Sulfhydryl Groups in Resting and Stimulated Rat Brain; Their Relationship with Protein Structure. (23729)

GEORGES UNGAR AND DOMINICK V. ROMANO
Department of Pharmacology, U. S. Vitamin Corp., N. Y. City

The possibility that some structural rearrangement of cellular proteins is involved in excitation has been suggested by some early observations (reviewed by Hydén(1)) and by later work(2,3). In a recent publication(4), it was shown that electrical stimulation of frog and rat sciatic nerve and of cat, dog, and rat brain produces structural changes in proteins of these tissues, as indicated by the increase in the number of ionized side-groups. The present paper supplies further evidence in support of the hypothesis that excitation is accompanied by changes in protein configuration similar to those commonly associated with reversible denaturation.

Methods. Male albino rats (C. F. Wistar strain), weighing 250 to 350 g, were used. The skull was opened under topical ethyl chloride anesthesia and the exposed brain surface was kept moist with saline. Stimulation was effected through electrodes attached to the central end of the sciatic nerves by means of a 40 v. 60 cycle current from a Harvard stimulator. The brain was frozen by the procedure of Geiger *et al.*(5). Resting brain specimens were secured by the same procedure from rats rested at least 20 min. after exposure of the brain. The extracts were prepared according to the technic described previously(4). *Sulfhydryl groups* were estimated by amperometric titration, using the technic of Benesch, Lardy, and Benesch(6). Results are expressed in terms of μM of –SH per g of wet weight of brain. The use of wet weight was judged satisfactory because there was no significant difference between the water content of resting and stimulated rat cortex: 79.2 $\pm$ 0.9% at rest and 79.5 $\pm$ 0.4% after 20 min. stimulation. When the –SH determination was not done immediately after the experiment, the extract was kept frozen at –20°C. *Spectrophotometric estimation* of the ionization of protein side-groups was described in detail(4). It is based on the observation of Crammer and Neuberger(7) that at high pH the ultraviolet spectrum of proteins undergoes a shift in the wavelength of maximum absorption and an increase in extinction coefficient. The change is related to the number of ionized –OH groups of tyrosine (in the 290-300 mμ range) and of –SH groups of cysteine (in the 240-250 mμ range)(8). Results obtained with this method are expressed in terms of the side-group ionization ratio (SGIR) obtained by dividing the optical density of the extract at pH 12 by the optical density at pH 7.

Results and discussion. Table I summarizes the experiments with saline extracts of brain. It is seen that both the –SH content and the SGIR are higher in extracts from stimulated brain than in those from resting brain. After stimulation of 1 min. the change is statistically not significant, but after 20 min. application of electrical current the differences are highly significant ($P<.001$) for both values. In previously published experiments(4), in rats

TABLE I. Changes in –SH Groups and Side-Group Ionization Ratio (SGIR) in Resting, Stimulated, and Recovering Rat Brain.

Stimulation	Recovery	–SH*	± S.D.	SGIR†	± S.D.	P	N
Resting		4.56	.57	1.80	.08		29
1 min.		5.08	.78	1.95	.07	>.05	7
1	1 min.	4.75	.83	1.90	.05		6
1	2	4.15	.56	1.80	.05		6
20		6.55	1.02	2.09	.11	<.001	26
20	10	5.20	.96	1.91	.08		6
20	20	4.17	.32	1.85	.04		6

* In μM/g wet wt. † At 245 mμ. Brain extracts were made up in 0.15 M NaCl.

TABLE II. Changes in –SH Groups and Side-Group Ionization Ratio in Resting and Stimulated Rat Brain.

Extract		–SH†	± S.D.	SGIR‡	± S.D.	P	N
Ringer sol.	Resting	5.38	.72	1.77	.09		8
	Stimulated*	8.90	1.21	2.05	.10	.01–.001	8
Isotonic sucrose	Resting	4.20	.57	1.72	.12		7
	Stimulated*	6.01	1.23	1.95	.07	.01–.001	7

* 20 min. stimulation. † μM/g wet wt. ‡ At 245 mμ.

anesthetized with nembutal, SGIR values were lower (1.52 in resting brain and 1.85 after 20 min. stimulation). Table I also shows that the changes are reversible after removal of the stimulus.

When brain extracts were prepared in Ringer solution (without glucose), the SGIR changes were the same as in saline extracts. The –SH values, however, were found to be considerably higher (Table II). In extracts prepared in isotonic sucrose, both values were somewhat lower than in saline. Differences between resting and stimulated brain, however, were highly significant in all types of extracts tried.

The values given in Tables I and II represent the sum of free and bound –SH groups. To determine the bound –SH, extracts from resting and stimulated brain were dialyzed against 25 volumes of saline in a shaking dialyzer. At equilibrium, the –SH groups were titrated in the non-dialyzable fraction. Table III shows that about 75% of the –SH groups are bound to non-dialyzable molecules, presumably to proteins. The Table also shows that the changes produced by stimulation affect mostly the bound –SH. Stimulation causes a greater change in bound (55%) than in total (47%) or free –SH (29%).

Available evidence(8,4) suggests that the SGIR changes at 245 mμ indicate an increase in the number of ionized –SH groups of cysteine. Comparison of the direct estimation of titratable –SH groups with the SGIR has confirmed this assumption. Fig. 1 shows the correlation between the 2 sets of values, as obtained in 62 experiments with saline extracts of brain. The correlation coefficient ($r = 0.72 \pm 0.062$ S.D.) is highly significant ($P = <.001$). This correlation, however, is

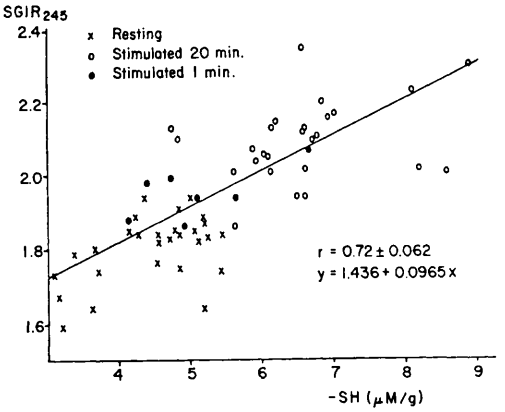

FIG. 1. Correlation between –SH groups and side-group ionization ratio. Abscissa: –SH groups in μM/g wet wt; ordinate: side-group ionization ratio at 245 mμ. Regression line calculated by least squares method. Significance of the correlation coefficient is $P = <.001$.

valid only in the given ionic environment. The relationship changes with the composition of the extraction medium. The influence of various electrolytes and pharmacological agents on the phenomena just described will be reported elsewhere.

It has been admitted that in native proteins only a fraction of the –SH groups present can be titrated directly. The increase in the

TABLE III. Changes in Free and Bound –SH Groups.

	–SH (μM/g)*					
	Resting	± S.D.	Stimulated†	± S.D.	t	P
Total	4.31	.73	6.34	.93	4.5	<.001
Non-dialyzable	3.15	.38	4.84	.41	8.1	<.001
Dialyzable‡	1.16	.41	1.50	.69	1.1	.3–.2

* Mean values of 8 extracts. † 20 min. stimulation. ‡ Estimated by difference.

titratable –SH groups has been considered as a sign of denaturation. It seems, therefore, legitimate to interpret the changes just described as indications of a structural rearrangement of some protein molecules. Similar changes in –SH groups have been described in different systems; in the egg following fertilization(9) and in rhodopsin stimulated by light(10). The latter observation confirms Mirsky's hypothesis that light produces a reversible denaturation-like change in the protein moiety of visual purple(11). Other protein sidegroups are also "unmasked" in the process of denaturation, but the carboxyl or amino groups are not as readily detectable as the –SH groups. Ionization of the phenolic hydroxyl of tyrosine was described earlier(7,4).

The possible role of changes of protein configuration in the mechanism of cellular excitation is fully discussed elsewhere(12). Reversibility is one of the essential conditions for any change to be directly associated with excitation. The problem of the comparatively slow restoration of protein structure, as compared with the prompt return of excitability has to be approached with more sensitive methods, capable of detecting changes caused by a small number of impulses. Another problem, that of the relation between protein changes and the shift of Na^+ and K^+ ions during excitation, is under investigation.

Summary. An increase in titratable –SH groups was observed in rat brain stimulated through its afferent nerves. The change is reversible when the resting state is restored. The increase produced by stimulation affects mainly the non-dialyzable, presumably protein-bound –SH groups. The change is interpreted as an indication of structural rearrangement of certain proteins which may participate in the mechanism of excitation.

1. Hydèn, H., *in* Elliott, K. A. C., Page, I. H., Quastel, J. H., *Neurochemistry*, Springfield, Ill., C. C. Thomas, 1952, p204.

2. Nassonov, D., *Isvest. Akad. Nauk USSR, ser. biol.*, 1948, v4, 381.

3. Segal, J., *in Internationales Symposium über den Mechanismus der Erregung*, Berlin, 1956, p12.

4. Ungar, G., Aschheim, E., Psychoyos, S., Romano, D. V., *J. Gen. Physiol.*, 1957, v40, 635.

5. Geiger, A., Dobkin, J., Magnes, J., *Science*, 1953, v118, 655.

6. Benesch, R. E., Lardy, H. A., Benesch, R., *J. Biol. Chem.*, 1955, v216, 663.

7. Crammer, J. L., Neuberger, A., *Biochem. J.*, 1943, v37, 302.

8. Benesch, R. E., Benesch, R., *J. Am. Chem. Soc.*, 1955, v77, 5877.

9. Rapkine, L., *Ann. Physiol.*, 1931, v7, 382.

10. Wald, G., Brown, P. K., *J. Gen. Physiol.*, 1951-52, v35, 797.

11. Mirsky, A. E., *Proc. Nat. Acad. Sci.*, 1936, v22, 147.

12. Ungar, G., *J. de Physiol.*, in press.

Received November 13, 1957. P.S.E.B.M., 1958, v97.

Pteroylglutamic Acid in Different Diseases. (23730)

SACHCHIDANANDA BANERJEE AND SUNIL KUMAR PAIN
Department of Physiology, Presidency College, Calcutta, India

Sauberlich and Baumann(1) observed that pteroylglutamic acid (PGA) is converted to citrovorum factor (CF) in the body and the latter is the active form of the vitamin PGA. The metabolism of PGA has been studied in normal persons(2-4) and in patients suffering from liver diseases(5), pernicious anemia(6, 7), megaloblastic anemia(8), gout(9), cancer and tuberculosis(10). Considering the various functions of PGA in the different physiological and pathological processes in the animal body it was of interest to study the metabolism of PGA in patients suffering from various diseases and in normal persons, by estimating urinary excretions of PGA and CF.

Methods. 17 cases of cirrhosis of liver, 10 of typhoid fever with positive blood culture,