References and Notes

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3. Methods: Pregnant and newborn hooded rats recently derived from two established lines, an albino and a black non-agouti selfed, were irradiated with x-rays in doses of 10, 20, 30, or 40 r, over the whole body, in lucite containers ⅛ in. thick, on the 16th, 18th, or 22nd day of pregnancy or on one of the first 3 days after birth. In addition, one litter was irradiated on the 18th day of pregnancy with 50 r and members of another litter were given 47 r on the day after birth. The irradiated offspring were killed at intervals of 24 hours, 72 hours, 1, 2, 3½, 5, 9, or 10 weeks later with precisely matched litter mate or cousin controls. We irradiated 137 animals during development, and there were 83 controls. Of these, 41 animals, irradiated on the 16th, 18th, or 22nd day of pregnancy or on the day after birth, were drawn as samples for histologic study with 31 of the controls. Ten of the 41 animals had received 20, 30, or 40 r on the day after birth, and these were compared 72 hours later with 12 litter mate controls. Another 8 animals from the same litters were compared with 10 matching controls at the other intervals up to 9 weeks. The forebrain from just in front of the anterior commissure back to the level of the habenular nucleus was sectioned serially either in paraffin at 8 μ or in frozen sections at 20 μ. Serial sections were made sagitally of one sagittal half of the cerebellum with attached brain stem, usually in frozen sections at 20 μ, but paraffin was used in four early-stage animals. In some animals both the forebrain and cerebellum were studied. Matching paraffin sections stained with cresyl violet and Luxol fast blue, or frozen sections stained with Cajal pyridine silver were compared directly on viewscopes, by direct observation of superimposed sections, and by photography. Pencil tracings of projected images of the dendrites of groups of corresponding normal and abnormal Purkinje cells were also compared. The radiation factors were Westinghouse Coronado therapy unit, 250 kv at constant potential, 9 ma, no added filter, 70 cm from source to middle of animal's body, 59.7 r per minute. The radiation was measured by cylindrical wax phantoms equivalent to the tissue and corresponding in size to the pregnant and newborn rats in their experimental surroundings.

   Cell counts were made with a ruled square eyepiece reticule, the magnification being adjusted to cover layers 2 and 3 without encroaching on layer 4, whose fuzzy outline in the one-day old irradiated animals made numerical comparisons of this layer difficult. For example, the number of neurons in three alternate columns of the seven columns in the reticule was counted in the dorsal and dorsolateral cortex in five to ten successive paraffin sections. The same thing was done in the precisely corresponding areas of the cortex to be matched. Several parts of different cortical regions were counted. At the magnification most often used (× 160), the reticule covered an area of 0.15 mm² and enclosed about 250 neurons in layers 2 and 3 of the dorsal cortex of a 3½-week-old normal rat.
4. We thank Dr. Charles S. Simons, department of radiology, for carrying out the radiation dosimetry.
5. The basophilic material of the cytoplasm has been described previously [S. P. Hicks, M. C. Cavanaugh, E. D. O'Brien, *Am. J. Pathol.* **40**, 615 (1962)] as being present diffusely in the cell body and the early apical dendrite when the primitive neuron first begins to differentiate under normal conditions. With subsequent postnatal growth and differentiation, it becomes progressively concentrated in special places: (i) at the bases of spurs which give the first evidence of dendrite branches along the main dendrites; (ii) in minute aggregates along the neurofibrils of the young main dendrites; and (iii) somewhat diffusely in the cell body cytoplasm up to the stage when aggregated Nissl substance is formed. This basophilic material absorbs ultraviolet light of 2630 angstroms and is removable by ribonuclease. It is considered by us and others (*6*) to be an indicator of cytoplasmic RNA. In the day-old rat, the cells of 2, 3, and 4 are the most immature members of the cortex, with simple, early, diffusely basophilic apical dendrites; in layers 5 and 6, which are more mature, the cytoplasmic basophilic material has begun to be distributed in the special places just mentioned. In the present experiments, we found that radiation on the day after birth retarded the normal progression of this distribution and, at the same time, the dendrite growth that parallels it was retarded. Although these first morphological changes following irradiation resembled those that follow anoxia of day-old rats, the consequences in later life were quite different.
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7. This work was supported by Atomic Energy Commission contract AT(11-1) 1201, U.S. Public Health Service grant B3861, and United Cerebral Palsy Associations, Inc.

17 June 1963

Flavin Sensitized Photoreactions: Effects of 3-(p-Chlorophenyl)-1,1-Dimethylurea

Abstract. *Photooxidation reactions, which were inhibited by high concentrations of oxygen, were affected by the addition of 3-(p-chlorophenyl)-1,1-dimethylurea (CMU), but the response varied with the substrate being oxidized. With ascorbic acid and 2,3-diketogulonic acid, CMU reversed the inhibition caused by high concentrations of oxygen. With ethylenediaminetetraacetic acid (EDTA), tetraethylene tetramine, or Mn^{++} as reductant, CMU itself inhibited the reactions. The photoreduction of flavins by EDTA and the bleaching of flavin mononucleotide under anaerobic conditions were also inhibited by CMU. Corresponding photoreactions sensitized by phthaleine dyes or methylene blue were completely insensitive to CMU. This compound therefore seems to change specifically the reactivity of excited flavin molecules.*

The stoichiometric formation of the end products of photosynthesis and the high efficiency of photochemical reactions in living cells are achieved with the aid of the complex molecular structure in which the sensitizing pigments are imbedded. In homogeneous solution, by contrast, the existence of alternate chemical pathways for seemingly simple photoreactions has frustrated many workers in their attempts to establish a clear-cut description of the process.

Habermann and Gaffron (*1*) observed that the flavin-sensitized photooxidation of ascorbic acid, in the presence of catalase and Mn^{++}, proceeds in two steps. When the amount of oxygen taken up during the first step approaches one equivalent, the rate of oxidation becomes very slow. Then the photooxidation suddenly starts again and a second equivalent of oxygen is consumed. Thus, with only a slight increase in the complexity of a mixture of reagents in solution, a pronounced order is established in the succession of photoreactions induced by a sensitizing dye. We have now studied how certain photooxidations are influenced by 3-(*p*-chlorophenyl)-1,1-dimethylurea (CMU), which is known to be a highly specific inhibitor of photosynthesis (*2*).

In the absence of catalase, the flavin-sensitized photooxidation of ascorbic acid has been reported to lead to an ascorbic acid hydroperoxide which slowly decomposes to threonic acid lactone and oxalic acid (*3*). It appears that, in the first step, the catalase decomposes the ascorbic acid hydroperoxide yielding dehydroascorbic acid, which, under the experimental conditions, hydrolyzes to 2, 3-diketogulonic acid. It is this latter compound which is photooxidized in the second step, provided Mn^{++} is present, to oxalic acid and threonic acid; but this oxidation does not start until all the ascorbic acid is used up. Such inhibitions of photochemical reactions by small amounts of ascorbic acid have been noted before (*4*). We observed that the time required for the oxidation of the last traces of ascorbic acid at the end of the first step could be markedly lengthened by increasing the oxygen concentration (see Fig. 1.) At low oxygen concentrations, the rate of oxidation of ascorbic acid remained almost constant. The rate of the second oxidation also decreased with increasing concentrations of oxygen. Similar inhibitive effects of oxygen on photooxidations have been reported by Bäckström and other investigators (*5*). We found that iodide also inhibits both steps in the photooxidation of ascorbic acid.

Addition of CMU had the effect of reversing the inhibition by iodide, or completely abolishing the inhibition by oxygen (Fig. 1). Thus, when the concentration of oxygen was 70 percent, and when CMU was present, the course of the two-step reaction was almost the

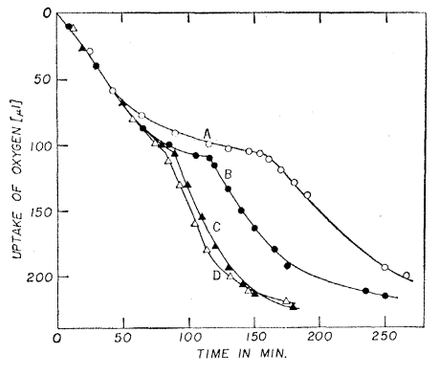

Fig. 1. The effect of the concentration of oxygen and of CMU on the two-step photooxidation of ascorbic acid. Experimental conditions: reaction mixture in rectangular Warburg vessels; illumination with 1980 lumen/m² (180 ft-ca) incandescent light; temperature: 21°C. Reaction mixture: 5 μmole ascorbic acid; 4 μmole $MnSO_4$; 3 mg catalase; 0.37 μmole flavin mononucleotide; 0.1 ml ethanol; 0.3 mmole maleate buffer, *p*H 6.6; water to make total volume 4.0 ml. Curve *A*, gas phase 70-percent O_2 in N_2; *B*, gas phase 30-percent O_2 in N_2; *C*, gas phase 10-percent O_2 in N_2; *D*, gas phase 70-percent O_2 in N_2, $10^{-3}M$ CMU. (Curves for $10^{-3}M$ CMU with 10-percent O_2 and 30-percent O_2 coincide with curve *D*.)

same as when the concentration of oxygen was 10 percent, but no CMU was present. Similar results were obtained with other substituted ureas, and, to a smaller degree, with acetylaniline. Of these compounds, acetylaniline is the weakest inhibitor of photosynthesis and, similarly, was the least effective substance in our system.

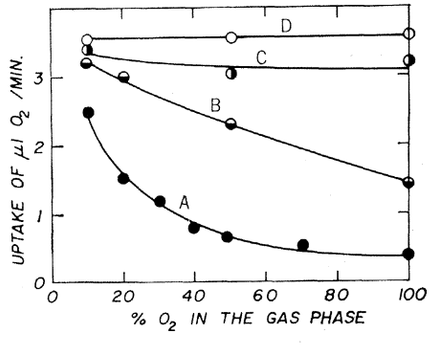

Fig. 2. Dependence of the rate of photooxidation of 2,3-diketogulonic acid on the concentration of oxygen, in the presence or absence of CMU. Experimental conditions and reaction mixture as described for Fig. 1, with the addition of 5 μmole of 2,3-diketogulonic acid as a starting substance. Curve *A*, control (no CMU); *B*, $10^{-4}M$ CMU; *C*, $5 \times 10^{-4}M$ CMU; *D*, $10^{-3}M$ CMU. (The plotted data represent the rate of the linear part of the reaction.)

High concentrations of oxygen also inhibited flavin-sensitized photooxidations of such substrates as ethylenediaminetetraacetic acid (EDTA), tetraethylene tetramine, and Mn^{++} (the last in the presence of catalase). These reactions were strongly inhibited by CMU itself. Thus the influence of CMU was quite different from its effect on the oxidations of ascorbic acid and 2,3-diketogulonic acid (Figs. 2 and 3). There remained a low rate of photooxidation in the CMU-treated reactions with EDTA (Fig. 3). Here the sensitivity to oxygen was reduced—that is, the inhibition by CMU was more pronounced at low oxygen concentrations.

Comparable experiments with fluoresceine and other phthaleine dyes have shown the same oxygen-sensitive, two-step pattern of the ascorbic acid photooxidation. But CMU had no effect with any of these sensitizers. Similarly, the fluoresceine-sensitized photooxidation of Mn^{++}, in the presence of catalase, was not affected by CMU. The same was true for the aerobic photooxidation of ascorbic acid by methylene blue. Its reaction mechanism is, however, different from that sensitized by flavin or phthaleine dyes. The effects of CMU on flavin-sensitized photooxidation reactions appeared, however, to be independent of the nature of the flavin: flavin-adenine dinucleotide, flavin mononucleotide, riboflavin, and lumiflavin gave essentially the same results.

To test the apparent specificity of the action of CMU we studied the anaerobic reduction of the sensitizers by spectroscopy. Flavin mononucleotide undergoes anaerobic decomposition in the presence of light (*6*). We tested a solution of buffered flavin mononucleotide under anaerobic conditions and found that the addition of CMU caused the photochemical bleaching to be markedly inhibited. The anaerobic photoreduction of flavin by EDTA (*7*) was also inhibited by CMU. No anaerobic bleaching of flavin and, correspondingly, no CMU effect were observed when ascorbic acid was added as a reducing agent (*1*). The rates of the anaerobic bleaching of methylene blue by ascorbic acid and EDTA were not affected by CMU. In an anaerobic system containing lumiflavin, methylene blue, and EDTA, CMU inhibited the photochemical dye reduction only when blue-green light was absorbed by the lumiflavin, and not when red light was absorbed by the methylene blue.

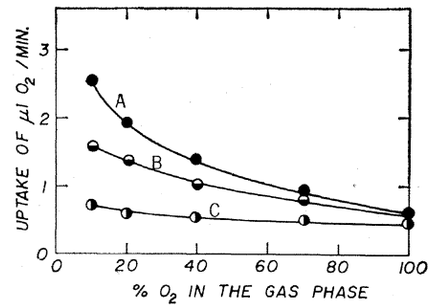

Fig. 3. Dependence of the rate of photooxidation of EDTA on the concentration of oxygen, in the presence or absence of CMU. Illumination with 5830 lumen/m² (530 ft-ca); other experimental conditions as described for Fig. 1. Reaction mixture: 5 μmole EDTA; 0.37 μmole flavin mononucleotide; 2 mg catalase; 0.1 ml ethanol; 0.3 mmole maleate buffer, *p*H 6.6 water to make total volume 3.0 ml. Curve *A*, control (no CMU); *B*, $10^{-4}M$ CMU; *C*, $5 \times 10^{-4}M$ CMU.

These observations support the view that the effects of CMU on the photooxidation of ascorbic acid and 2,3-diketogulonic acid, on other aerobic photooxidations, and on anaerobic photoreductions of dyes are specific for reactions sensitized by excited flavin molecules. The results obtained with the other sensitizers make it difficult to envisage how CMU might influence the secondary reactions—that is, any reactions other than those which result directly from the primary process involving excited flavin molecules.

The inhibition of the anaerobic reduction of flavin by EDTA suggests that at least one of the actions of CMU is the blocking of an electron or hydrogen transfer from the substrate to a flavin molecule. This action of CMU is also suggested by its inhibitive effect on the anaerobic photochemical bleaching of flavin mononucleotide, since in this reaction an inter-molecular oxido-reduction between the flavin molecules is supposed to occur (*6*).

Measurements of absorption and fluorescence gave no indication that a flavin-CMU complex is formed. While our studies were in progress Sweetser (*8*) reported that illumination of plants in the presence of flavin mononucleotide (but not other dyes) reverses the CMU inhibition of carbon dioxide assimilation in photosynthesis. We confirmed this observation. When an excess of flavin mononucleotide was added to an illuminated suspension of algae which had been inactivated by CMU, the normal rate of photosynthesis was restored.

It is likely that the inhibitor was removed by a photooxidative reaction. Sweetser also illuminated a solution of flavin mononucleotide and CMU for several hours and was able to isolate a reaction product of high molecular weight; we consider this to be the result of an irreversible photooxidative destruction of an initial CMU-flavin complex, the formation of which may be highly favored by the similarity in structure between CMU and flavin (see *8*). An association between these molecules might be responsible for the effects we observed. For example, such an association should facilitate a "chemical quenching" process, as has been discussed by Matsumoto and by Eigenmann for other systems (*9*).

The significance of our results with CMU in relation to photosynthesis is not immediately apparent, since we observed changes in the reactivity of light-excited flavin molecules, and so far, these are not believed to be part of the photosynthetic mechanism. Yet the properties of chloroplast flavin may be changed by its forming a complex with CMU so that it diverts the precursors to free oxygen from their regular pathway (*10*).

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1 August 1963

Bradykinin: Vascular Relaxant, Cardiac Stimulant

Abstract. *Bradykinin infusion causes an increase in cardiac output in rats whether the autonomic nervous system is blocked or not. After autonomic blockade, bradykinin causes a lesser decrease in the total peripheral resistance but a greater increase in cardiac output, resulting in an elevation of arterial pressure. The increase in cardiac output is caused by a small increase in heart rate and a substantial increase in stroke volume. The fact that these increases are observed after autonomic blockade suggests that bradykinin increases cardiac output by direct stimulation of the heart.*

Bradykinin is known primarily as a potent relaxant of vascular smooth muscle, having a hypotensive effect. The results of our study, however, indicate that this effect is greatly modified or even reversed by its direct stimulating action on the heart. It is known that bradykinin causes an increase in cardiac output in man (*1*) and in the intact dog (*2*). This might be caused by (i) a secondary neurogenic compensation for the hypotensive effect, or (ii) by a direct positive inotropic action or a direct positive chronotropic action, or both, on the heart. The second possibility is in accordance with the observation that the polypeptide causes an elevation in arterial pressure when administered to rats that have had their autonomic nervous systems blocked (*3*). However, this pressor response could be caused either by an increase in cardiac output or by a reversal of the usual vasodilator effect of bradykinin. The current study was undertaken to evaluate the possibility that the pressor effect of bradykinin is evidence of its direct stimulating action on the heart.

The mean arterial pressure (MAP), cardiac output (CO), and heart rate (HR) were measured during infusion of synthetic bradykinin (*4*) into rats before and after ganglionic blockade. From these values, the total peripheral resistance (TPR) and stroke volume (SV) were calculated. Ten male rats weighing 195 to 280 g were used. They were anesthetized with sodium pentobarbital (40 mg/kg by intraperitoneal injection) and heparinized (10 mg/kg by intravenous injection). Figure 1 shows the details of the technique. The MAP was recorded directly from the femoral artery; the CO was measured by a thermodilution technique (*4, 5*). The indicator (0.1 ml saline at room temperature) was injected into the central venous pool through a cannula in the right external jugular vein. The resultant thermodilution curve was monitored from a thermistor placed in the ascending aorta via the right carotid artery. The CO measurements were made at 2-minute intervals during the control and infusion periods. Bradykinin was infused into the right femoral vein at 12.5 (slow rate) and 25.0 (fast rate) μg/kg per minute. The duration of infusion was 5 minutes and another period of 5 minutes was allowed after each infusion for the destruction of bradykinin. The autonomic nervous system was blocked by pentolinium bitartrate (5 mg/kg by intraperitoneal injection). In each experiment, responses to the two infusion rates of bradykinin were measured before and after pentolinium was administered.

Results of one experiment are given in Fig. 2 and the average of the results of ten experiments are given in Table 1. Without ganglionic blockade, infusion of bradykinin caused an initial decrease in MAP; this decrease was transient, the pressure always returning toward control levels early in the infusion period. The CO increased, reflecting a slight increase in HR and a substantial increase in SV. The TPR fell precipi-

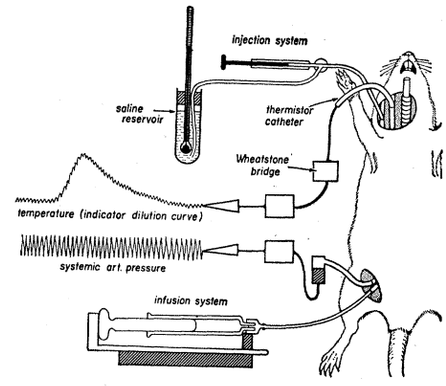

Fig. 1. Thermodilution technique for measurement of cardiac output. The closed system for injection of the indicator is at room temperature (20-25°C). Saline is withdrawn from the reservoir where its temperature is known to the nearest 0.1°C, into a Hamilton microliter syringe; it is then injected rapidly into the rat. Dilution of the indicator (change in temperature) is recorded by a thermistor (Fenwall Electronics, GC32J1) which has a time constant in saline of 0.12 second. Changes are recorded on a Grass model 5 polygraph.