance of relatively stable antiviral activity required the continued presence of the

TABLE I. Effect of Inhibition of Functional Cellular Protein Synthesis by FPA on Established Antiviral Activity.

Pretreatment for 24 hr	2nd treatment	Time of 2nd treatment before wash & VSV challenge (hr)	Yield of VSV $\log_{10}$	Reduction of VSV yield $\log_{10}$	% Reduction of VSV yield
Medium	None	0	6.7	—	—
Interferon	"	0	5.1	1.6	97.5
Medium	Medium	2	6.7	—	—
Interferon	Interferon	2	5.1	1.6	97.5
Medium	FPA*	2	6.6	—	—
Interferon	Interferon + FPA	2	4.8	1.8	98.3
Medium	Medium	4	6.7	—	—
Interferon	Interferon	4	5.2	1.5	97.0
Medium	FPA	4	6.7	—	—
Interferon	Interferon + FPA	4	4.7	2.0	99.0
Medium	Medium	6	6.8	—	—
Interferon	Interferon	6	4.9	1.9	98.7
Medium	FPA	6	6.5	—	—
Interferon	Interferon + FPA	6	4.6	1.9	98.7
Medium	Medium	24	7.0	—	—
Interferon	Interferon	24	4.8	2.2	99.4
Medium	FPA	24	6.4	—	—
Interferon	Interferon + FPA	24	5.8	.6	75.0

* FPA = 60 μg/ml fluorophenylalanine in phenylalanine-free medium.

TABLE II. Effect of Inhibition of Cellular Protein Synthesis by Cycloheximide on Established Antiviral Activity.

Pretreatment for 24 hr	2nd treatment	Time of 2nd treatment before wash & VSV challenge (hr)	Yield of VSV $\log_{10}$	Reduction of VSV yield $\log_{10}$	% Reduction of VSV yield
Medium	None	0	7.0	—	—
Interferon	"	0	4.8	2.2	99.4
Medium	Medium	4	6.6	—	—
Interferon	Interferon	4	4.4	2.2	99.4
Medium	Cycloheximide	4	6.6	—	—
Interferon	Interferon & cycloheximide	4	4.4	2.2	99.4
Medium	Medium	7	6.8	—	—
Interferon	Interferon	7	4.4	2.4	99.6
Medium	Cycloheximide	7	6.4	—	—
Interferon	Interferon & cycloheximide	7	4.4	2.0	99.0
Medium	Medium	16	6.4	—	—
Interferon	Interferon	16	4.6	1.8	98.3
Medium	Cycloheximide	16	6.0	—	—
Interferon	Interferon & cycloheximide	16	5.0	1.0	90.0
Medium	Medium	24	6.2	—	—
Interferon	Interferon	24	4.5	1.7	98.0
Medium	Cycloheximide	24	5.9	—	—
Interferon	Interferon & cycloheximide	24	5.0	.9	87.0

initial concentration of interferon in the extracellular fluid of ME.

Effect of inhibition of cellular protein synthesis on established antiviral activity. To help determine whether protein synthesis was required for maintenance of established antiviral activity, ME were treated with interferon (100 units/ml) overnight at 37°C so that antiviral activity had reached a constant level. Inhibitors of protein synthesis were

TABLE III. Effect of Inhibition of Cellular RNA Synthesis by Actinomycin D on Established Antiviral Activity.

Pretreatment for 24 hr	2nd treatment	Time of 2nd treatment before wash & VSV challenge (hr)	Yield of VSV $\log_{10}$	Reduction of VSV yield $\log_{10}$	% Reduction of VSV yield
Exp 1					
Medium	Medium	24	7.5	—	—
Interferon	Interferon	24	5.4	2.1	99.2
Medium	Actinomycin D*	24	5.3	—	—
Interferon	Interferon + actinomycin D	24	4.7	.6	75.0
Exp 2					
Medium	Medium	21	7.4	—	—
Interferon	Interferon	21	5.3	2.1	99.2
Medium	Actinomycin D	21	5.8	—	—
Interferon	Interferon + actinomycin D	21	4.8	1.0	90.0
Medium	Medium	24	7.4	—	—
Interferon	Interferon	24	5.4	2.0	99.0
Medium	Actinomycin D	24	5.6	—	—
Interferon	Interferon + actinomycin D	24	4.4	1.2	93.7

* 0.1 μg/ml.

added to the cultures, and at the intervals shown in Tables I and II, the cultures were washed 3 times to remove the inhibitor and interferon and then challenged with a high multiplicity of VSV. The results demonstrate that inhibition of (functional) protein synthesis by 60 μg/ml DL-p-fluorophenylalanine (FPA) (in phenylalanine-free medium) or cycloheximide for 16 or more hours in the continued presence of interferon decreased the degree of established resistance to virus. Multiplication of VSV after removal of the inhibitors indicated that the effect of inhibitors could be fully reversed for virus multiplication up to 7 hours after application. At later times reversal of inhibitors was not complete although the yield of VSV was still high.

Effectiveness of the inhibitors against protein synthesis was shown biochemically and biologically. As measured by incorporation of ^{14}C-phenylalanine into protein, FPA (60 μg/ml) inhibited protein synthesis 60% at 4 hours, 61% at 6 hours, and 74% inhibition at 24 hours. At the same times there was no inhibition and sometimes enhancement of incorporation of ^{3}H uridine. Similarly 10 μg/ml of cycloheximide was followed by 93% inhibition of incorporation of ^{14}C-phenylalanine at 1 hour. In a biological system both inhibitors suppressed multiplication of VSV more than 1000-fold in a single growth cycle.

Similar FPA experiments with chicken interferon in chick embryo cultures showed that preestablished resistance of 0.7 $\log_{10}$ inhibition of VSV was entirely abolished by treatment for 6 hours with 120 μg/ml FPA for 6 hours.

Effect of inhibition of cellular RNA synthesis on established antiviral activity. To help determine whether RNA synthesis was required for maintenance of antiviral activity, ME were treated with 100 units/ml interferon overnight at 37°C so that resistance to virus had reached a constant level. Actinomycin D (0.1 μg/ml) was then added to the cultures, and at the intervals shown in Table III the cultures were washed 3 times to remove extracellular inhibitor and interferon. The cultures were challenged with a high multiplicity of VSV. Treatment with actinomycin D up to 8 hours did not lead to a measurable decrease in cellular resistance to VSV. Treatment for 21-24 hours did significantly decrease resistance to VSV as shown in Table III.

Challenge of ME with VSV at intervals after addition of actinomycin D indicated that the cells lost the ability to support fully multiplication of VSV after 21 hours' treatment with actinomycin D (Table III), although treatment for up to 5 hours did not inhibit viral multiplication. Biochemical con-

trols, using the incorporation of labeled uridine or phenylalanine showed that by 8 hours and 24 hours after treatment with actinomycin D, RNA synthesis was inhibited more than 95%. Protein synthesis was inhibited 46% eight hours after actinomycin D, and 87% twenty-four hours after actinomycin D. These results are consistent with the current viewpoint that actinomycin D acts primarily to inhibit the synthesis of DNA-primed RNA, including messenger RNA, and as a consequence of decay of preexisting messenger RNA, a secondary inhibition of protein synthesis occurs(19). The assumption is made that the decay of ribosomes during this period would not be sufficient to account for the observed decrease of protein synthesis. These findings imply that the decrease in established resistance by actinomycin D, probably reflects a decreased production of the antiviral protein due to decreased availability of its messenger RNA. As a result the resistant cell, treated with interferon and actinomycin D, would not adequately replace decaying antiviral protein.

Discussion. Any interpretation of the mechanism of induction and maintenance of antiviral activity by interferon must account for the observed variables. (a) Reaction of interferon with cell cultures is followed by an initial rise of resistance and subsequently a leveling off of resistance, (b) Development and maintenance of antiviral activity requires the continued presence of interferon, (c) The main determinant of the degree of resistance was the concentration of interferon in the extracellular fluid, (d) Development and maintenance of interferon-induced resistance required protein synthesis, (e) A requirement for RNA synthesis for the development and also probably for maintenance of antiviral activity.

The main question is whether these findings can lead to an understanding of some of the mechanisms which govern the development and maintenance of antiviral activity in cells exposed to interferon. Development of resistance seems most simply explained by the previous suggestion that interferon induces the production of an antiviral substance (protein) which accumulates intracellularly and accounts for the increasing resistance(2,20-23).

Maintenance of established resistance could be due to a refractoriness of cells to respond further to interferon. However this possibility is not supported by the findings that: (a) resistant cells respond appropriately to an increased concentriation of interferon and (b) that maintenance of antiviral aactivity requires the continued presence of interferon in the extracellular fluid. A more likely interpretation is that established resistance is maintained by continual induction by interferon of new antiviral protein which serves to replace decaying antiviral protein. This interpretation is supported by (a) the requirement for continued presence of interferon in the extracellular fluids (b) the requirement for continued protein synthesis and (c) the probable requirement for continued RNA synthesis.

Summary and conclusions. The results of the present study indicate that several factors affect the interferon-induced resistance to viruses in tissue culture. Reaction of cells and interferon results in rapidly increasing antiviral activity over about 7 hours. Thereafter antiviral activity remains relatively stable in the presence of an unchanging amount of interferon in the extracellular fluid. Continued presence of the initial concentration of interferon was required to maintain the established level of resistance. Addition of more interferon resulted in a further rise of resistance. The level of cellular antiviral activity was determined mainly by the concentration of interferon rather than by the amount applied. Maintenance of resistance required continued cellular protein synthesis and also probably required continued cellular RNA synthesis. These findings support the previous proposal that development of antiviral activity by cells exposed to interferon is due to the induction of a cellular antiviral protein. The results also suggest that maintenance of stable antiviral activity in the presence of interferon could be the result of continued induction by interferon of enough antiviral protein to offset decay.

1. Isaacs, A., Adv. Virus Res., 1963, v10, 1.
2. Taylor, J., Biochem. Biophys. Res. Comm.,

1964, v14, 447.
3. Baron, S., Buckler, C. E., Science, 1963, v141, 1061.
4. Levy, H. B., Virology, 1964, v22, 575.
5. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., J. Biol. Chem., 1951, v193, 265.
6. Lockart, R. Z., J. Bact., 1963, v85, 996.
7. Buckler, C. E., Baron, S., Levy, H. B., Science, 1966, v152, 80.
8. Youngner, J. S., Taube, S. E., Stinebring, W. R., personal communication, 1966.
9. Baron, S., Buckler, C. E., Dianzani, F., Ciba Foundation Symposium, 1967, in press.
10. Lindenmann, J., Burke, A., Isaacs, A., J. Exp. Path., 1957, v38, 551.
11. Wagner, R. R., Virology, 1961, v13, 323.
12. Sellers, R. F., Fitzpatrick, M., Brit. J. Exp. Path., 1962, v43, 674.
13. Gifford, F. E., J. Gen. Microbiol., 1963, v33, 437.
14. Youngner, J. S., Hallum, J. V., personal communication, 1966.
15. Lockart, R. Z., personal communication, 1966.
16. Ke, Y., Armstrong, J., Ho, M., personal communication, 1966.
17. Isaacs, A., Westwood, M. A., Nature, 1959, v184, 1232.
18. Pauker, I., Cantell, K., Virology, 1963, v31, 22.
19. Reich, E., Cancer Res., 1963, v23, 1428.
20. Friedman, R. M., Sonnabend, J. A., Nature, 1964, v203, 366.
21. Lockart, R. Z., Biochem. Biophys. Res. Comm., 1964, v15, 513.
22. Levine, S., Virology, v24, 229.
23. Rita, G., Russi, M., Dianzani, F., Quercioli, A., Proc. Ital. Soc. Micro.-Symp. Arboviruses, 1965.

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A Sexual Influence on the Biosynthesis and Storage of L-Ascorbic Acid in Rats.* (32348)

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Previous investigations have demonstrated a marked hypophyseal influence on the biosynthesis of ascorbic acid in rats(5); an influence shown to be mediated by somatotrophic hormone(6). Perusal of some of the older literature reporting concentrations of ascorbate in tissues of male and female rats has given indication of a possible sexual influence as well. Several authors have reported higher concentrations of ascorbate in a few tissues of male rats in comparison with those from females of similar age(2,4,7). Although there has been no uniform agreement as to which tissues show the higher concentrations in males, such data do indicate a possible sexual influence on the biosynthesis and storage of ascorbic acid. To the best of our knowledge no comparison of specific cellular enzymes in the biosynthetic pathway has been made for the two sexes. This paper reports the first analyses of 4 hepatic enzymic activities involved in ascorbic acid synthesis in both males and females, and confirms the sexual difference in tissue ascorbate concentrations for several tissues.

Methods. Male and female rats of the Sprague-Dawley strain between 45 and 65 days of age were used. Animals were decapitated, exsanguinated, and tissue samples removed for analysis. Blood was collected during the exsanguination. Liver was divided into two parts; one part was used for determination of ascorbate concentration, and the other was homogenized and separated into microsomal and cytoplasmic fractions for enzymic assay. Tissues were weighed to the nearest milligram and homogenized in 10 ml of 10% trichloroacetic acid. One milliliter of serum was added to 2 ml of 10% trichloroacetic acid. Following removal of precipitated residue, all supernates were analyzed for total ascorbic acid content by a modification of the method of Lowry *et al*(3).

Enzymic assay conditions, substantially as described previously(6), were slightly modified to give linear initial reaction rates. The

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