Biochemical Pharmacology, Vol. 18, pp. 253–256. Pergamon Press. 1969. Printed in Great Britain

Vitamin C as a requirement for the storage of norepinephrine by the iris*

(*Received* 24 *June* 1968; *accepted* 16 *August* 1968)

PRESSOR responses and cardiac inotropic responses to scorbutic guinea pigs are potentiated by exogenously administered catecholamines.[1] Further investigations suggest that uptake or binding (or both) of exogenously administered ^{3}H-norepinephrine by the hearts of scorbutic animals *in vivo*[2] as well as by scorbutic atria *in vitro*[3] may be defective. The object of the present study is to determine whether ascorbic acid is required for the storage of norepinephrine. Turnover data for iridial norepinephrine were obtained by measuring the endogenous concentration of norepinephrine in the irises of normal and scorbutic animals whose biosynthesis of norepinephrine has been blocked[4] and by determining the decline in specific activity of the iris after labeling with ^{3}H-norepinephrine.[5,6]

Pigmented weanling guinea pigs weighing between 150 and 200 g were divided into two groups. One group received a diet deficient in vitamin C. Ascorbic acid was determined by titrating with 2,6-dichlorophenolindophenol in 4% metaphosphoric acid with ascorbic acid as a standard. After 14 days no ascorbic acid could be detected in the plasma or aqueous humor. The second group had vitamin C added to its diet. All animals were studied between 17 and 20 days, at which time only a few animals in the deficient group had developed clinical evidence of scurvy. Both groups were then given an i.p. injection of an aqueous solution of 1-α-methyl-*p*-tyrosine (20 mg/ml) in the amount of 200 mg/kg.[7] Groups of animals were then sacrificed 4, 8, 16 and 20 hr later. The irises were removed and assayed for norepinephrine.[8] At 12 hr, the remaining animals received a second injection of 1-α-methyl-*p*-tyrosine to assure adequate tissue levels of the inhibitor.

Two similar groups were given subconjunctival injections of 1 mμmole of *d,l*-norepinephrine-7-^{3}H-acetate (sp. act., 10·1 mc/μmole) obtained chromatographically pure from New England Nuclear Co., Boston, Mass. The animals were sacrificed at various intervals after injection. The irises were rapidly removed, rinsed, blotted and weighed. Each iris was then minced and homogenized in 0·4 M perchloric acid. The grinding tubes were rinsed and the rinse was added to the homogenate. After centrifugation at 13,000 rpm at 0° for 5 min, the extract volumes were measured and an aliquot was placed in counting vials containing 10 ml Bray's phosphor.[9] Radioactivity was measured in a Nuclear-Chicago liquid scintillation counter. Quenching was determined by a channels ratio method.[10] ^{3}H-norepinephrine and its metabolites[11] and total iridial norepinephrine[8] were determined after isolation on alumina. Any neutral or acidic metabolites not remaining on the column were extracted in ethyl acetate, counted, and the counts subtracted from the counts in the total eluate so that the specific activity of ^{3}H-norepinephrine/μg iridial norepinephrine/g iris could be determined.

After blockade of synthesis, the disappearance of endogenous norepinephrine from the iris demonstrated a single exponential function (Fig. 1). In steady state the rates of norepinephrine synthesis and removal are equal. Therefore, after complete and rapid blockade of synthesis, the concentration of norepinephrine declines at a rate proportional to the concentration. Thus a semilogarithmic plot of the concentration of norepinephrine against time yields a straight line, the slope of which is the rate constant for efflux of norepinephrine. Turnover times and rates may be calculated from these considerations of steady state kinetics[3] and are shown in Table 1. Tissue norepinephrine concentrations at zero time, i.e. prior to blockade of synthesis, indicate that scorbutic irises have about 50 per cent of the concentration of norepinephrine present in normal irises, a percentage decrease similar to that observed for the scorbutic heart.[2] The turnover time in the "scorbutic" group is short, 4·35 hr as compared to 8·7 for the normal. Evidence for the rapid turnover time demonstrated by this method was supported by following the decline in specific activity of iridial ^{3}H-norepinephrine after administration of small amounts of labeled amine. The entry of exogenous norepinephrine into various intraneuronal pools and its release therefrom are probably complex and multiphasic. However, if the turnover times derived from the semilogarithmic plot of the decline in specific activity of iridial ^{3}H-norepinephrine calculated by the method of least squares are compared for the normal and

* This research was supported in part by United States Public Health Service Grant No. NB 03871, the Connecticut Lions Eye Research Foundation, Inc. and Research to Prevent Blindness, Inc.

"scorbutic" group, the latter has a turnover twice that of the former. This is so for time intervals of 3–12 hr, 3–18 hr or 3–24 hr. A plot of the 3–24 hr interval is shown in Fig. 2. For this interval, a Student *t*-test of the slopes is significant at the 2·5 per cent level. (At the shorter intervals, the P values are better than 1 per cent.) Turnover times for this interval are 6·3 hr for scorbutic pigs and 10·7 hr for normals. These rapid turnover times suggest that scorbutic irises have a reduced storage capacity for norepinephrine.

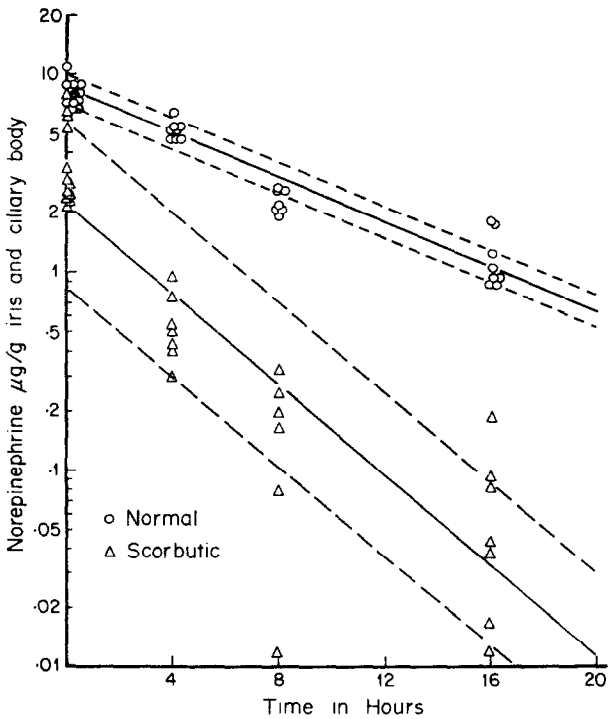

FIG. 1. Levels of norepinephrine in the irises pf normal (○) and scorbutic (△) guinea pigs after i.p. administration of 1-α-methyl-*p*-tyrosine (200 mg/kg). Dashed lines represent ± S.E. of each slope. Slopes were calculated by the method of least squares.

A study in progress of net accumulation of norepinephrine by irises *in vitro* has served as a model for the steady state condition of storage of norepinephrine by irises *in vivo*. In this study the finding of reduced net accumulation of norepinephrine by scorbutic irises supports the idea that the high concentration of ascorbic acid (10^{-3} M) normally present in the anterior uveal tissues and aqueous

TABLE 1. TURNOVER DATA CALCULATED FROM THE DECLINE OF ENDOGENOUS IRIDIAL NOREPINEPHRINE AFTER BLOCKADE OF NOREPINEPHRINE BIOSYNTHESIS*

Animals	Initial levels (μg/g ± S.E.)	Rate constant (k) (k(hr^{-1}) ± S.E.)	Turnover time (hr)	Turnover rate (μg/g/hr)
Normals	8·01 ± 0·54 (12)	0·115 ± 0·006 (35)	8·7	0·92
Scotbutics	3·91 ± 0·59 (12)	0·23 ± 0·027 (35)	4·4	0·899

* Turnover rate is the product of steady state level and of k, the rate constant of catecholamine decline; turnover time is $1/k$.

humor of the mammalian eye may serve to stabilize the relatively large amounts of norepinephrine present in the iris.

The question of impaired entry or uptake of norepinephrine by scorbutic tissues referred to in other works[2,3] cannot be answered by our data. At (extrapolated) zero time, the number of counts of ^{3}H-norepinephrine taken up per gram of scorfutic tissue is about twice normal (Fig. 2). This discrepancy cannot be used to decide the question of differences in uptake by normal and scorbutic

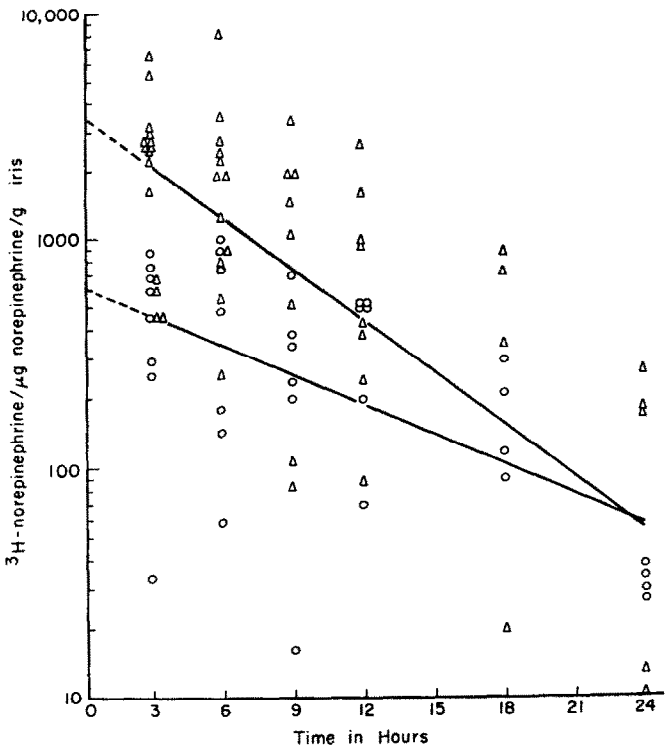

FIG. 2. Slopes of decline in specific activity of ^{3}H-norepinephrine, cpm per μg norepinephrine/g of normal (○) and scorbutic (△) iris after subconjunctival administration of 1·0 mμmole *dl*-^{3}H-norepinephrine. Slopes were calculated by the method of least squares.

tissues, since the rates and degree of penetration of small water-soluble molecules through the sclera into the iris from the site of injection under the conjunctiva are probably increased in scorbutic tissues. In any event, the process of uptake of norepinephrine by scorbutic tissues as compared with normal ones could be augmented, the same or reduced, but would not necessarily affect the finding of an increased rate of egress of norepinephrine from scorbutic tissues, a phenomenon related to storage rather than to uptake.

Acknowledgement—Mr. E. Lerner did the statistical analysis. B. Ladd and S. Hemperly gave expert technical assistance. Dr Clement Stone of Merck, Sharp & Dohme Research Laboratories, West Point, Pa., kindly provided the 1-α-methyl-*p*-tyrosine.

Section of Ophthalmology,
Yale University School of Medicine,
New Haven, Conn., U.S.A.

M. L. SEARS

REFERENCES

1. N. B. THOA and W. M. BOOKER, *Fedn Proc.* **22**, 448 (1963).
2. N. B. THOA, R. J. WURTMAN and J. AXELROD, *Proc. Soc. exp. Biol. Med.* **121**, 267 (1966).
3. B. BHAGAT, W. L. WEST and I. M. ROBINSON, *Biochem. Pharmac.* **15**, 1637 (1966).
4. B. B. BRODIE, E. COSTA, A. DLABAC, N. H. NEFF and H. G. SMOOKLER, *J. Pharmac. exp. Ther.* **154**, 3 (1966).
5. R. MONTARINI, E. COSTA, M. A. BEAVEN and B. B. BRODIE, *Life Sci.* **4**, 232 (1963).
6. L. L. IVERSEN and J. GLOWINSKY, *Nature, Lond.* **210**, 1006 (1966).
7. S. SPECTOR, A. SJOERDSMA and S. UDENFRIEND, *J. Pharmac. exp. Ther.* **147**, 86 (1965).
8. A. BERTLER, A. CARLSSON and E. ROSENGREN, *Acta physiol. scand.* **44**, 273 (1958).
9. T. BRAY, *Analyt. Biochem.* **1**, 279 (1960).
10. G. A. BRUNO and J. E. CHRISTIAN, *Analyt. Chem.* **33**, 650 (1961).
11. I. J. KOPIN, J. AXELROD and E. GORDON, *J. biol. Chem.* **236**, 2109 (1961).

Biochemical Pharmacology, Vol. 18, pp. 256–260. Pergamon Press. 1969. Printed in Great Britain

Inhibition of antibody synthesis by cycloleucine

(*Received* 16 *May* 1968; *accepted* 9 *August* 1968)

DURING the past decade, such immunosuppressive drugs as 6-mercaptopurine, thioguanine, methotrexate and cyclophosphamide have been identified and studied in detail.[1–5] Maximal inhibition of the immune response usually results when these and other agents are introduced within 18 to 48 hr after primary antigenic stimulus, i.e. during the induction phase. An exception to this general rule is the sulfonic acid esters, which mimic radiation effects and which are most inhibitory if administered prior to immunization.[2, 6, 7] The results of a study of another such immunosuppressant, the amino acid analogue cycloleucine (1-amino-cyclopentane-carboxylic acid), constitute the subject of this report. Pretreatment of Swiss mice with this drug prevents the synthesis of hemagglutinins and hemolysins for sheep erythrocytes by reducing the number of plaque-forming cells in the splenic pulp.

METHODS

The methodology, which has been described in detail elsewhere, consisted of administering the drug, suspended in methylcellulose, intraperitoneally and the immunization of mice with a single dose of washed sheep erythrocytes given via the same route. The animals were bled from the orbital sinus at selected intervals and the hemagglutinin titers determined.[4] Hemolytic titers were measured by adding an excess of guinea pig complement to the hemagglutination tubes and incubating for an additional hour at 37°. The number of plaques in the spleen was determined by the method of Jerne *et al.*[8] Statistical analysis of the titration data in the tables was carried out by using the t = test. In each instance the results were compared with those obtained from the control animals.

RESULTS

In the first three experiments maximal single doses of cycloleucine were given at various times in relation to the intraperitoneal injection of sheep erythrocytes. These data have been combined in Table 1. In part A, significant immunosuppression was achieved when cycloleucine was injected 3 days before, but not 3 days after, antigen. Additional experiments in parts B and C indicated that inhibition could still be obtained if pretreatment with cycloleucine was carried out up to 12 days prior to antigenic stimulation.