each dosage of 5-FU were compared with the appropriate saline or glucose control value and plotted log-dose against probit percent. The regression lines obtained indicated that 50-percent inhibition of tumor growth is caused by a dose of about 32 mg of 5-FU per kilogram when given with saline and by a dose of about 18 mg with concurrent glucose treatment. Since the pK_a values of 5-FU nucleosides and nucleotides are 7.6 to 7.7, at the physiological *p*H of 7.4 these compounds are about 60 percent undissociated; at *p*H 6.3 the level reached in tumors after glucose treatment (*9*, *10*), the 5-FU ribose derivatives would be 95 percent un-ionized. Thus the percentage increase in the antitumor activity may be related to the increase in the percentage of un-ionized species of nucleotide that is present.

An experiment was performed to test whether 5-FU antitumor action can be enhanced by galactose administration (in the same regimen as for glucose in Table 1). Treatment with this hexose was tested because, in contrast to the effect of glucose treatment, it does not cause lactate accumulation and a lowered *p*H in the Flexner-Jobling carcinoma (*9*). The tumor sizes after 7 days of treatment were (in grams wet weight ± S.D.): saline alone, 6.5 ± 1.5; galactose alone, 6.5 ± 1.5; saline + 5-FU (10 mg/kg), 6.6 ± 1.6; galactose + 5-FU (10 mg/kg), 6.5 ± 1.9; glucose + 5-FU (10 mg/kg), 4.8 ± 0.9. Thus galactose had no measurable effect on the tumor response to 5-FU while the expected response to concurrent glucose therapy was observed ($0.01 > P > 0.001$).

The question arises whether glucose treatment increases the toxicity of 5-FU as well as its antitumor effect; that is, does the therapeutic index remain unchanged? Present indications are that the toxicity is not significantly affected in the rat by added glucose treatment. At the various doses (Table 1), the average weight change was comparable in the saline and glucose-treated group pairs. Fatalities were observed only at 40 mg of 5-FU per kilogram, where two animals of six in the saline group died and one of six in the glucose group died. Groups of normal rats were administered 5-FU in seven daily doses of 40, 60, 80, and 100 mg per kilogram of body weight with concurrent saline or glucose treatment. All animals that received 60 mg of 5-FU or more per kilogram of body weight per day died within the 7 days of the experiment and those given 40 mg died within 3 days after the end of the treatment. The average times of death at the various 5-FU doses were similar in the saline and glucose-treated group pairs.

The major site of action of 5-FU is the enzyme thymidylate synthetase (*14*), which is profoundly inhibited by 5-fluoro-2′-deoxyuridine-5′-monophosphate. Since this analogue nucleotide is formed from 5-FU in a "lethal synthesis" in which at least four different enzymes participate, *p*H alterations might affect the reaction at thymidylate synthetase, or one or more of the enzymes in the pathway of 5-fluoro-2′-deoxyuridine-5′-monophosphate synthesis. Although enzyme studies showing the analogue nucleotides to be more effective in an acidic environment led to the present experiments, it may not be assumed that glucose potentiation is occurring at the enzyme level. Transport of 5-FU into the tumor cell may be enhanced by the lowered *p*H. Future studies will be needed to determine which, if any, of these explanations is valid.

The pK_a values of other antitumor compounds, such as 8-azaguanine, 6-azauracil, 6-thioguanine, and 6-mercaptopurine and their nucleosides and nucleotides are significantly below those of their respective natural congeners (*15*), but it is not known whether the antitumor action of these compounds may also be enhanced in an acidic tumor environment. Perhaps the therapeutic index can be improved still further by decreasing the toxicity to normal tissues by alkalinization through bicarbonate administration or hyperventilation (*16*).

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11. In preliminary studies the intraperitoneal administration of 5 g glucose per kilogram of body weight in mice caused a high mortality rate, thus rat tumors seemed more suitable for study than mouse tumors.
12. The line of Flexner-Jobling carcinoma used is that carried in the Wisconsin Alumni Research Foundation laboratories.
13. The 5-fluorouracil was a gift of Dr. James Price.
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15. Reported pK_a values are: 8-azaguanosine, 8.5 (R. M. Bock, private communication); 6-azauridine, 6.8 (*5*); 6-thioguanosine, 8.3 (*4*); 6-mercaptopurine riboside, 7.8 (*4*).
16. Supported by grant CY 2686 of the National Cancer Institute.

26 April 1963

"Microsome" Fraction of Brain: Structural Changes Induced by Ascorbic Acid

Abstract. *The usual vesicular configuration of membrane fragments in the brain "microsome" fraction is radically altered by treatment with ascorbic acid and adenosine monophosphate. Light-scattering measurements and electron micrographs show that the treated membranes assume predominantly planar forms. This change in structure appears to be contingent upon a continued transport of electrons from ascorbic acid.*

Although the ascorbic acid content of neurons in certain parts of the brain such as hypothalmus is remarkably high (*1*), the nature of its role in neural function is completely unknown. Ascorbic acid was found to induce gross structural changes in vitro in the membranous component of the so-called "microsome" fraction of rat brain. Although these structural changes are associated with an oxidation of ascorbic acid, no other potential electron donor, for example, glucose, tricarboxylic acid cyclic intermediates, glutathione, or hydrosulfite, could be substituted for ascorbic acid or D-isoascorbic acid in producing this effect.

This investigation actually began as an attempt to duplicate with microsomes from the brain the finding that ascorbic acid, in the presence of microsomes from several non-neural tissues such as adrenal, liver, and kidney, can activate the oxidation of reduced nicotinamide adenine dinucleotide ($NADH_2$) (*2*). However, there was an interference with the optical measurement of this oxidation because of an unexpected change in light scattering in brain microsome fractions that had been treated with ascorbic acid.

This suggested an alteration in particulate structure. Since such changes had not been described, a further investigation of this ascorbate effect in brain microsomes was undertaken. Small changes in light scattering in response to various reagents including Mg^{++} and adenosine triphosphate have been observed in liver microsomes (*3*).

Microsomes were prepared from rat brain (*4*). A single brain was homogenized in 10 volumes of 0.25*M* sucrose at 0°C with a Teflon pestle (cylinder diameter 2.54 cm; clearance 0.012 to 0.018 cm) at 1500 rev/min for 30 sec (6 strokes). The supernatant of an initial 12-minute spin at 18,000*g* was decanted and centrifuged at 30,000*g* for 25 minutes. The resulting white pellet was "washed" in 0.25*M* sucrose and resedimented. The microsomes obtained were virtually free of mitochondrial and ribosomal contamination as indicated by electron microscopy and by the absence of succinic dehydrogenase and ribonucleic acid. The basic medium used in all experiments consisted of 0.05*M* tris-Cl buffer (*p*H 7.4) to which microsomes and specified reagents were added.

In the presence of this brain microsome fraction, ascorbic acid seemed to activate the oxidation of $NADH_2$ judging by the decrease in optical density at 340 mμ. Although microsomes derived from brain appeared to behave similarly to those of other tissues, nevertheless at low concentrations of $NADH_2$ the apparent extent of oxidation exceeded that expected even if all the $NADH_2$ initially present were oxidized. Therefore, the assumption that the change in optical density represented a specific absorbency shift due to $NADH_2$ oxidation had to be reexamined. The total decrease in optical density was actually accounted for by a decrease in particulate light scattering induced by ascorbate. This was indicated by the fact that the change was completely independent of the presence of $NADH_2$, that it occurred uniformly throughout the visible spectrum, and that the shift in the direct light path was opposite in direction to that at 90°. This change in light scattering is grossly visible—it appears as a clearing of an initially turbid microsome suspension.

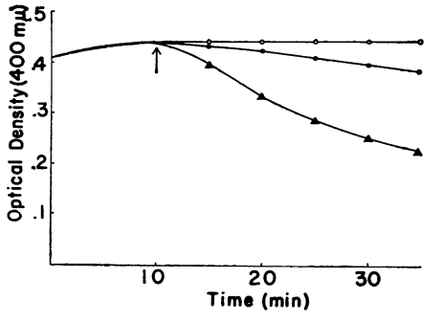

Fig. 1. Optical-density changes in suspensions of rat-brain miscrosomes after addition of 1m*M* AMP and 0.1m*M* ascorbate either separately or in combination. Initial optical density reading represents turbidity of microsomes (0.16 mg protein/ml, 1-cm light path) in a medium of 0.05*M* tris-Cl, *p*H 7.4 (25°C). ○—○, No additions or 1m*M* AMP; ●—●, 0.1m*M* ascorbate; ▲—▲, 0.1m*M* ascorbate + 1m*M* AMP.

Figure 1 shows a typical experiment in which the optical density of a brain microsome suspension (0.16 mg protein per milliliter) gradually decreases upon the addition of ascorbic acid. The ascorbate-induced change is greatly accelerated by adenosine monophosphate (AMP), and within 30 minutes there is a 50-percent drop in optical density. Alone AMP has no effect. Adenosine triphosphate (ATP), except in the presence of magnesium ion, is not as effective as AMP in promoting the action of ascorbate. The optimal molar ratio of Mg^{++} to ATP is 2:1, although this is not critical. Adenosine diphosphate, even with Mg^{++}, is actually inhibitory. The rate or extent of the changes in light scattering are not influenced by Na^+ and K^+, but after a 30-minute exposure of microsomes to ascorbate and AMP there is a 50-percent reduction in the rate of Mg^{++}- and $Na^+ - K^+ - Mg^{++}$-activated ATP hydrolysis. This inhibition is proportional to the degree of change of light scattering rather than to the presence of ascorbate or AMP per se. Alone, Mg^{++}, Ca^{++}, and Mn^{++} induce small increases in light scattering; while Mg^{++} and Ca^{++} do not interfere with the ascorbate-induced changes, Mn^{++} is a potent inhibitor even at $10^{-6}M$.

When control microsomes and those treated with ascorbate and AMP are centrifuged, the resulting pellets reflect the optical-density changes that have occurred in the suspended state. Because of intense light scattering, pellets from the controls have a white, translucent appearance, whereas pellets from treated microsomes have become virtually transparent. In addition to the change in light scattering, pellets from treated microsomes exhibit a distinct yellow-brown color, perhaps indicating the presence in the brain of a hemochromogen similar to that in liver microsomes (*5*).

That ascorbic acid and AMP together induce a large decrease of light scattering in suspensions of brain microsomes, as well as a comparable change in the appearance of derived pellets, suggested a structural change in the membrane fragments. This was confirmed by electron micrographs obtained from both control and treated pellets. In Fig. 2*A* a representative section from a control pellet shows many "smooth" vesicular membrane fragments characteristic of microsome preparations from brain. These "vesicles" are essentially artifacts, arising from the endoplasmic reticulum and other membranes torn during homogenization (*4*, *6*).

A section of microsomes treated with ascorbate and AMP is shown in Fig. 2*B*. There is virtually a complete loss of the vesicular forms. The membranes appear thinned and spread out

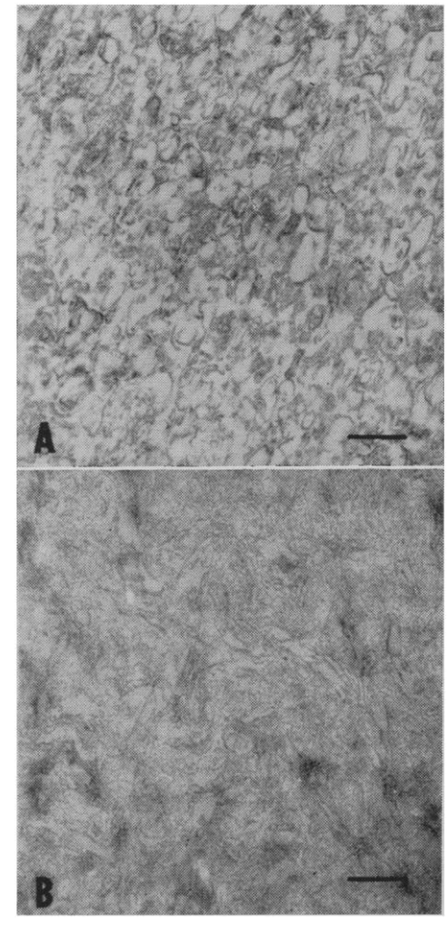

Fig. 2. *A*, An electron micrograph of an untreated brain microsome fraction showing typical vesicular membrane fragments. *B*, A considerable alteration of the vesicular forms after treatment with 0.1m*M* ascorbate and 1m*M* AMP for 30 minutes. The treated membranes appear "flattened," and are very closely packed in the pellet. Scale: 0.15 μ.

largely into planar forms. Such "flattened" membranes would clearly tend to scatter light much less readily than those with curved surfaces which predominate in the control material. Thus there is a correspondence between the measurements of light scattering and the structural change visualized in electron micrographs.

Preliminary studies reveal a correlation between the structural change and a cyanide-insensitive oxidation of ascorbic acid. When the ascorbate in a reaction cuvette is exhausted, the change in light scattering ceases. Similarly, if at any point the ascorbate is rapidly degraded by the addition of an equivalent of an oxidant which by itself has no effect on the system, for example, ferricyanide or cytochrome *c*, there is an immediate cessation of the change. Moreover, under anaerobic conditions there is complete inhibition of the ascorbate effect, suggesting that the change is dependent on a continuous transport of electrons, rather than on a static reduction, with oxygen as the final acceptor. If anaerobiosis is introduced after a decrease in light scattering has occurred there is considerable reversal of the change. As yet, no other conditions for reversal have been found. The possible "coupling" of ascorbate oxidation to the structural transformation appears to be very complex since chlorpromazine (50 μM) will completely inhibit the change in light scattering but allow the oxidation of ascorbate to continue unhampered.

Although changes in cellular conformation occur in tissue culture it remains a question whether the structural changes in isolated microsomal membranes have a counterpart in intact cells or in vivo. Several substances thought to affect brain function—namely serotonin, norepinephrine, phenothiazines, and thyroxine—are potent inhibitors of the ascorbate-induced changes in brain microsome structure (*7; 8*).

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Eyeball Retraction: Classical Conditioning and Extinction in the Albino Rabbit

Abstract. *A comparison of the percentage-performance curve of a classical conditioning group with those of three control groups provided unequivocal evidence of successful conditioning of the retractor bulbi response to an auditory conditioned stimulus.*

Stimulation of the cornea of the rabbit's eye with a puff of air is accompanied by a closure of the outer eyelids, retraction of the eyeball into its orbit, and a sweeping of the nictitating membrane across the surface of the cornea. Although the mechanism of movement and the innervation of the nictitating membrane remain subject to controversy, eyeball retraction is accomplished by the retractor bulbi or choanoid muscle, activated by the sixth cranial nerve (*1*). With regard to the rabbit's nictitating membrane, Last (*2*) contends it has no connection with any muscle and simply moves passively across the cornea when the eyeball retracts. On the other hand, Lierse (*3*) reports the presence of a striated muscle connected to the upper temporal portion of the membrane which acts to pull the membrane forward.

Although we have conditioned the eyelid (*4*) and nictitating membrane response (*5*) in the albino rabbit, the conditioning of eyeball retraction may possibly offer certain advantages. If, in fact, the membrane moves across the eye only as a concomitant of the retractor bulbi response, it follows that the latter is the more direct and perhaps more stable of the two response systems. Furthermore, it is improbable that attenuation of the air puff (that is, the unconditioned stimulus) could occur from anticipatory retractions of the eyeball (that is, conditioned responses), thus aborting an argument which is sometimes used against eyelid conditioning.

The conditioning apparatus and the manner in which the rabbit was restrained have been described (*4–6*). To record eyeball retractions, a transducer was constructed of a balanced lever, on one end of which was mounted a 9-mm diameter loop made of PE 60 polyethylene tubing. A nylon string fastened to the other end of the balanced lever was connected to the shaft of a gravity-return rotary potentiometer. To permit unimpeded recording of eyeball retractions the upper and lower eyelids of the rabbit's right eye were taped open and the nictitating membrane held back by a metal hook fastened to a loop of nylon that had been sutured in the membrane. The polyethylene loop was then placed on the cornea of the prepared eye, and retractions of the eyeball caused the shaft of the potentiometer to rotate. The signals from the potentiometer were then amplified and graphically recorded by an ink-writing penmotor.

The conditioned stimulus (CS) was a 600-msec 3500-cy/sec tone at a sound pressure level of 72 db (relative to 2×10^{-4} dyne/cm^2) and the unconditioned stimulus (UCS) was a puff of compressed nitrogen of 100-msec duration with an intensity of 80 mm-Hg at its point of delivery to the right eye. The orifice of the air jet was adjusted to deliver the air puff through the center of the polyethylene loop at a distance of about 5 mm from the cornea.

Twenty-four albino rabbits, 80 to 100 days of age, were assigned to one of four groups for 2 days of adaptation, 8 days of acquisition training, and 3 days of extinction. On each of the 2 days of adaptation neither conditioned nor unconditioned stimuli were presented and during these sessions a measure of spontaneous eyeball movement was obtained for all groups by recording the frequency of responses in time intervals corresponding to the 70 CS-UCS acquisition trials presented to the classical conditioning group. In acquisition, one control group received the CS alone (group CS-Alone), another received the UCS alone (group UCS-Alone), and a third group received random presentations of the CS alone and the UCS alone (group CS-UCS Mixed).