of copper. Indirect evidence for this theory may be present in the relationship of hereditary deficiency or absence of ceruloplasmin to hepatolenticular degeneration (Wilson's disease), as has been previously outlined (*7*), but proof of this concept is still to be obtained.

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10. Fraction IV from human plasma was obtained through the kind cooperation of James A. McComb (director), John M. Newell, and Lewis H. Larsen, of the Massachusetts Public Health Biologic Laboratories, and of Sam T. Gibson, director, American National Red Cross Blood Program. Ceruloplasmin AK-81 was prepared from fraction IV and was generously supplied to us by H. O. Singher and Alan Keltz of the Ortho Research Foundation, Raritan, N.J.
11. The primary aim in using ion-exchange resins in this procedure was to remove the excess ascorbic acid present in C-1 by anion exchange. However, for reasons that are not known, an apoprotein solution capable of being converted into blue ceruloplasmin was never obtained unless C-1 was also passed over a cation-exchange resin.
12. IR-120 and IRA-400 resins were used here both to remove excess copper and to demonstrate that the copper of the regenerated ceruloplasmin, like that of the original material, cannot be removed by a cation exchange resin.

4 October 1957

Severe Arteriosclerosis and Other Diseases in the Rat Produced by Corticotrophin

In older rats repeatedly injected with corticotrophin (ACTH), we have observed several disease syndromes: in the female, extremely severe arteriosclerosis; in the male, polyarteritis nodosa, gastric ulcers, testicular atrophy, and renal calculi, but no arteriosclerosis. Both sexes developed a marked hypertension (only systolic pressures were measured).

Male and female Sprague-Dawley rats, approximately 1 year old, were injected with ACTH subcutaneously three times a week for periods of up to 7 weeks. The dose was 0.333 of a unit per 100 g of body weight. The animals were maintained on a standard rat diet without fat supplements or any added salt in the drinking water. Some groups of rats were unilaterally nephrectomized. The older animals were all discard breeders. Equivalent groups of male and female weanling rats were treated identically.

Female rats. After administration of ACTH, a fulminating type of arteriosclerosis, involving the entire aortic tree, was observed in the female rats (Fig. 1). The large arteries showed a senile type of ectasia, in which the aorta was so severely stiffened that it could be made to stand by itself when dissected free of its bed. The arteriosclerotic changes extended into the cerebral vessels, the peripheral vessels, and the coronary arteries. In the more severe cases, saccular aneurysms were encountered in the arch of the aorta.

Microscopic examination demonstrated that several types of arterial damage were proceeding simultaneously in various segments of the aorta. Many areas were characterized by intimal swelling and proliferation, accompanied by medial hypertrophy and alteration of the elastic tissues. In some areas of intimal proliferation, the adjacent media showed necrosis and calcification, and in others, calcification was of the metastatic variety, without prior necrosis. Cartilaginous metaplasia with formation of bars of cartilage were frequently seen in the same animals. The aneurysms occurred in areas where the elastic tissue had become swollen and fragmented, permitting herniation of the arterial wall. The intimal plaques contained minute droplets of intracellular, and some extracellular, fat. Histochemical stains revealed profound changes in the mucopolysaccharides, or ground substance, of all layers of the arterial wall. The elastic fibers were disrupted, particularly above the intimal vegetations, and the normally regular elastic lamellae were stretched and distorted. Under tension, the interlamellar elastic fibrils were accentuated.

In some vessels, including the coronary arteries, thromboses were encountered, along with subsequent recanalization.

The coronary vessels stood out in abnormally sharp relief against the myocardium. The arteries were kinked and stiffened and showed intimal calcification, medial swelling, and fragmentation of the elastic tissue. Multiple infarcts were found in the ventricular portions of the heart. The cerebral vessels showed swollen tunica media, derangement of elastic tissue, and intimal calcification.

Male rats. Entirely different lesions were encountered in the males. In approximately 30 percent of these animals we observed polyarteritis nodosa, with severe aneurysmal dilatation, especially in the mesenteric arteries. Deep, penetrating gastric ulcers developed in more than half of the animals, usually two large ulcers and several smaller ones.

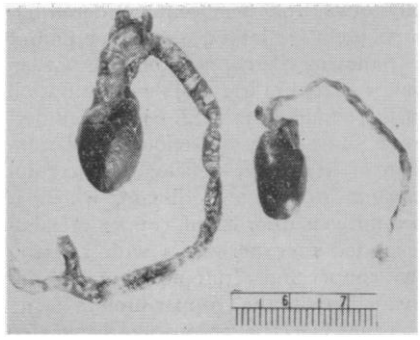

Fig. 1. Heart and aorta of ACTH-treated (left) and control (right) 1-year-old female rats. The ACTH-treated rat shows cardiac hypertrophy, ectasia, small saccular aneurysms, and silvery-white placodes throughout.

Testicular atrophy, too, occurred very frequently. In 85 percent of the animals the testes were unusually hard and firm and showed snow-white areas suggestive of fibrosis. The tubules appeared to be contracted. These tissues are now being studied microscopically.

Multiple renal calculi were encountered in about one-third of the animals. The lithiasis was intense; as many as 70 hard stones were found in one kidney. Chemical analyses of these stones are being made, and the problem of abnormal calcium metabolism in the animals is being studied.

The high incidence of polyarteritis, gastric ulceration, and testicular fibrosis can be ascribed to the administration of the ACTH. We are not yet certain whether this is equally true of the renal lithiasis, for kidney stones have also been found in some of the control male rats. This problem is being studied further.

In the older male and female rats another finding of interest is that of unusually rapid aging, evidenced by the lethargic behavior of the animals and the worn condition of their coats.

In contrast, the young, weanling rats, male and female, showed remarkably little gross pathology. Microscopic sections from these animals are now being studied.

Discussion. Experimental production of arteriosclerosis has, up to the present time, been restricted almost entirely to the rabbit and chicken (*1*). Our studies (*2*) suggest that a more suitable animal, the rat, can be employed. Further experiments will be required to determine whether the female rat is susceptible only under the special conditions we

have employed—that is, whether only the discard breeders are susceptible (*3*).

If this is the case it focuses attention on the stress of repeated pregnancy in these animals as a sensitizing factor in the subsequent development of arteriosclerosis. Preliminary experiments we have performed on the effect of exposure to cold and heat suggest that stressful situations other than exogenous administration of ACTH will also induce arteriosclerosis in these discard breeders.

Although unilateral nephrectomy is not essential to the production of arteriosclerosis, it does appear to intensify the lesions. However, the age of the rats is clearly a critical factor. Experiments are in progress to ascertain the youngest age at which rat arteriosclerosis can be initiated by ACTH injections. In addition, the effects of smaller amounts of ACTH and of less frequent injections are being studied in an effort to detect the earliest lesions of arteriosclerosis.

Because so much attention has been centered on the role of fat in the genesis of human arteriosclerosis, it should be emphasized that our rats were fed a normal diet not supplemented with fats. Furthermore, the serum cholesterol levels of our animals did not rise during the development of vascular disease. This does not, however, exclude the possibility that fat metabolism in the arterial wall itself is altered, and that this alteration is responsible for the changes observed in the rats.

The characteristic lesions observed in male rats may also provide some interesting experimental tools. The deep, penetrating peptic ulcers and the multiple renal calculi produced by ACTH administration may prove to be extremely useful in the study of peptic ulcer and kidney stones in man. The apparent speeding up of the aging process, observed in both male and female rats, also suggests research applications.

It is of interest to note that the dosage of ACTH we have administered (0.333 of a unit per 100 g body weight) is the equivalent of approximately 200 units in an average-sized human being. This is close enough to therapeutic levels to make one question the possible toxic effects of ACTH on the human vascular system in susceptible patients. Indeed, when one surveys the spectrum of lesions obtained in both male and female rats —arteriosclerosis, hypertension, senescent changes, renal stones, and gastric ulcerations—it is tempting to compare these changes with those seen in Cushing's disease. The possibility that the severe arteriosclerosis observed in our rats may actually result from hyperfunctioning of the adrenal glands is suggested by the fact that we have found these glands to be enlarged and the thymus glands to be involuted.

If further experiments prove that the arteriosclerotic lesions in the rat closely resemble those in human beings, the effects of an overactive pituitary-adrenal axis on arteriosclerosis in man will demand evaluation. If, as Selye believes, the stresses of life are channeled through the pituitary-adrenal system (*4*), stress may conceivably be an important determinant in human arteriosclerosis.

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25 November 1957

Use of Arginine to Eliminate Medium Changes in Tissue Culture Systems

Conventional tissue-culture techniques require that the nutrient medium be changed at frequent intervals. This methodology presents no difficulty in the instance of the small volumes handled in flask or test-tube type cultures. On the other hand, it presents a major obstacle to successful and continuous cultivation of mammalian cells in fluid agitated suspension (submerged culture), in volumes of 3 to 20 liters, as commonly employed in our laboratories (*1–3*). This report presents the results obtained when L-arginine is employed as a means of circumventing the necessity for making frequent changes of medium in such submerged culture systems. This is important not only from the standpoint of economy or ease of manipulation but also in relation to certain types of biochemical and biological experiments wherein it is desirable to maintain, as closely as possible, a somewhat constant cellular environment.

On the basis of the studies of Westfall *et al.* (*4*) it was known that, in certain tissue culture systems, histidine, arginine, isoleucine, methionine, and phenylalanine were rapidly depleted. It occurred to us that these substrates might be limiting and, as such, might be used to maintain active rates of cellular proliferation without renewal of media. Subsequent experiments (*5*) have demonstrated the validity of this assumption and, further, the fact that use of arginine alone is sufficient for this purpose.

In the basic investigations, Earle's L cell was used in Spinner culture (*1*). The nutrient medium routinely used was a modified Eagle's mixture (*3*). Rates of cellular proliferation were appraised by direct enumeration in a hemocytometer. Cell viability was determined through use of the vital stain trypan blue (*1*).

Submerged-culture cells of the L strain procured from a New Brunswick fermentor were centrifuged and resuspended in equal volumes of nutrient media in three Spinner vessels. Thereafter, the three Spinner cultures received additions of substrate every other day as follows: the control culture received saline; the second vessel received 21 μg of L-arginine per milliliter of culture; 42 μg of L-arginine per milliliter was introduced into the third vessel.

In this typical experiment there were uniform rates of cellular proliferation in all three Spinners during the first 2 days after initiation of the test (Fig. 1). After the 4th day, the cell concentration of the control culture decreased rapidly; this was followed by a loss in cell viability of from 98 percent on the 6th day to 30 percent by the 11th day.

Cells in the Spinner vessel that had received 21 μg of arginine per milliliter of culture continued to proliferate until the 4th or 5th day, when stabilization of the cell population occurred; this was followed by a gradual drop in cell concentration. The cell viability of this culture was maintained at a level above 90 percent through the 11th day. Active cellular proliferation in the 42 μg/ml culture was maintained, in this experiment, until the 8th or 9th day. Viability of this culture was maintained at a level above 95 percent throughout the 11th day (Fig. 1).

Addition of concentrations of 84 to

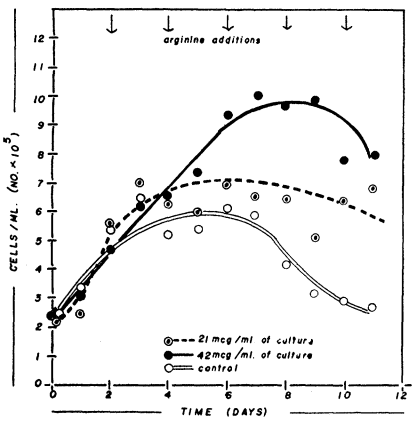

Fig. 1. The effect of arginine on the proliferation of L cells in submerged culture.