allata are also a link in the humoral cycle initiating ovarian development in mosquitoes.

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19 November 1957

Testing a Servoanalytic Hypothesis for Pupil Oscillations

Oscillations are a common and important instance of the malfunctioning of a servomechanism. Oscillations are also a common pathological abnormality in a wide variety of neurological diseases and are manifested in such clinical signs as tremor, ataxia, clonus, and nystagmus. This report indicates how a biological system has been analyzed by linear servoanalytic methods and experimentally justifies this approach by quantitatively verifying a prediction.

The pupil response to light—an example of neurological servosystem—has been studied by means of servoanalytic concepts and techniques (*1*). A sinusoidally varying intensity of light was applied, and the pupil area was continuously recorded. When intensity increases, the pupil contracts. The resultant effect of light falling on the retina can be resolved into two factors: (i) an increase due to increase in applied intensity and (ii) a decrease due to contraction of the pupil. System gain is here defined as the ratio of (ii) to (i). An open-loop transfer function

$$G(s) = 0.16e^{-0.18s}/(1 + 0.1s)^3$$

was computed from data graphically displayed in Fig. 1. This, the Nyquist diagram, is a vector plot of the relationship between gain and phase shift at various frequencies. For example, at 1.2 cy/sec, the gain is 0.12, with a phase lag of 180°. This gain means that the pupil compensates for 12 percent of the change in applied light intensity.

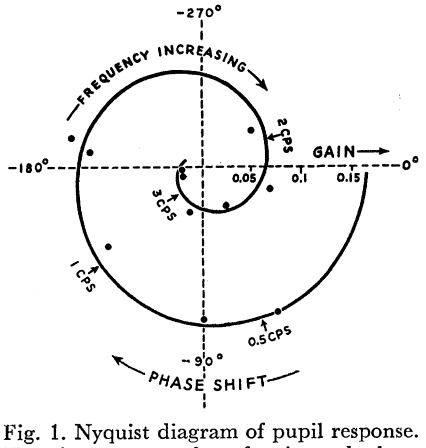

Fig. 1. Nyquist diagram of pupil response. This is a vector plot of gain and phase shift. The scale of the modulus is shown, and a few frequencies are indicated. The curve is derived from fitted lines from gain and phase frequency-response graphs, while the points are experimental.

If the gain were to be increased to 1.0 or more, the system would become unstable, and the pupil would oscillate at its natural frequency—that is, the frequency at which the phase lag is 180°. From the figure we see that this frequency is 1.2 cy/sec. Thus, from our servoanalysis of the normal, low-gain, stable pupil system, we are led to predict that a large increase in gain would produce sustained oscillations and that the frequency of these oscillations would be 1.2 cy/sec (72 cy/min). A test of the validity of the servoanalytic method would be the production of pupil oscillations in this manner and at the predicted frequency. Observations which comprise such a test are already available for evaluation.

Clinical studies have been reported in which sustained oscillations of the pupil have been produced and in which the frequency of the oscillations has been measured. Stern (*2*) pointed out that a series of oscillations of the pupil could be induced by imaging a small point of light just on the margin of the pupil. This causes the iris to contract, and all the light is cut off in the early phase of contraction. The iris therefore redilates, and full light intensity again enters the eye. The gain is thus increased to more than 1.0, and sustained oscillations result. Campbell and Whiteside (*3*) made a careful study of parameters affecting this induced pupil oscillation, using quantitative techniques on a group of normal subjects. A third report is that of Wybar (*4*), who studied a large number of normal subjects and also patients with multiple sclerosis (*5*). In Table 1 the frequencies of the sustained oscillation observed are summarized.

There is good agreement between the frequency of pupil oscillations observed in normal subjects and our prediction. Further experiments have been carried out by Stark and Baker (*6*) in which the transfer function of the pupil system has been altered by drugs. For example, when the 180°-phase cross-over frequency was changed from 1.5 to 0.9 cy/sec, the high-gain oscillation frequency shifted in a parallel fashion from 1.5 to 1.0 cy/sec (*6*).

Table 1. Observed frequencies of pupil oscillations (references in parentheses).

Group studied	Mean frequency (cy/min)
10 Normal subjects (*2*)	80
1 Normal subject (*3*)	71
37 Normal subjects (*3*)	69
34 Normal subjects (*4*)	62
Patients with multiple sclerosis: 70 pupils (*4*)	41
Predicted value (*1*)	72

The qualitative value of the servoanalytic approach is demonstrated by the clarification introduced through explanation of the nature of these pupil oscillations. The quantitative nature of the method is illustrated by the accuracy of the prediction (*7*).

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5. It is interesting to note that oscillation of the pupil in the multiple sclerosis group differs from that in the normal groups in mean frequency. This difference must be a result of a change in system parameters produced by the neurological lesions.
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4 November 1957

Preparation of an Apoprotein from Ceruloplasmin by Reversible Dissociation of Copper

The blue copper-protein of plasma, ceruloplasmin, with a molecular weight of 151,000, contains eight atoms of copper per molecule (*1*). The physiologic significance of this protein, while not clear, has usually been implicitly associated with its oxidase activity (*2–5*). On the other hand, the possibility that reversible binding and release of copper by the protein may be the basis of its

physiologic role has been considered improbable (*4*), largely because attempts at removing the copper from the protein have resulted in irreversible denaturation of the ceruloplasmin (*2, 6*). A consideration of the possible etiologic relationship of hereditary deficiency of ceruloplasmin to Wilson's disease, which is primarily a disorder of copper metabolism, led to experiments with radioactive copper and ceruloplasmin which indicated that the copper-protein bond can, in fact, be reversibly dissociated under certain conditions (*7*). These results suggested that it might prove possible to remove copper from ceruloplasmin and prepare an apoprotein which would be capable of recombining with copper to form ceruloplasmin. This report describes experiments in which such an apoprotein was obtained (*8*).

The reversible separation of the copper and protein was effected under conditions which were as similar as possible to those under which exchange of ceruloplasmin copper and radioactively labeled copper ions had been observed. The procedure was carried out, therefore, in an acetate buffer and in the presence of sufficient ascorbic acid to keep the ceruloplasmin copper in the monovalent state throughout its removal from the protein. Advantage was taken of the fact that the diethyldithiocarbamate ion combines tightly with copper ions to form a colloidal suspension (*9*). This made it possible to remove by ultracentrifugation the copper which had been separated from ceruloplasmin.

Solution A (Table 1) was made by bringing ceruloplasmin preparation AK-81 (*10*) to a *p*H of 5.2 and an ionic strength of 1.2 with acetate buffer. To 35.5 ml of solution A, which contained 8.5 mg of ceruloplasmin per milliliter, 58.5 mg of crystalline ascorbic acid was added, which instantly decolorized the blue solution. During the next 5 hours the solution was kept at 0°C and was stirred while 9.3 ml of 0.4-percent sodium diethyldithiocarbamate in water was added, dropwise. The final mixture, B—golden brown and slightly cloudy—was centrifuged in a Spinco model L ultracentrifuge for three hours at 40,000 rev/min, at 0°C. The black precipitate was analyzed quantitatively for copper, and 32.0 ml of slightly yellow, but clear, supernatant solution, C-1, was passed at 0°C through a column of ion-exchange resins containing 10 ml of IR-120 (sodium) resin and 15 ml of IRA-400 (acetate) resin (*11*). By rejecting the dilute fore- and after-runs, we obtained 31.0 ml of a clear, water-white solution, C-3, which contained 69.9 percent of the protein of solution A. The C-3 solution, which contained the apoprotein, was kept frozen at −30°C in several aliquots. Ceruloplasmin was regenerated after thawing by the addition of 1.3 ml of ascorbic acid solution (120 μg/ml), 3.0 ml of cupric sulfate solution (20 μg of Cu^{++} per milliliter), and 7.7 ml of water to 3.0 ml of C-3. After passage of this solution through a column which contained 7 ml of acetate resin and 14 ml of sodium resin (*12*), and which had been previously equilibrated with a 0.2*M* acetate buffer of *p*H 5.25, 14.8 ml of a clear blue solution, C-4, which contained ceruloplasmin, was collected.

Table 1 lists comparative analytical data on the original ceruloplasmin, the apoprotein, and the regenerated ceruloplasmin. It is clear that about 95 percent of the blue color, enzymatic activity, and ceruloplasmin copper possessed by the protein in solution A have been removed from C-3.

The analyses of C-4 indicate that nearly 60 percent of both the blue color and tightly bound ceruloplasmin copper and 86 percent of the enzymatic activity present in solution A were restored to C-3. Addition of up to ten times as much copper and ascorbic acid as were used originally resulted in no greater intensity of blue color or greater yield of tightly bound copper or enzymatic activity.

We have observed that the addition of cupric ions without ascorbic acid to a solution of apoprotein does not result in the formation of blue ceruloplasmin. However, enzymatic activity equal to that of C-4 is found in this solution if the substrate paraphenylenediamine dihydrochloride is added after addition of the cupric ions. Oxidation of this substrate produces dark-colored products which have prevented us from determining if the paraphenylenediamine dihydrochloride has restored blue color to the apoprotein-cupric ion mixture in addition to restoring enzymatic activity.

The percentage of enzymatic activity restored to C-4 is disproportionately higher than the amounts of blue color and tightly bound copper which were regenerated. It has consistently been found to be the case that each atom of copper which is recombined with apoprotein is about 1.5 times as active enzymatically as an atom of copper in the original ceruloplasmin. Yet this enhancement is not found with respect to the blue color. Current work is directed toward determining the reason for this discrepancy.

The physiologic significance of the demonstration that ceruloplasmin can exist as an apoprotein remains to be clarified. It is at least consistent with the hypothesis that ceruloplasmin functions by reversibly releasing and binding copper at various sites in the organism, possibly thereby regulating the absorption

Table 1. Quantitative comparison of some characteristics of ceruloplasmin (solution A), the apoprotein (solution C-3), and regenerated ceruloplasmin (solution C-4). (O.D., optical density.)

Characteristic	Solution		
	A	C-3	C-4
Total protein			
O.D.$^{280\ m\mu}_{1\ cm}$	15.25	10.64	2.00
Nitrogen (mg/ml)	1.4	1.09	0.195
Blue color			
Δ O.D.$^{610\ m\mu}_{1\ cm}$ *	0.635	0.011	0.048
Blue color, as percentage of that in solution A, corrected for content of total protein†	100	2.48	57.5
Copper			
Total (μg/ml)‡	30.7	1.46	2.54
Free (μg/ml)§	1.7	0.00	0.32
Ceruloplasmin copper (μg/ml)‖	29.0	< 1.46	2.22
Ceruloplasmin copper, as percentage of that in solution A, corrected for content of total protein†	100	< 7.2	58.4
Enzymatic activity			
Δ O.D.$^{530\ m\mu}_{1\ cm}$/min ml¶	0.832	0.0334	0.0935
Enzymatic activity, as percentage of that in solution A, corrected for content of total protein†	100	5.7	86.0

* Difference in O.D. before and after decolorization of ceruloplasmin by ascorbic acid. All ΔO.D.'s$^{610\ m\mu}_{1\ cm}$ were higher than ΔO.D.'s at other wavelengths between 540 and 660 mμ (see *7*).

† The calculation for blue color is as follows:

$$(\Delta O.D.^{610\ m\mu}_{1\ cm}/O.D.^{280\ m\mu}_{1\ cm}) \times [(O.D.^{280\ m\mu}_{1\ cm}/\Delta O.D.^{610\ m\mu}_{1\ cm})]_{soln\ A} \times 100.$$

Percentages of ceruloplasmin copper and of enzymatic activity are calculated in an analogous manner.

‡ By wet digestion (see 7). Of the 1090 μg of copper in solution A, 983 μg were found, by analysis, in the precipitate and supernatant (C-1) from the ultracentrifugation. Of the copper accounted for, 914 μg, or 93 percent, was in the precipitate.

§ Copper which will react directly with dicyclohexanoneoxalyldihydrazone to form a blue compound at *p*H 7 (see 7).

‖ Total minus free. The amount of ceruloplasmin copper in solution C-3 has not been determined with certainty, but it is less than 1.46 μg/ml, since any copper-diethyldithiocarbamate which is present in solution will not react with dicyclohexanoneoxalyldihydrazone.

¶ Measured spectrophotometrically by effect on rate of oxidation of paraphenylenediamine (see 7).

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

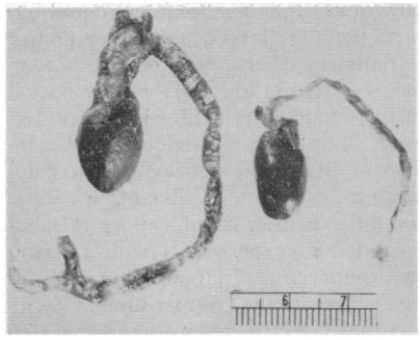

Fig. 1. Heart and aorta of ACTH-treated (left) and control (right) 1-year-old female rats. The ACTH-treated rat shows cardiac hypertrophy, ectasia, small saccular aneurysms, and silvery-white placodes throughout.

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.

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