THE ABSENCE FROM THE URINE OF PERNICIOUS ANEMIA PATIENTS OF A MOSQUITO GROWTH FACTOR PRESENT IN NORMAL URINE

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The larvae of the mosquito, *Aëdes aegypti*, require for their normal growth and development certain accessory growth substances which they obtain, in nature, from living microorganisms. All attempts to grow the larvae in the absence of living microorganisms proved unsuccessful, until it was found (1) that a medium containing heat-killed yeast and 0.5 per cent Lilly liver extract No. 343 (a partially purified preparation used for the treatment of pernicious anemia) would support normal growth under sterile conditions. The growth factor, designated as factor A, which is present in liver extract, cannot be supplied (2) by the highly purified anti-anemic preparations of Dakin and West (3) and Jacobson and Subbarow (4), showing that factor A and the antipernicious anemia principle are not identical. Factor A does, however, resemble the anti-anemic substance in the following ways. It is abundant in liver and kidney and less so in heart, while body muscle contains very little (2, 5). It can be adsorbed by charcoal and dialyzed through a collodion membrane (2, 6). It is heat-stable in neutral or slightly acid solution, but is destroyed by ashing, by boiling for 1 hour with 0.5 N sulfuric acid, or by exposure at room temperature for 24 hours to 0.5 N sodium hydroxide (2, 3). It is present in small amount in that portion of aqueous liver extract precipitated by 70 per cent alcohol, and in large amount in the precipitate obtained when the 70 per cent alcoholic filtrate is brought to a concentration of 95 per cent alcohol (2, 7).

These resemblances seemed to warrant further investigation. Wakerlin's finding (8) that normal human urine contains a substance

effective against pernicious anemia suggested the assay for mosquito growth factor of urine extracts from normal persons and from patients with aplastic anemia and with pernicious anemia before and after adequate treatment.

Two of us prepared the urine extracts, while the other performed the mosquito growth tests, usually in ignorance of the type of person from whom the extract was obtained.

Methods

1. Preparation of the Urine Extracts.—All the urine passed during a 24 hour period was collected with a few drops of toluene as a preservative and stored in a refrigerator. It was then measured, evaporated under reduced pressure at 55°C. to a volume of about 150 cc., and poured into a volume of 95 per cent alcohol sufficient to give a final concentration of 70 per cent alcohol. The mixture was allowed to stand overnight and was then filtered. The filtrate was concentrated under reduced pressure to about 100 cc. and was poured into enough absolute alcohol to give a concentration of 95 per cent alcohol. After vigorous shaking, a fine flocculent precipitate settled out. This was filtered off and dissolved in 100 cc. of distilled water. The pH was adjusted to 6.0 and the material was autoclaved ½ hour at 120°C.

2. The Mosquito Growth Test.—Significant results can be obtained only if the larvae are reared in the absence of living microorganisms. As in previous work on the nutrition of mosquito larvae (2), 1 to 2 day old eggs of *Aëdes aegypti* were sterilized on the outside and placed in tubes of sterile 0.5 per cent Lilly liver extract No. 343. 4 days later the young larvae were washed in sterile distilled water and inoculated into the experimental tubes. These were prepared by making suitable dilutions of the urine extract with sterile distilled water. Each tube contained a total of 6 cc. of medium and received 0.3 cc. of washed killed yeast suspension. Three larvae were inoculated into each tube. Each urine extract was tried in at least four different dilutions, using at least two tubes for each dilution. Some of the extracts were tested several different times with concordant results. The tubes containing the larvae were held in an incubator at 28° ± 1°C. and were observed daily at first and then every other day for a period of about 20 days, the number of larvae in each instar being noted.

RESULTS

(*a*) *In Urine Extracts.*—

It has been shown (2) that in the presence of killed yeast (0.1 cc. per larva) and 0.5 per cent Lilly liver extract No. 343 (or other suitable source of growth factor A) (2) all the larvae reach the third instar on the 3rd day, and nearly all reach the fourth instar on the

TABLE I

The Growth of Aëdes aegypti Larvae in Urine Extracts

Extract*	Concentration (fraction of total volume)	Reaching 3rd stage in 20 days	Average time to reach 3rd stage	Reaching 4th stage	Rating
		per cent	*days*	*per cent*	
C:1	1/3	0	—	0	
	1/6	0	—	0	−
	1/12	0	—	0	
	1/20	17	13	0	
C:2*a*	1/3	Toxic	—	0	
	1/6	33	8	0	+++
	1/12	33	9	0	
	1/20	17	6	0	
C:2*b*	1/6	33	9.5	0	
	1/12	33	7	0	++++
	1/20	66	9	0	
	1/60	17	11	0	
G:1	1/3	17	9	0	
	1/6	17	6	0	+
	1/12	17	6	0	
	1/20	0	—	0	
G:2	1/3	0	—	0	
	1/6	83	7	0	+++++
	1/12	67	5	33	
	1/20	50	8	0	
A:1	1/3	0	—	0	
	1/6	0	—	0	−
	1/12	0	—	0	
	1/20	17	11	0	
A:2	1/6	Toxic	—	0	
	1/12	17	9	0	+
	1/20	17	4	0	
	1/60	0	—	0	
A:3	1/3	83	6	0	
	1/6	67	7	0	
	1/12	33	5	0	++++
	1/20	0	—	0	
	1/60	17	6	0	

* The capital letter refers to the patient, the number to the extract, and the small letter to the trial as described in Table II.

TABLE II

Mosquito Growth Test of Various Urine Extracts

Patient	Description of case	Urine extract	Mosquito growth test
A	Housewife of 61. Paresthesia of extremities, 2 yrs. Gastrointestinal disturbance and anemia, 1 yr. Diminished perception of vibratory sense in the extremities. Erythrocytes 4,720,000; hemoglobin 100%; leukocytes 3,700. No free HCl in gastric juice. Treated twice weekly by intramuscular injection of liver extract (Lederle Laboratories) 1 cc. Full remission effected	1. Before treatment 2. Before treatment 3. After treatment	− + ++++
B	Housewife of 50. Pallor and weakness, 3 yrs. Paresthesia and difficulty in walking, 8 mos. Inadequate therapy had been given. Pallor, lingual atrophy, icteroid sclerae, and absent vibratory sense of the extremities. Erythrocytes 3,900,000; hemoglobin 87%; leukocytes 4,950; mean corpuscular volume 89. No free HCl in the gastric juice. Treated twice weekly with intramuscular injection of 10 cc. liver extract (Eli Lilly and Co.). Complete disappearance of symptoms	1. Before treatment 2. Before treatment 3. Before treatment 4. After treatment	− − + +++
C	Housewife of 39. Weakness and loss of weight, 1 yr. Diagnosis of pernicious anemia. Sore tongue and mouth. Paresthesia of extremities for 2 mos. Erythrocytes 2,080,000; hemoglobin 58%; leukocytes 5,000; mean corpuscular volume 121. No free HCl in gastric juice. Treated every 2 wks. with 10 cc. liver extract (Lilly) intramuscularly. Full remission effected	1. Before treatment 2. After treatment, trial *a* 2. After treatment, trial *b*	− +++ ++++

TABLE II—*Continued*

Patient	Description of case	Urine extract	Mosquito growth test
D	Married clerk of 48. Pallor and weakness, 6 yrs. Diagnosis of pernicious anemia for 5 yrs. with inadequate oral therapy. Paresthesia of extremities, 2 wks. Icteroid sclerae, atrophic glossitis, and diminished vibratory sense. Erythrocytes 1,380,000; hemoglobin 42%; leukocytes 3,100; mean corpuscular volume 140. No free HCl in gastric juice. Treated twice weekly with 10 cc. liver extract (Lilly) intramuscularly. Full remission effected	1. Before treatment, trial *a*	+
		1. Before treatment, trial *b*	++
		2. After treatment	+++++
E	Italian-born male cigar worker of 54. Weakness and loss of weight, 2½ yrs. Oral liver extract, 1 yr. Paresthesia and loss of coordination, 1 yr. Marked loss of perception of vibration and position. Erythrocytes 2,490,000; hemoglobin 76%; leukocytes 4,400; mean corpuscular volume 133. No free HCl in gastric juice. Treated twice weekly with concentrated liver extract (Lederle) intramuscularly. Full remission effected	1. Before treatment	−
		2. Before treatment	−
		3. After treatment	++
		4. After treatment	+
F	Single seamstress of 55. Pallor and weakness, 5 yrs. Diagnosis of pernicious anemia for 4 yrs. with inadequate treatment. Paresthesia for 4 mos. Icteroid sclerae, atrophic glossitis, and diminished vibratory sense. Erythrocytes 1,400,000; hemoglobin 50%; leukocytes 4,350; mean corpuscular volume 149. No free HCl in gastric juice. Treated twice weekly with 10 cc. liver extract (Lilly) intramuscularly. Full remission effected	1. Before treatment	+
		2. After treatment	++

TABLE II—*Continued*

Patient	Description of case	Urine extract	Mosquito growth test
G	Housewife of 65. Stomatitis, pallor, weakness, 8 mos. Paresthesia, 2 mos. 30 lbs. loss of weight. Atrophic glossitis, icteroid sclerae, and diminished vibratory sense of the extremities. Erythrocytes 710,000; hemoglobin 22%; leukocytes 1,950; mean corpuscular volume 129. No free HCl in gastric juice. Treated twice weekly with 10 cc. liver extract (Lilly) intramuscularly. Full remission effected	1. Before treatment 2. After treatment	+ +++++
H	Married stationary engineer of 64. Pallor and weakness, 2 yrs. Diagnosis of pernicious anemia with inadequate therapy. Icteroid sclerae. Vibratory sense absent in lower extremities. Erythrocytes 1,400,000; hemoglobin 40%; leukocytes 2,700; mean corpuscular volume 122. No free HCl in gastric juice. Treated twice weekly with 10 cc. liver extract (Lilly) injected intramuscularly. Full remission effected	1. Before treatment 2. After treatment	++ ++++
I	Married unemployed male of 40 with stomatitis, weakness, and pallor for 7 yrs. Inadequate oral treatment with liver extract. Icteroid sclerae and very slightly diminished vibratory sense in the extremities. Erythrocytes 2,600,000; hemoglobin 74%; leukocytes 6,650; mean corpuscular volume 112. No free HCl in gastric juice. Treated by ultraviolet light for 2 wks. with moderate improvement of the blood. Treated twice weekly with 10 cc. liver extract (Lilly) injected intramuscularly. Full remission effected	1. Before treatment 2. After ultraviolet treatment 3. After liver extract treatment	− ++ ++++

TABLE II—*Concluded*

Patient	Description of case	Urine extract	Mosquito growth test
J	Cancer of intestine	1	+
K	Ulcerative colitis	1	+
L	Aplastic anemia	1	+++
M	" "	1	+++++
N	" "	1	++++
O	" "	1	++++
P	" "	1	+++
Q	Probable aplastic anemia	1	++
R	" " "	1	++++
S	Leukemia	1	++
T	"	1	++++
U	Normal	1	+++
V	"	1	+++++
W	"	1	++++
	"	2	+++

4th day and emerge as adult mosquitoes on the 9th day. With the same amount of killed yeast in distilled water (or in various other media not containing factor A) the larvae never get beyond the second instar, and they eventually die in this stage.

Preliminary trials with normal urine extract showed that while many of the larvae reached the third instar, only a few reached the fourth and none emerged as adults. Thus normal urine extract either does not contain enough factor A to bring about normal growth, or else contains only some of the substances which are responsible for the factor A activity. Concentrations of urine extract higher than 40 per cent by volume were generally toxic, all the larvae being dead 1 day after inoculation. In the middle range of concentrations, depending on the urine extract used, the larvae either survived for a long time in the second instar or reached the third instar and then survived at this stage.

Accordingly, the percentage of larvae reaching the third instar within 20 days was taken as the chief criterion of growth. The other criteria were the average time to reach the third instar and the percentage reaching the fourth instar (very small even in the most favorable cases). On the basis of these criteria the urine extracts were rated with respect to their growth factor content as −, +, ++,

etc. Table I gives some of the actual data and illustrates the method of rating.

The results with the various urine extracts tested and rated in this manner are given in Table II.

TABLE III

Mosquito Growth Test of Urine Extracts in the Presence of the Calcium-Filtrate Fraction†*

Medium		$N \times \frac{1}{T}$
Calcium-filtrate fraction only		5.1
Calcium-filtrate + flavine-purine complex (100 cc. of solution has material from 50 gm. liver)		23.1
Calcium-filtrate + urine extract W:2‡	1/6	16.0
	1/12	15.0
	1/24	12.2
" " " " C:2	1/6	13.6
	1/12	15.8
	1/20	10.3
" " " " D:1	1/6	7.4
	1/12	11.5
	1/20	7.1
" " " " D:2	1/12	17.1
	1/20	15.4
	1/60	10.1
" " " " A:2	1/12	9.2
	1/20	0
	1/60	0

* Concentration of urine extract expressed as fraction of total volume.

† Concentration of calcium-filtrate fraction always such that 100 cc. of solution contains the material derived from 50 gm. of liver.

‡ See Table II for description.

(*b*) *In Urine Extracts Supplemented with Certain Liver Fractions.*—Work, as yet unpublished, has shown that the mosquito growth factor A consists of at least two components.[1] One of these was iso-

[1] The work was done in collaboration with Dr. Y. Subbarow of The Harvard Medical School.

lated as a flavine-purine complex (1.2 per cent flavine-phosphate). The other was present in a fraction (designated as calcium-filtrate fraction) derived from the material obtained from liver extract by adsorption on charcoal and elution with alcohol (6). In the presence of killed yeast, neither of these two fractions alone supported normal growth of the larvae. But both together, in a concentration such that 100 cc. of solution contained that amount of each which was derived from 50 gm. of liver, gave entirely normal growth and metamorphosis. The method employed for the quantitative assay of factor A has been previously described (2). A growth index is obtained as a number, $N \times \frac{1}{T}$, which is determined by the percentage of larvae reaching the fourth instar in 10 days and the average time required to reach the fourth instar. When growth proceeds at an optimum rate, the value of $N \times \frac{1}{T}$ is $100 \times \frac{1}{4}$ or 25. Five of the urine extracts were tested in the presence, first, of an optimum concentration of flavine-purine complex and second, of an optimum concentration of calcium-filtrate factor. Growth in all the urine extracts plus flavine-purine complex was the same as in the urine extract alone, so that $N \times \frac{1}{T}$ equalled zero. But normal urine extract plus calcium-filtrate fraction gave growth almost as good as in flavine-purine complex plus calcium-filtrate fraction. These results are shown in Table III. It is worth noting that in this test, as well as in the test shown in Table II, extracts C:2, rated as +++, and D:2, rated as +++++, gave growth as good as did the normal extract W:2, rated as +++, while the growth obtained with D:1 and A:2, both rated as +, was markedly less.

DISCUSSION

The data of Table II demonstrate that normal urine, as well as urine from persons with aplastic anemia or leukemia, contains a substance which, under the described conditions, will enable many *Aëdes aegypti* larvae to reach the third instar, and a few to reach the fourth instar. In the urine of nine pernicious anemia patients this substance was absent or present in much smaller amount. Following a full remission of symptoms produced by liver extract therapy the

urine from all of these patients showed an increased amount of the mosquito growth factor. In the urine of seven of the nine patients the amount of this substance was greatly increased, reaching or slightly exceeding that present in normal urine. Interestingly enough, the urines from a patient with cancer of the intestine and from one with ulcerative colitis also showed a low content of growth factor.

The data of Table III give some indication as to the nature of this growth substance. Normal urine extract, or extract from the urine of adequately treated pernicious anemia patients, can replace the flavine-purine complex which is necessary for the growth of the mosquito larvae. Extract from pernicious anemia patients who show symptoms cannot replace the flavine-purine complex, giving growth but little better than that obtained with the calcium-filtrate fraction alone. Neither normal nor pernicious anemia urine extracts can replace the calcium-filtrate fraction. Hence we can tentatively conclude that the flavine-purine complex, or some material endowed with its potentialities for mosquito development, is excreted in much smaller amount by pernicious anemia patients showing symptoms than by normal individuals, persons with aplastic anemia, or adequately treated pernicious anemia patients. Normal urine extract, since it does enable a few larvae to reach the fourth instar, must also contain very small amounts of substances having effects like those of the calcium-filtrate fraction. The data thus far obtained give no information concerning the presence or absence of these substances in pernicious anemia urine extracts.

Several workers have shown that appreciable amounts of free flavine are excreted in normal human urine (9–13). No work has yet been reported on the flavine excretion of pernicious anemia patients. There is sufficient evidence that riboflavine is neither the anti-anemic factor (14), the "extrinsic factor" (15) or the pellagra preventive factor (16, 17). Nevertheless, it is still possible that there is, in pernicious anemia, an upset in the flavine metabolism. In this connection, the work of Laszt and Verzár (18) on chronic iodoacetate poisoning of rats is of especial interest. These investigators found that rats fed on a complete diet containing suitable amounts of iodoacetate failed to grow, and developed steatorrhea, osteoporosis,

skin symptoms, a decided anemia, and great hypertrophy of the suprarenals. All the effects could be completely counteracted if the rats were fed flavine-phosphate, but not if they were fed lactoflavine, indicating that the poisoning interfered with the phosphorylation of lactoflavine, a reaction necessary in the formation of yellow enzyme (19).

Miller and Rhoads (20) have shown that the livers of swine fed a modified Goldberger diet are deficient in antipernicious anemia substance. They have also found (21) that guinea pigs kept on this diet lose weight rapidly and die in 2 to 3 weeks unless the diet is supplemented with adequate amounts of liver extract or vegex, when the animals remain in normal health. An extract from the liver of a swine on this diet, and three extracts from the livers of groups of guinea pigs on this diet, were found to contain very much less mosquito growth factor A than normal swine and guinea pig liver extracts respectively. Unfortunately, these deficient extracts were not tested in such a manner as to determine whether they were lacking in flavine-purine complex or in calcium-filtrate fraction or in both components of factor A.

SUMMARY

Extracts prepared from the urine of normal persons or patients with aplastic anemia or leukemia contain a substance, possibly flavine or a flavine compound, which under suitable conditions of test enhances the growth of larvae of the mosquito, *Aëdes aegypti*. This substance is lacking, or is present in much smaller amount, in extracts from the urine of pernicious anemia patients showing symptoms of the disease. Extracts from the urine of the same patients after adequate treatment contain as much of the substance as normal urine extracts.

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