in yeast. Moreover, proteose-peptone and Edamine S, both of which are requirements for growth and maintenance of kappa of stock 51, may be substituted for each other without loss of lambda in stock 299.

The complexity of the growth requirements of killers as they differ from sensitives and the differences in these requirements between stocks serves as an indication of the intricate relationships between these remarkable particles and the cells of the host. More detailed analysis of nutritional requirements of the killers is presently being undertaken, to develop a chemically-defined medium. In this way, greater insight into the nature of the particles themselves as well as identity of the factors responsible for their maintenance may be obtained.

Summary. Two killer stocks of *Paramecium aurelia*, 51 and 299, have been cultivated axenically in a medium consisting of vitamins, a salt mixture, stigmasterol, proteose-peptone, an enzymatic digest of lactalbumin and the nondialysable fraction of a hot water extract of yeast. All components are necessary for growth of the ciliates and for maintenance of the kappa character. Differences in nutritional requirements of the two stocks have been noted. As far as is known, this is the first instance of cultivation of a kappa-bearing strain of *Paramecium* in bacteria-free medium.

Since this paper was submitted for publication, stocks 138 and 139 of *P. aurelia*, containing respectively μ and π particles, have been placed in axenic culture (medium No. 8 as given in Table II). The organisms have been maintained in tube culture for two months without loss of particles.

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Influence of Dietary Protein Levels on Ascorbic Acid Status of the Rat.* (26195)

M. SRINIVASAN, M. V. L. RAO AND H. N. BHAGAVAN†
(Introduced by E. L. Severinghaus)
Central Food Technological Research Institute, Mysore-2, India

There appears to be no report regarding the influence of dietary protein on biosynthesis of ascorbic acid in the rat. Prompted by a chance observation that rats receiving a high protein diet excreted relatively large amounts of ascorbic acid in urine, we investigated the influence of dietary protein on the Vit. C status of the rat. In the first instance, the effect of variations in dietary protein levels was examined.

Materials and methods. Adult male albino rats (Wistar strain) with body weights in the range 180-200 g were employed in all experiments. Animals were distributed according to the randomized block design for the various test treatments. Diets fed had the following composition: Protein (Polson's lactic casein)—according to choice; fat (refined peanut oil)—10%; vitaminized starch—1%; salt mixture‡—2%; and corn starch—to 100.

* A preliminary report of this work was presented at the Symposium on Proteins held at this Institute Aug. 14-16, 1960.

† Present address: Dept. of Animal Nutrition, Univ. of Illinois, Urbana.

‡ Hubbell, Mendel and Wakeman mixture.

The vitaminized starch contained thiamine hydrochloride 40 mg, riboflavin 80 mg, niacin 100 mg, pyridoxine hydrochloride 40 mg, calcium pantothenate 100 mg and folic acid 5 mg per 100 g. The rats were given necessary amounts of diluted shark liver oil twice a week to provide the requirements of Vit. A and D.

Cages with wide mesh wire bottoms which were well covered with resistant paint were used to house the animals individually. Urine was collected in brown glass bottles containing 10% trichloracetic acid (TCA) over 3-day periods. Collecting funnels and bottoms of cages were rinsed with 10% TCA solution at least thrice a day during the collection period. Urine collections were made up to known volume and aliquots used for estimation of total Vit. C (ascorbic acid, dehydroascorbic acid and diketogulonic acid) according to the dinitrophenylhydrazine (DNPH) method of Roe and Keuther(1). The same procedure was employed for estimation of ascorbic acid in tissues and blood.

To ensure that the above photometric procedure did not include an appreciable proportion of chromogenic derivatives other than those related to ascorbic acid, DNPH derivatives were prepared(2) from the urine as well as the tissues of test animals (especially in the case of those giving high excretions) and their chromatographic behaviour and absorption spectra studied. On paper chromatograms developed with a number of solvents the derivatives from urine as well as the tissues gave only a single pink coloured band or spot identical in position and absorption characteristics with the derivative prepared from standard L-ascorbic acid (Merck). Also, the derivatives in 12.5 M sulphuric acid solution gave absorption spectra identical with that of the standard derivative under similar conditions (in the range 310-600 mμ, determined with the Beckman spectrophotometer, Model DU).

TABLE I. Urinary Ascorbic Acid Excretion of Rats Transferred from Stock Ration to Synthetic Casein Diets. Six rats in each group. Period of feeding, 3 weeks.

Group	% casein in diet	Avg urinary ascorbic acid excretion, mg/day	
I	10	1.18	±.19 (15 d.f.)
II	20	1.82	
III	30	2.10	
IV	50	2.87	
Statistical significance		I ~ II *	I ~ III***
		II ~ III N.S.	II ~ IV**
		III ~ IV *	

Differences significant at: *5%, **1% and ***0.1% level. N.S., not significant.

Results. Effect of protein quantity. Stock rats were transferred to diets containing 10, 20, 30 and 50% casein. After being fed these rations for 3 weeks their urinary ascorbic acid excretion was measured (Table I). There was a progressive increase in excretion of ascorbic acid as protein content of the diet increased; rats on the 50% casein diet excreted more than twice as much ascorbic acid in the urine as rats on the 10% protein ration.

Effect of protein depletion-repletion. Rats were fed a protein-free diet for 2 weeks and urinary ascorbic acid excretions were measured. They were then distributed into appropriate groups and fed diets containing 10, 30 and 50% casein for 2 weeks and their urinary excretion and tissue levels of the vitamin were

TABLE II. Urinary Excretion and Tissue Levels of Ascorbic Acid in Rats Maintained for 2 Weeks on Protein-free Diet and Re-fed Casein Diets for 2 Weeks. Five rats in each group.

Group	Diet	Urinary ascorbic acid excretion, mg/day	Liver ascorbic acid (total), mg	Adrenal ascorbic acid (total), μg	Blood ascorbic acid levels, mg/100 ml
I	Protein-free diet	.48 ±.12 (4 d.f.)	—	—	—
II	Re-fed 10% casein diet	1.28	2.06	114.8	.78
III	" 30% " "	2.44 ±.30 (8 d.f.)	4.46 ±.15 (8 d.f.)	153.0 ±6.9 (8 d.f.)	1.40 ±.07 (7 d.f.)
IV	" 50% " "	2.92	4.82	189.0	1.42
Statistical significance		I ~ II *	II ~ III***	II ~ III**	II ~ III***
		II ~ III*	II ~ IV ***	II ~ IV ***	II ~ IV ***
		II ~ IV **	III ~ IV N.S.	III ~ IV **	III ~ IV N.S.
		III ~ IV N.S.			

Differences significant at: *5%, **1% and ***0.1% level. N.S., not significant.

TABLE III. Effect of Sulfaguanidine on Urinary Excretion and Tissue Levels of Ascorbic Acid in Rats. Ten rats in each group.

Group	Diet	Urinary ascorbic acid excretion, mg/day			Liver ascorbic acid avg		Liver conc., mg/g	Adrenal ascorbic acid avg, μg	Blood ascorbic† acid avg, mg/100 ml
		5 wk feeding	10 wk feeding		Total, mg				
I	10% casein diet	1.6	†		3.38	†	.38	† 96.0	1.24
II	*Idem*, with 2% sulfaguanidine	.35	***		1.46	***	.25	*** 95.7	.76
III	50% casein diet	1.5	±.16	1.5 ±.14	2.7	(8 d.f.)	.38	(8 d.f.) 113.4	N.S. 1.2
IV	*Idem*, with 2% sulfaguanidine	1.1	(9 d.f.) N.S.	1.3 (9 d.f.) N.S.	2.4	N.S.	.37	N.S. 108.4	1.4

† As range of variation of values in each group was proportional to the average, logarithmic transformations have been used for purposes of statistical evaluation.

In group I one rat died in the 4th wk. In group II adrenal ascorbic acid was extremely low in one rat. In group III adrenal ascorbic acid value was very low in one case and in group IV one of the rats died during the experiment; for one rat, adrenal sample was lost. Corresponding values for statistical analysis were estimated by the missing plot method (*Experimental Designs* by W. G. Cochrane and G. M. Cox, 1950, pp. 99-100).

‡ Not statistically evaluated as 2 or 3 specimens in each group were lost or could not be obtained.

Statistical significance: *5%, **1% and ***0.1% level. N.S., not significant.

determined. Again, progressive increase in urinary excretions of ascorbic acid with increase in protein content of the diet was evident (Table II). Significant changes in liver, adrenal and blood ascorbic acid levels paralleling urinary excretions were also observed.

Effect of sulfaguanidine. In view of the findings of Schwartz and Williams(3-5) and Schwartz *et al.*(6), that ingestion of antibacterial agents like aminopterin and sulfasuxidine along with the diets markedly reduces the liver level and urinary excretion of ascorbic acid in the rat, the effect of including 2% sulfaguanidine in the low (10%) as well as in the high protein (50%) diets was examined (Table III). In the low protein group there was a marked decrease in urinary excretion of rats receiving the sulpha drug at the end of 5 weeks. These rats were sacrificed for tissue Vit. C determination. In the high protein group, on the contrary, there was no significant difference in urinary excretion as between rats fed the sulpha drug and the corresponding controls. Therefore, they were continued on the same diet for 10 weeks when urinary ascorbic acid excretion was again determined. As even on prolonged feeding the sulfa drug did not affect urinary excretion, these animals were then sacrificed for tissue analysis. In subsequent experiments similar results have been obtained with sulfasuxidine.

Discussion. The evidence presented indicates that changes in protein content of the diet markedly influence urinary excretion and tissue levels of ascorbic acid in rats during short-term feeding. Since the increased excretion on high protein diets is not accompanied by any decrease in tissue levels but, on the contrary, by an increase, it is justifiable to infer that increase in amount of protein in the diet favorably influences the biosynthesis of the vitamin presumably by increasing the liver content of the enzymes involved in Vit. C biogenesis or by increased provision of essential precursors. The former appears plausible in view of the well known dependence of liver cytoplasmic proteins upon the plane of protein nutrition in the rat.

As contrasted with short-term experiments, prolonged feeding (over 3 weeks) levels off the differences in urinary excretion and tissue content of the vitamin between rats receiving high and low protein diets. This may involve an adaptive process.

The adverse effects of the sulfa drug in animals receiving low protein diets appear to implicate the intestinal microflora in biosyn-

thesis of Vit. C in the rat. The protective effect of high levels of dietary protein may be interpreted as the effect of excess protein *per se* on the biosynthetic mechanisms or on the precursors or indirectly on the microflora. Further experiments are in progress to test these hypotheses.

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Effect of Premarin on Survival in Men with Myocardial Infarction.* (26196)

JESSIE MARMORSTON, FREDERICK J. MOORE, OLIVER T. KUZMA, OSCAR MAGIDSON AND JOHN WEINER

Departments of Medicine and Public Health, University of Southern California School of Medicine, Los Angeles County General Hospital, and Cedars of Lebanon Hospital, Los Angeles

Considerable epidemiological and experimental evidence suggests that estrogens may be antiatherogenic(1-11). However, only 2 clinical trials of the effect of estrogens on survival in men with myocardial infarction have been reported. Oliver and Boyd(12) failed to observe any effect of ethinyl estradiol on survival but Stamler, Pick and Katz in an interim report(13) found suggestive evidence that mixed, conjugated estrogens (Premarin) increased survival. The latter used doses causing manifest feminization and the results lacked statistical significance.

This paper reports results of a continuing study of Premarin in men using small, well tolerated doses, in which survival in the Premarin group is significantly higher than in the controls.

Material and methods. Clinical material consists of men who had recovered from a myocardial infarction in the Los Angeles County General Hospital or the Cedars of Lebanon Hospital, who were ambulatory, cooperative and considered able to attend our clinics regularly, who were willing to take estrogen if prescribed, who had no requirement for hormone therapy (other than insulin or thyroid), and who were free of concomitant illness threatening survival. Acceptability was determined during the first few visits to the clinic, which was staffed entirely by project personnel. Upon acceptance, patients were randomly allocated to control or treatment with Premarin or one of 2 other estrogens (results with these being reported elsewhere(14)). In those receiving Premarin, dosage was regulated solely by tolerance of the individual and in most cases was 1.25 to 2.5 mg daily. These doses usually gave rise, after some months, to minor breast changes that were not objectionable to the patient. In the event of objectionable side effects, dosage was reduced to the point of ready tolerance.

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