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BIO-ORGANIC CHEMISTRY QUARTERLY REPORT

March through May 1963

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*Preceding Quarterly Reports: UCRL-10743, UCRL-10634

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March through May 1963

M. Calvin, Director

Lawrence Radiation Laboratory and Department of Chemistry
University of California, Berkeley, California

July 29, 1963

1. FERTILITY AND LITTER SIZE OF NORMALLY OVULATED AND ARTIFICIALLY OVULATED MICE

Ann M. Hughes

In the course of some of our studies on D_2O-induced sterility in mice, we have induced ovulation artificially by hormone injections in female mice. Three I. U. of pregnant-mare serum were injected 48 to 72 h before the desired ovulation time, and were followed by 6 I. U. of human chorionic gonadotropin 12 h before the desired ovulation time. Casual observations indicated that mating of artificially ovulated females did not result in pregnancy as regularly as did the mating of those allowed to ovulate normally, nor were the litters of those that did become pregnant as large with artificial as with natural ovulation. We therefore conducted a controlled experiment to compare the two types of ovulation.

Twelve C_{57} male mice and five Swiss males were used in the first experiment, and eight Swiss males were used in the second. All males were tested for fertility prior to the start of the experiment, and only those males which sired at least two litters were used. Female Swiss mice were used in both experiments. Those females induced to ovulate were injected so that they should ovulate at 2 a.m. Males were caged individually. On alternate nights two normal females or one artificially ovulated female were placed with each male at midnight. At 9 a.m. females were removed from the male cages and examined for vaginal plugs (as evidence that copulation had taken place). Those females in which plugs were observed were then housed 10 to a cage until the 16th day of gestation, when they were individually caged. When a litter was born the date was noted, but the mother and litter were not disturbed for the first week after parturition. Litters were counted and sacrificed at 2 weeks of age. In the tabulation of results, those litters which disappeared during the first 2 days (eaten by the mother, and therefore presumably born defective) were classified as dead. Those females in which a plug was seen but which never appeared obviously pregnant and never reached parturition were classified as nonpregnant. Results are given in Tables 1-I and 1-II.

The second experiment showed that the gestation time of the artificially ovulated females tended to be longer than that of those ovulating normally. Normally ovulated females had an average gestation time of 19.4 days, with a range from 18 to 24 days, while those in which ovulation had been induced had an average time of 21.3 days, with a range from 17 to 29 days.

Table 1-I. Comparison of fertility and litter size of normally and artificially ovulated mice, mated with both C_{57} and Swiss males.

	C_{57} Males				Swiss Males			
	Females showing plugs (No.)	Non-pregnant (%)	Dead litters (%)	Offspring per litter	Females showing plugs (No.)	Non-pregnant (%)	Dead litters (%)	Offspring per litter
Normal ovulation	43	16	12	8.3	22	14	5	7.2
Induced ovulation	17	47	12	6.3	17	24	24	6.3

Table 1-II. Comparison of fertility and litter size of normally and artificially ovulated mice, mated only with Swiss males.

	Females showing plugs (No.)	Non-pregnant (%)	Dead litters (%)	Offspring per litter
Normal ovulation	100	17	7	8.1
Induced ovulation	107	44	12	6.2

2. FURTHER STUDIES ON STERILITY PRODUCED IN MALE MICE BY DEUTERIUM OXIDE

Ann M. Hughes (in collaboration with Laurel E. Glass*)

We previously reported that an examination of eggs from female mice mated to males rendered sterile with D_2O showed few normal multicellular eggs.[1, 2] In that study, the eggs were examined 1 to 3 days after copulation; thus it was difficult to determine whether the eggs had been fertilized and then had degenerated, or whether fertilization had not occurred. We have continued the study, therefore, examining eggs at much shorter intervals after copulation, in an attempt to determine whether sperm entry and fertilization occurred.

As in previous experiments, the initial fertility of the males was determined by mating each with two or three females prior to the start of the experiment. Only those males which sired two or more normal litters were used in the experiments. The experimental males were then supplied with 30% D_2O in place of tap water for the duration of the experiment. Control males were maintained under similar conditions, except that they received tap water.

In the first series of experiments, beginning during the first week of D_2O administration and continuing for 6 weeks, two female mice were placed in the cage with each male mouse between 10 and 12 each night. The females were then examined at 4-h intervals for the presence of a vaginal plug. Thus the time of copulation could be determined within 4 h. All females were removed from the males' cages at 8 a. m. Twelve hours after observation of plugs, females were sacrificed and the two oviducts removed.[3] They were fixed in Bouin's solution, embedded in paraffin, sectioned at 10 μ, and stained with haematoxylin and eosin. The sections were examined with a light microscope at magnifications as high as 400 ×. In sections of oviducts from females mated to control males, most of the eggs had two pronuclei, an indication of fertilization. In females mated to males that had received D_2O for 2 or more weeks, most of the eggs had metaphase spindles, an indication that they were not fertilized. However, some eggs contained one pronucleus, and many contained an unidentifiable mass of dark-staining material, which might possible be a degenerated sperm.

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*Department of Anatomy, University of California Medical School.

1. Ann M. Hughes and Edward L. Bennett, in Bio-Organic Chemistry Quarterly Report, UCRL-9208, June 1960, p. 114.

2. Ann M. Hughes, Edward L. Bennett, and Melvin Calvin, Ann. N. Y. Acad. Sci. 84, 763 (1960).

3. In each experiment representative females were allowed to go to term, instead of being sacrificed, to verify that males which we classed as sterile were indeed incapable of siring viable offspring.

Such observations suggested that in some cases the sperm penetrated the egg but failed to transform into a pronucleus. To investigate this possibility further, the preceding experiment was repeated, with the modifications that females were sacrificed 6 h after plugs were observed, and live eggs were examined by means of phase-contrast microscopy.[3] Eggs from control females usually showed a sperm head within the cytoplasm. Many of the eggs from experimental females had sperm among the cumulus cells, and in some cases a sperm head was seen within the cytoplasm, indicating that sperm from sterile males are capable of penetrating the egg.

As further verification of sperm penetration of the egg, an experiment is in progress in which females were mated to males whose sperm had been labeled with thymidine-^{3}H. The oviducts were removed 12 h after copulation, and are now being studied autoradiographically.

3. PLANARIAN DISAGGREGATION

Jon Palmer

In hopes of finding a way to dismantle planarians, on a cellular level without lysing the cells, we attempted to disaggregate them. When and if we are successful, the cellular material may be fractionated and characterized.

Enzyme methods were first tried, using old stocks of papain, trypsin, and pancreatin. A worm added to a solution of 2 mg enzyme per 4 ml of water was still active after 24 h. The worm was then gently ground up in a 0.8-ml homogenator and left in the enzyme solution at room temperature. Subsequent microscopic examinations of the suspension revealed no free-floating cells. Three new enzymes--collagenase, papain, and lysozyme--were obtained and used in concentrations ranging from 0.8 to 3.2 mg/ml. After incubation in these solutions most of the worms were nearly dead after one day, and microscopic examination at 200 and 400 power showed pieces of tissue and smaller aggregations of cells, but very few isolated cells. When examined after 4 or 5 days, the suspensions were infested with bacteria, and in one case were infested with paramecia.

In nonenzyme attempts, it was found that in 10.9 mg/ml sodium lauryl sulfate solution, a planarian's underside mucus covering was quickly loosened, and that after 1 day the worm was completely dissolved--no remains were visible. A 0.5 mg/ml solution of the same detergent seemed to dissolve out the inside of the worm after 1 day and leave a shell-like piece of skin. When approximately 100% D_2O was used, the effects were very similar to those obtained with 0.5 mg/ml lauryl sulfate solution and a large piece of the worm was left on the bottom of the test tube. NaCl (0.25 M) solution had only a slight effect after 1 day, but at 3 days caused the worm to curl up. Distilled water had only slight effect on a worm after 3 days' soaking. The worms in both the NaCl solution and the distilled water died after 1 week, and mucus was greatly loosened from the underside of the body. These worms were easily broken up with a stirring rod. No whole cells were visible in the centrifuged suspension.

A search for staining methods was then made; and some of Ann Hughes's stained slides of planarian sections were viewed in order to learn the general size and shape of planarian cells.

A cell-separation method of Ansevin and Buchsbaum was tried.[1] This involved soaking the worm in penicillin solution to induce shedding of the mucus, cutting into small pieces with a scalpel, and soaking for 1.5 h in 0.004% EDTA solution. Repeated passage into and out of a 5-ml pipette was effective in breaking up the tissue. Microscopic examination at 260 and 120 power again showed no free cells.

A worm disintegrated after soaking for 1 day in 0.4 mg/ml hyaluronidase solution; the sediment appeared partially structured and not as completely lysed as it did after 5-h treatment of a worm in 2 mg/ml hyaluronidase solution, in which disintegration was effected when the test tube was shaken.

Several uv spectra were run on a Cary Model 11 spectrophotometer. Sodium lauryl sulfate solution showed no maximum nor minimum, only a scattering curve. EDTA solution (0.004%) showed only a slight rise in the region from 350 to 210 mμ. A fresh preparation of planarian made by the Ansevin and Buchsbaum method,[1] when centrifuged at maximum speed in a clinical centrifuge for 2 min or for 15 min, showed an absorption maximum at 253 mμ. However, two worms in a solution of 4.8 mg sodium lauryl sulfate plus 1.0 mg hyaluronidase per 4 ml showed a maximum at 265 to 270 mμ. The supernatant from a solution in which two worms died and disintegrated through natural hazards gave only a scattering spectrum. When several worms were minced with razor blades and the centrifuged mince washed twice with 0.25 M sucrose and resuspended in 1.0 M sucrose, the spectra from the washed suspension made after settling and centrifuging several times showed only low, broad peaks at 275 to 280 mμ. The supernatant from the original unwashed mince of some worms gave a peak at 260 to 265 mμ.

As an aid in identifying material resulting from these disaggregation attempts several stains were tried. Dilute Giemsa, toluidine blue, eosin, and hematoxylin were used with and without buffered formalin fixative. When used together, eosin and hematoxylin not only gave the best results, but showed some differential staining.

Several disaggregation attempts were made with 0.2% and 0.3% trypsin in combination with 0.3% and 0.7% NaCl. Although complete disaggregation was not achieved, raising the pH from 4 to 5 (the initial range) to 7 to 8 (the optimum range for trypsin activity) increased the amount of disaggregation. Similarly, raising the pH increased disaggregation when hyaluronidase (0.1% and 0.7%) was used in combination with 0.003% EDTA and 0.5 to 1.5% NaCl. Small amounts of red-stained, large-sized heterogeneous material, looking like cell walls or shredded tissue, and much larger quantities of material, stained light to dark purple and consisting of small particles, perhaps cell nuclei, were visible. Many individually isolated particles were seen in several preparations from trypsin, hyaluronidase, and a supernatant from a dead-worm solution, and were tentatively identified as neoblasts.

A trypsin-EDTA preparation was viewed through the phase-contrast microscope. This instrument permitted better viewing, as it has higher magnification and shows more detail than the non-phase-contrast microscopes. Most of the suspended material appeared to be broken tissue or cells with several intact "neoblasts."

Barnes, in his attempts to isolate individual planaria cells, used a much higher concentration of trypsin (4%) and a lower concentration of salt (containing Ca^{++}), and adjusted the pH to 7.8.[2] We tried to duplicate his methods, using fresh, more highly purified trypsin (Worthington Biochemical Corp.). The worm was put into 1 ml of a 4% trypsin solution and viewed

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1. K. D. Ansevin and R. Buchsbaum, J. Exptl. Zool. 142, No. 2, March 1961.

2. Charles Barnes (University of California Medical Center, San Francisco), personal communication.

under a dissecting microscope. About 30 min later, when the worm was dead and broken into pieces, the trypsin solution was decanted, a further 1 ml of 4% trypsin solution was added, and the solution was repeatedly drawn through a small-bore pipette. This yielded the best results ever obtained. Examination at magnifications of 440 and 900 revealed much less broken or shredded material, more round particles called neoblasts, and several rod-shaped particles that may be rhabdite. The old bottle of trypsin used earlier would not work as well and was only partially soluble at this concentration of 40 mg/ml.

In an attempt to characterize or separate the suspension obtained by using this trypsin method, a sucrose-gradient separation was tried. One ml each of 1.50, 1.25, 1.00, and 0.75 M sucrose was layered in a plastic centrifuge tube and allowed to diffuse for 1 day at 5°. The previous good results from the high-concentration trypsin disaggregation could not be duplicated. One ml of a cell suspension was put on top of the gradient, and the tube was spun for 30 min at 20,000 × g. Most of the material sedimented to the bottom of the tube, but the top layer contained a fine suspension, and small amounts of filamentous material were concentrated at the boundaries between the 0.75 and the 1.00 M layers, and between the 1.00 and the 1.25 M layers. This run put the material too far down in the tube, although both speed and time were less than in a previous unsuccessful try.

One fractionation experiment was done with ^{14}C-labeled worms. Fifty-seven worms were put into 15.0 ml of spring water containing 4.0 mg of sodium propionate 2-C^{14} (10.6 μC/mg). The solution contained 68.5×10^6 dis/min initially, 52.0×10^6 dis/min after 1 day, and 31.8×10^6 dis/min after 2 days. The worms were then removed, rinsed repeatedly, and thoroughly homogenized in 5 ml of 0.1 M tris buffer, pH 7.4, in a glass homogenizer. The homogenate was centrifuged for 10 min at 1,800 × g, and the supernatant was again centrifuged at 20,000 × g after enough NaCl solution was added to bring the supernatant to 0.1 M. This clear supernatant was next dialyzed against 0.01 M tris buffer, pH 7.6, by use of a spiral string drip method.[3] The nondialyzable material was concentrated to about 0.2 ml by use of "Aquacide," and then washed from the dialysis tubing and centrifuged again. This supernatant was put onto a 1×20-cm DEAE Sephadex column and eluted with a gradient elution apparatus, running from 0.01 M tris buffer, pH 7.6, to 1.8 M NaCl. About 120 1-ml tubes were collected.

Each tube was scanned from 290 to 250 mμ on the Cary 14 spectrophotometer. A plot of the optical density at 280 and 260 mμ revealed a peak at tube number 15, one at number 25, and one at tubes 60 to 80; that had much trailing on the right side. The 280-mμ absorption is lower than the 260-mμ absorption, but the relative differences remain nearly constant.

Kjeldahl and Folin analyses were made of the various precipitates and supernatants throughout the procedure, and a good correlation was obtained between the two methods when the Kjeldahl μg N per ml was plotted against the Folin optical density for each sample.

Samples were taken at various stages of the procedure and counted by liquid scintillation counting. Not all the counting and calculation is complete yet.

3. V. D. Hospelhorn, Anal. Biochem. 2, 182 (1961).

4. UPTAKE OF ORGANIC COMPOUNDS BY PLANARIANS. II

Edward L. Bennett, Marie Hebert, and M. Calvin

In the previous Quarterly Report,[1] some preliminary observations on the uptake and use of organic compounds by planarians were reported. In the present report, additional observations are summarized on the uptake of sodium propionate-2-^{14}C, adenine-8-^{14}C, cytosine-^{3}H, and glycine-2-^{14}C. The methods have been similar to those previously described, and only major changes in procedures are noted.

In the preceding Quarterly, results were presented indicating that adenine and cytosine were extensively taken up and utilized by planarians. The uptake of adenine during 2 days was concentration-dependent in the external concentration range of 0.1 to 1.2 μM/ml. Preliminary data were presented on the relative uptake of adenine and cytosine. An experiment in which the external concentrations of the bases were high (10 μM/ml) indicated that cytosine was considerably better utilized in synthesizing nucleic acids than is adenine, whereas a comparison of experiments in which the external concentrations were lower (2.5 μM/ml) indicated that utilization of the two bases was more nearly equal.

In Table 4-I are presented the results of an experiment to test the effect of concentration on the uptake and distribution of adenine-8-^{14}C, adenine-^{3}H, cytosine-^{3}H, and propionate-2-^{14}C. In this same table, the validity of our techniques using ^{14}C and ^{3}H can be evaluated by comparing the results obtained from the isotopically labeled adenine. The radioactivity taken up by the planarians was fractionated into that extracted by 10% trichloroacetic acid (TCA), and that not extracted by 10% TCA. The former fraction includes purines, pyrimidines, nucleosides, and nucleotides; the latter fraction includes the nucleic acids and proteins.

The data in Table 4-I indicate that during the first 2 days, when the planarians are living in the radioactive organic bases, uptake is approximately (but not absolutely) proportional to the external concentration. Thus, with a fourfold increase in concentration, adenine uptake increased about threefold. Approximately 2 to 4 times as much cytosine as adenine was incorporated into the TCA-insoluble fraction when the planarians were in the radioactive solution. As previously noted, the 10% TCA-soluble pool derived from adenine is much larger than that derived from cytosine. As a result, the radioactivity in the 10% TCA-insoluble fraction (RNA-DNA) continued to increase as much as 2 weeks after planarians were removed from the radioactive adenine solution, whereas the activity decreased in this fraction within 1 week after removal from the cytosine solution. Owing to this large pool of adenine nucleotides, the amount of adenine incorporated into the TCA-insoluble fraction became equal to or greater than that incorporated after cytosine labeling. After planarians were removed from the radioactive solution, as much as 80% of the activity derived from cytosine was 10% TCA-insoluble, whereas never more than 40% of the activity derived from adenine was TCA-insoluble.

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1. Bio-Organic Chemistry Quarterly Report, UCRL-10743, March 1963, p. 76-88.

Table 4-I. Comparison of uptake of adenine-8-^{14}C, adenine-^{3}H, cytosine-^{3}H, and propionate-2-^{14}C by planarians (mμM incorporated per mg planarian)

Time in radioactive solution	Adenine-8-^{14}C 2.4 μM/ml			Adenine-8-^{14}C 8.3 μM/ml			Adenine-^{3}H 10.0 μM/ml		
	Total uptake	TCA-insol.	% TCA-insol.	Total uptake	TCA-insol.	% TCA-insol.	Total uptake	TCA-insol.	% TCA-insol.
20 h	2.4	0.10	4.4	5.7	0.28	5.1	6.0	0.39	6.4
2 days	4.5	0.16	3.6	10.9	0.20	1.8	11.3	0.46	3.9
2 days in 3 days out	4.6	0.65	14	6.1	0.75	12.3	7.0	0.8	11.4
2 days in 7 days out	3.3	0.65	19	5.4	1.5	28	4.8	0.95	21
2 days in 13 days out	2.7	0.9	33	5.0	2.0	40	4.8	1.75	37
2 days in 28 days out	1.6	0.5	31	2.7	1.0	38	2.4	0.85	35

	Cytosine-^{3}H 2.4 μM/ml			Cytosine-^{3}H 9.9 μM/ml			Propionate-2-^{14}C 10.0 μM/ml		
	Total uptake	TCA-insol.	% TCA-insol.	Total uptake	TCA-insol.	% TCA-insol.	Total uptake	TCA-insol.	% TCA-insol.
20 h	0.62	0.27	44	2.4	1.15	48	17.2	6.2	36
2 days	0.82	0.41	50	4.3	2.1	49	30	11.2	38
2 days in 3 days out	0.58	0.40	69	3.0	1.9	64	30	15	50
2 days in 7 days out	0.41	0.32	82	1.9	1.5	79	18	10.5	57
2 days in 13 days out	0.35	0.29	83	2.3	2.0	85	14.7	9	61
2 days in 28 days out	0.12	0.10	83	0.6	0.5	83	9.2	4.7	49

The uptake into planarian of adenine-8-^{14}C and adenine-^{3}H was similar after correction was made for the fact that 40% of the radioactivity present in the stock adenine-^{3}H solution is lost upon evaporation by an air stream. Therefore, this volatile activity is not bound to the adenine, but presumably exists as a contaminant ($^{3}H_2O$). No similar test has yet been made with the cytosine-^{3}H; if a similar contaminant were present in the cytosine solution, correction would result in increased relative utilization of cytosine.

Propionate-2-^{14}C is taken up by planarians rapidly, but is lost slowly. About 10 times as much propionate as cytosine is taken up under comparable conditions of 2 days in precursor, followed by 1 day without precursor. At least 50% of the propionate is converted to 10% TCA-insoluble material, presumably macromolecules. Other experiments have indicated that about 65% of the material derived from propionate-2-^{14}C is either insoluble at pH 7.6 or nondialyzable.

In a second experiment, the uptake into planarians of adenine-8-^{14}C, adenine-^{3}H, glycine-2-^{14}C, and propionate-2-^{14}C at concentrations of approximately 1, 3, and 10 μM/ml was again investigated. The planarians were subsequently fractionated into (a) material extracted by 80% ethanol–20% H_2O, (b) that extracted by 30% ethanol–70% H_2O, and (c) the insoluble residue. The results are summarized in Table 4-II. By this method of extraction, we obtained relative distributions between soluble and insoluble material different from those obtained with TCA. The difference was particularly marked when radioactive adenine was used. A much larger fraction of the radioactive compounds was insoluble; the proportion was dependent upon the external concentration and the time the planarian had been in adenine. With a low concentration of adenine (0.6 μM/ml), as much as 50% of the radioactive materials was not extracted by either the 80% or 30% ethanol; at higher external concentrations of adenine, about 20% remained unextracted. In comparison, 5% of the radioactivity was not extracted by 10% TCA. Paper chromatograms after alkaline hydrolysis should indicate the nature of the alcohol-insoluble radioactive material. These chromatograms are now being prepared. The radioactive material left by 10% TCA appears to be predominantly nucleic acid; it is probable that the alcohol-insoluble fraction contains additional classes of radioactive compounds.

Almost all the soluble material resulting from glycine-2-^{14}C uptake was extracted by 80% ethanol, with less than 10% of the soluble material generally being extracted by the subsequent 30% ethanol extraction. About 50% of the radioactivity was aqueous-ethanol insoluble, and this proportion did not change greatly with the concentration of the external glycine.

With propionate-2-^{14}C as the radioactive precursor, about 25 to 30% of the total radioactivity was extracted by 80% ethanol, 10 to 15% by 30% ethanol, and 50 to 60% remained insoluble, presumably as higher-molecular-weight compounds.

The uptake of adenine was comparable when measured with either adenine-8-^{14}C on adenine-^{3}H. The uptake increased, but not linearly, with external concentration and with time in the radioactive solution. The uptake of glycine-^{14}C and propionate-^{14}C also increased with time and external concentration. However, during the course of this series of experiments,

Table 4-II. Uptake of adenine-8-^{14}C, adenine-^{3}H, glycine-2-^{14}C, and propionate-2-^{14}C into planarians (mμM incorporated per mg planarian)

Time in radioactive solution	Ext. by 80% EtOH	Ext. by 30% EtOH	Aqueous-EtOH Ins.	Ext. by 80% EtOH	Ext. by 30% EtOH	Aqueous-EtOH Ins.	Ext. by 80% EtOH	Ext. by 30% EtOH	Aqueous-EtOH Ins.
	Adenine-8-^{14}C								
	0.63 μM/ml			4.1 μM/ml			12.0 μM/ml		
1 day	0.27	0.08	0.35	2.0	0.4	1.0	5.0	0.85	1.6
2 days	0.65	0.27	0.50	2.9	0.55	2.0	7.0	1.25	1.75
4 days	1.0	0.4	0.85	5.0	1.7	2.0	12.0	2.1	2.2
4 days in 4 days out	0.14	0.48	0.95	1.0	1.15	3.5	---	---	---
4 days in 21 days out	0.22	0.12	0.67	1.82	0.53	2.16	---	---	---
4 days in 7 weeks out	0.08	0.11	0.26	---	---	---	---	---	---
	Adenine-^{3}H								
	1.1 μM/ml			3.5 μM/ml			10.8 μM/ml		
1 day	0.5	0.2	0.45	2.0	0.55	1.0	4.2	1.1	1.1
2 days	1.35	0.2	0.65	3.0	0.85	1.45	7.9	0.8	2.4
4 days	1.75	0.65	0.85	3.9	1.2	2.4	8.7	1.4	2.9
4 days in 4 days out	0.75	0.5	1.2	1.3	0.75	3.2	1.9	1.5	3.6
4 days in 21 days out	0.60	0.20	0.50	---	---	---	1.08	1.04	2.7
4 days in 7 weeks out	---	---	---	---	---	---	0.27	0.40	0.47
	Glycine-2-^{14}C								
	1.0 μM/ml			3.1 μM/ml			10.1 μM/ml		
1 day	0.10	0.025	0.13	0.23	0.015	0.25	0.75	0.07	0.6
2 days	0.30	0.03	0.333	0.95	0.075	0.9	1.5	0.11	1.45
	Propionate-2-^{14}C								
	1.0 μM/ml			3.1 μM/ml			10.2 μM/ml		
1 day	0.9	0.25	1.5	1.9	0.7	3.8	5.2	3.5	11.4
2 days	---	---	---	3.5	1.9	7.8	7.5	6.9	15.2

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the radioactivity in the external solutions was determined daily. It did not change appreciably in the adenine solutions, but considerable amounts (as high as 60 to 80%) of the radioactivity were lost in the glycine and propionate solutions within two days. This loss was attributed to bacterial action. The percentage loss of radioactivity was greater in the more dilute solutions. This bacterial action has probably contributed to the poor health of planarians in our labeling procedure and has been particularly troublesome when glycine has been used. In a current experiment, the loss of radioactivity from a propionate-2-^{14}C solution has been completely suppressed for one week by the addition of penicillin G (50 μg/ml) and dihydrostreptomycin-streptomycin (50 μg/ml) to the external solution. The planarians have remained healthy. The uptake of the radioactive adenine or propionate has been similar to or greater than previously indicated.

During the period covered by this Quarterly, the following additional compounds derived from fatty-acid uptake have been tentatively identified: glucose, glutamine, and leucine. Previously glutamic acid, aspartic acid, and alanine were identified. The compound previously reported as serine has now been identified as glucose.

Additional experiments are in progress to determine the distribution of radioactivity in planarians that have cannibalized portions of other planarians labeled with adenine-^{3}H or with propionate-^{14}C.

5. EFFECTS OF ENVIRONMENTAL COMPLEXITY AND TRAINING ON ACETYLCHOLINESTERASE AND CHOLINESTERASE ACTIVITY IN RAT BRAIN*

Edward L. Bennett, Marie Hebert, Hiromi Morimoto, and Barbara Olton

(in collaboration with Mark R. Rosenzweig and David Krech, Department of Psychology, University of California)

Last year we reported some experiments showing that Environmental Complexity and Training (ECT) (and we will define shortly what we mean by this term) cause, in rats between 25 and 105 days of age, an increase in both the total weight and the acetylcholinesterase (AChE) activity of the cortex; the total AChE activity of the subcortex, on the other hand, was shown to increase without simultaneous increase in weight.[1,2] Therefore, if AChE activity is expressed per unit weight of tissue, the activity in the cortex shows a relative decrease, while that in the subcortex shows an increase. A convenient way of expressing the results, then, is the ratio of cortical to subcortical AChE activity per unit weight of tissue; this value is smaller for an ECT rat than for an Isolated Control (IC) rat.

This report concerns further studies in which we have investigated cholinesterase (ChE) activity (often referred to as butyrylcholinesterase or pseudocholinesterase), as well as AChE activity. The results show: (a) in the cortex, AChE and ChE respond differentially to the ECT conditions, the former showing a decrease in activity per unit weight, the latter showing an increase; (b) in the subcortex, whereas AChE shows a significant increase in activity per unit weight, no significant change is found with ChE; (c) confirmation that ChE activity comprises less than 5% of our usual measure of AChE activity; (d) blood in the brain can contribute less than 0.5% of the total AChE activity assayed, and less than 1% of the ChE activity determined; and (e) no changes in protein content or hexokinase activity per unit tissue weight as a result of the ECT conditions.

Methods

Behavioral Conditions

At weaning (25 days of age), one rat, chosen at random from each pair of litter mates, was assigned to the ECT condition and the other rat to the IC condition. In the ECT condition, a group of ten to twelve animals lived together in a large cage. They were provided with "toys," were handled daily by the experimenter, daily explored the Hebb-Williams apparatus, and, from about 50 days of age, received training in the Lashley III maze, the Dashiell maze, and the Krech hypothesis apparatus.

*This research was supported in part by grants from U.S. Public Health Service, Surgeon General's Office, and National Science Foundation.

1. M. R. Rosenzweig, D. Krech, and E. L. Bennett, Fed. Proc. 21, 358 (1962).

2. M. R. Rosenzweig, D. Krech, E. L. Bennett, and M. C. Diamond, J. Comp. Physiol. Psychol. 55, 429 (1962).

The IC animals lived in individual cages under reduced illumination without contact or sight of other animals. They had a minimal amount of handling for biweekly weighing, were given no opportunity for exploration, and had no formal training. All animals had free access to unlimited supplies of food and water. These conditions were maintained until the animals were sacrificed at about 105 days of age.

Twelve litter-mate pairs were used in each experiment. In the first experiment, designated as I, S_1 rats from the U. C. Psychology Department colony were used. In the second experiment, designated as IIA, S_1 rats also were used; at the same time, a group of S_3 rats was used and was designated as IIB.

Analysis

To obtain the brain samples for analysis, the animals were killed by decapitation, the brain exposed, and five areas removed.[2] These areas included the visual and somesthetic cortex, the remaining dorsal cortex, the ventral cortex, and associated tissues. These sections can be cleanly separated from the subcortical brain (termed Subcortex II here). The subcortex includes the cerebellum, medulla, and pons. After removal, the samples were immediately weighed to the nearest 0.1 mg, and placed on a block of dry ice until frozen. Subsequently, samples were stored in a deep freeze at -20° until analyzed.

Two independent methods were used and compared for the assay of AChE in homogenates prepared from the frozen sections. In the first method, 0.0075 M acetylcholine perchlorate was used as substrate, and the rate of hydrolysis was determined by the rate of addition of base required to maintain the pH constant at 7.95. This is the method we have used in all our previous studies. The second method was based upon a spectrophotometric method described by Ellman et al.[3] Acetylthiocholine is used as substrate in the presence of 5, 5' -dithio bis-(2-nitrobenzoic acid), abbreviated "DTNB." The product of the enzymatic reaction, thiocholine, reacts essentially instantaneously with the DTNB to produce an intensely colored anion, 2-nitrothiobenzoate; the formation can be measured at 412 mμ. Four samples can be analyzed simultaneously; their absorbance as a function of time is recorded on a strip chart using a Beckman spectrophotometer equipped with a Gilford absorbance indicator and automatic cuvette positioner. These assays were done in 0.1 M phosphate buffer, pH 7.9. ChE activity has been determined in an analogous manner by using 10^{-3} M butyrylthiocholine as substrate in the presence of DTNB and 2×10^{-5} 1.5-bis-(4-trimethylammonium-phenyl) pentane-3-one diiodide (Burroughs Wellcome's Code 62C47). This compound was shown by Bayliss and Todrick to be a highly selective inhibitor of AChE.[4]

In Table 5-I, we have summarized the relative activity of AChE and ChE against acetylcholine and butyrylcholine and their thio analogues. AChE is a highly specific enzyme; it is 94% as active with acetylthiocholine as

3. G. L. Ellman, K. D. Courtney, V. N. Androes, Jr., and R. M. Featherstone, Biochem. Pharmacol. 7, 88 (1961).

4. B. J. Bayliss and A. Todrick, Biochem. J. 62, 62 (1956).

with acetylcholine under similar conditions of 7×10^{-3} M, and pH of 8.0. The activity against butyrylcholine is about 2% for rat brain AChE, and somewhat less than 2% for butyrylthiocholine. ChE is a much less specific enzyme. It is about one and one-half times as active with acetylthiocholine as it is with acetylcholine, against butyrylcholine about two and one-half times as active, and against butyrylthiocholine about three and one-half times as active.

Table 5-I. Relative activity of acetylcholinesterase and cholinesterase against selected substrates

	AcCh	AcSCh	BuCh	BuSCh
AChE	100	94	2.3	< 2
ChE	100	140-160	250	350

Abbreviations: AcCh, acetylcholine; AcSCh, acetylthiocholine; BuCh, butyrylcholine; BuSCh, butyrylthiocholine.

In Table 5-II, we have summarized (a) the activity of rat brain against these substrates and (b) the effect of various inhibitors. In addition, we have indicated the activity of rat blood, serum, and cells. These latter values are expressed per µl (approximately per mg) of whole-blood equivalent. Thus, the activity of whole brain against either acetylcholine or acetylthiocholine is about equivalent, and the activity is at least 95% inhibited by 2×10^{-5} M 62C47. The activity against acetylthiocholine is completely inhibited by 10^{-5} eserine; this is a characteristic property of cholinesterases. The activity of brain against butyrylcholine or butyrylthiocholine is very low and is 4 to 6% of the activity against acetylcholine or acetylthiocholine. About 35% of the activity against butyrylthiocholine is inhibited by 2×10^{-5} M 62C47, and at least 98% by 10^{-5} M eserine. The AChE and ChE activities of whole blood are also very low compared with whole rat brain.

We are often asked to what degree variation of the blood within a brain can contribute to the variations in AChE or ChE activities in the brain. Since the blood values are about one-tenth those of whole brain, more blood will decrease the values reported per unit weight of tissue. If we assume that 5% of the brain weight represents blood, we can calculate that less than 1% of the total activity in brain represents a contribution from the blood included in the sample. In other words, the difference between the total activity in a perfused brain and that in a nonperfused brain would be less than 1%.

Now let us look at some of the results obtained with groups exposed to different experimental conditions. In Table 5-III, we have summarized the effect of differential experience on weights of brain tissue. The visual cortex was heavier in the ECT animal in 70% of the pairs, with the increase ranging from 5.7 to 8.5%; the somesthetic cortex was heavier in 67% of the cases, with the increase ranging from 1.4 to 4.3%. In 75% of the cases the total cortex was heavier by 3.0 to 4.5%, and the P value (probability) reached 0.05 in each group. Only small changes were seen in the weight of the subcortex or total brain. If we express the ratio of cortical weight to subcortical weight, we find that this ratio increases by 3.5 to 5% and that this increase is highly significant.

Table 5-II. Activity of rat brain and blood with acetyl and butyryl choline and thio analogues

Enzyme source	Substrate	Conc. (M)	pH	Activity (moles $\times 10^{10}$ per min/mg)	Inhibition by 2×10^{-5} M 62C47	Inhibition by 1×10^{-5} M Eserine
Whole brain	AcCh	7.5×10^{-4}	7.95	159	96%	
	BuCh	1.0×10^{-2}	7.95	9.4	0%	
	AcSCh	7.0×10^{-4}	7.9	150	95%	99%
	BuSCh	1.0×10^{-3}	7.9	6.3	35%	98%
Whole blood	AcCh	7.5×10^{-4}	7.95	9.5		
	AcSCh	7.0×10^{-4}	7.9	~13	75%	90%
	BuSCh	1.0×10^{-3}	7.0	0.5—1.0	0%	
Serum[a]	AcCh	7.5×10^{-4}	7.95	2.3		
	AcSCh	7.0×10^{-4}	7.9	2.1	72%	> 97%
	BuSCh	1.0×10^{-3}	7.0	0.45	15%	> 97%
Cells[a]	AcCh	7.5×10^{-4}	7.95	6.6		
	AcSCh	7.0×10^{-4}	7.9	6.0—8.5	90%	95%
	BuSCh	1.0×10^{-3}	7.0	not measurable reproducibly		

[a]Serum and cell values are expressed in terms of mg equivalent to whole blood. It is assumed that blood is 50% serum.

Abbreviations: See Table 5-I.

Table 5-III. Effect of differential experience on weights of brain tissue (expressed as percentage of difference between means, ECT minus IC)

Expt.	Strain	Visual cortex	Somesthetic cortex	Remaining dorsal cortex	Total cortex	Subcortex	Total brain	Total cortex / subcortex
I	S_1	5.7[a]	2.7	4.7[a]	4.5[b]	-1.1	1.2	5.6[b]
IIA	S_1	8.5	4.3	4.4	3.2[c]	-0.2	1.2	3.5[b]
IIB	S_3	6.9	1.4	2.0	3.0[c]	-0.4	1.0	3.5[b]
% cases, ECT > IC		70	67	67	75	42	70	86

N (number = 12 litter mate pairs in each experiment

[a] $P < 0.10$

[b] $P < 0.01$

[c] $P < 0.05$

Table 5-IV summarizes the effects of differential experience on the AChE and ChE activities of the subcortex and cortex of ECT animals in comparison with their IC counterparts. The top two sections of the table compare the results obtained by the two methods of assay for AChE. We can summarize this comparison by saying that the results are nearly equivalent, although we did not get as high a significance by the spectrophotometric method as we did by the pH-stat method. The results indicate that the activity of AChE per unit weight decreased in the cortex, increased in the subcortex, and changed only slightly for the entire brain. A large and significant decrease was shown when the ratio of activity in the cortex to activity in the subcortex was calculated.

If we look at the ChE activity as assayed with butyrylthiocholine, in the presence of 62C47 to inhibit any AChE activity toward butyrylthiocholine, we see a somewhat different pattern of effects produced by the ECT conditions. A general increase of activity in the cortex is statistically highly significant, but there is little or no change in activity in the subcortical brain. The ratio of activity in the cortex to that in the subcortex now shows an increase in the ECT animals, in contrast to a decrease for the activity against acetylcholine or acetylthiocholine. We would like to emphasize that the total activity against butyrylthiocholine is low, and we are planning further replications and extensions of these experiments to confirm these results.

As for possible functional implications, the glial cells are said to be relatively high in ChE activity,[5] and this may indicate an increased activity or even an increased size or number of these cells as a result of the ECT conditions. To support this suggestion, studies in our Laboratory indicate that a larger glial-neural ratio may result from these ECT conditions.

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5. G. B. Koelle, Cholinesterases and Anticholinesterase Agents, in Handbuch der Experimentellen Pharmakologie , G. B. Koelle, Ed. (Springer-Verlag, Berlin, 1963) 15, p 265.

Table 5-IV. Effects of differential experience on AChE and ChE activity per mg (expressed as percentage of difference between means, ECT minus IC)

		AChE activity (with AcCh)				AChE activity (with AcSCh)			
Expt.	Strain	Total cortex	Sub-cortex	Total brain	Total cortex subcortex	Total cortex	Sub-cortex	Total brain	Total cortex subcortex
I	S_1	-2.6	3.5[a]	1.0	-6.0[b]	-2.6[a]	2.6	0.8	-5.4[c]
IIA	S_1	-0.7	2.6[a]	1.1	-3.1[a]	-0.9	2.2[a]	0.7	-2.8[a]
IIB	S_3	-3.0[a]	3.1[a]	1.1	-5.9[b]	-2.6[c]	1.8	0.0	-4.2[a]
% cases, ECT IC		42	79	55	17	29	56	50	21

		ChE activity (with BuSCh)			
Expt.	Strain	Total cortex	Sub-cortex	Total brain	Total cortex subcortex
I	S_1	5.7[a]	-1.0	0.0	7.1[a]
IIA	S_1	5.7[b]	0.2	1.5	5.6[b]
IIB	S_3	4.9[b]	-3.3	-1.3	8.1[b]
% cases, ECT > IC		82	44	58	78

N = 12 litter-mate pairs in each experiment

[a] $P < 0.05$

[b] $P < 0.01$

[c] $P < 0.10$

Abbreviations: AChE, acetylcholinesterase; AcCh, acetylcholine; AcSCh, acetylthiocholine; BuSCh, butyrylthiocholine.

6. EFFECTS OF ENVIRONMENTAL COMPLEXITY AND TRAINING ON BRAIN CHEMISTRY AND ANATOMY AMONG MATURE RATS*

Edward L. Bennett, Hiromi Morimoto, and Barbara Olton
(in collaboration with Mark R. Rosenzweig and David Krech,
Department of Psychology, University of California)

Last year we reported on the effects of experience and training on the brains of young rats.[1,2] Litter mates raised under conditions of enriched experience were shown to develop certain characteristics to a higher degree than litter mates raised under impoverished conditions. The enriched-experience animals developed (a) significantly greater weight of the cerebral cortex but no change in the weight of the rest of the brain, and (b) significantly greater total activity of acetylcholinesterase, both in the cortex and in the rest of the brain.

The results reported last year have been replicated and extended by subsequent experiments. To the 33 litter-mate pairs of the S_1 strain studied last year, we have added another 34 pairs, essentially duplicating the original results. Beyond this, we have found that not only acetylcholinesterase but also cholinesterase shows significant changes with differential experience, and the two enzymes show different patterns of change among brain regions.[3] With the collaboration of a neuroanatomist, Dr. Marian C. Diamond, we have also found that the increased weight of cortical tissue of our enriched-experience rats reflects a measurable increase in the depth of the cortex, and that there are further histological differences between the cortices of experimental groups.

All these results have been obtained with young animals exposed to the experimental conditions for about 80 days, starting from weaning at about 25 days of age. The results are therefore consistent with two possible interpretations: (a) These effects could be attributed simply to alterations in the rate of early growth and development and therefore would be expected to occur only among young anaimals; or (b) these cerebral changes may be consequences of differential opportunity to learn, and therefore could occur equally well among older animals.

We have attempted to test these interpretations by keeping rats of the S_1 strain under normal colony conditions until they were 105 days old, and then assigning them either to the enriched or the impoverished condition for 80 days. (Rats of 105 days can be considered young adults, since their brain growth, which never stops completely, has fallen to a low rate, and since rats mature sexually at about 70 days).

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*This research was supported in part by grants from U. S. Public Health Service, Surgeon General's Office, and the National Science Foundation.

1. M. R. Rosenzweig, D. Krech, and E. L. Bennett, Fed. Proc. 21, 358 (1962).

2. M. R. Rosenzweig, D. Krech, E. L. Bennett, and M. C. Diamond, J. Comp. Physiol. Psychol. 55, 429 (1962).

3. E. L. Bennett, M. Hebert, H. Morimoto, B. Olton, M. R. Rosenzweig, and D. Krech, this Quarterly Report, page 13.

Methods

The behavioral and chemical methods used with the adult rats were the same as those previously employed with weanling animals.

Behavioral Methods

Two replicate experiments were performed on adult male rats of the Berkeley S_1 strain. In each experiment, three animals were taken from each of twelve litters, and thus gave 36 animals in each experiment. At about 105 days of age, the animals were assigned at random so that one animal from each litter fell into one of the following three groups: (1) Environmental Complexity and Training (ECT), (2) Social Control (SC), and (3) Isolated Condition (IC). Food and water were available ad libitum in the cages of all groups.

The 12 ECT animals were housed in a large hardware cloth cage. The cage contained wooden "toys" and a small wooden maze that the rats used as a nest. Each day the rats explored an open field (the Hebb-Williams apparatus), with the pattern of barriers changed daily. Beginning at about 130 days of age, the animals were trained for one or two trials a day in three devices in succession--the Lashley III maze, the Dashiell checkerboard maze, and the Krech hypothesis apparatus. Training was for small sugar-pellet rewards.

The SC animals lived in ordinary colony cages, three animals to a cage. They lived in the same room as the ECT animals, but no attempt was made to enrich their experience beyond that of other colony animals.

The IC animals lived in individual cages with solid metal walls on three sides so that they could not see or touch another animal. In the first replication, the IC animals lived in the same room as their ECT and SC litter mates, but behind an opaque screen and under reduced illumination. In the second replication, they lived in a separate room.

Dissection of Brain Samples

At the end of the 80-day period, the animals were delivered under code numbers to the chemical laboratory and sacrificed by decapitation. Litter mates were sacrificed in immediate succession, but in a prearranged order so that the technicians doing the dissection and chemical analyses could not tell to which group any animal belonged.

Each brain was dissected into five parts: (a) a defined section of the occipital cortex of both hemispheres; (b) a sample of somesthetic cortex from both hemispheres; (c) the remaining dorsal cortex of both hemispheres; (d) the ventral cortex of both hemispheres and associated tissue including the hippocampus, amygdaloid nuclei, and corpus callosum; and (e) the remaining brain, including the medulla, the cerebellum, and the olfactory bulbs. In the rat, the cortex can be peeled off rather cleanly from the underlying white matter. As each section of brain was removed, it was weighed to 0.1 mg and then was frozen on dry ice and stored frozen until chemical analysis.

Chemical Analysis

The values reported here include all enzymatic activity that hydrolyzes acetylcholine; we have shown that more than 95% of this activity is due to acetylcholinesterase (AChE).[3] The analyses were done with an automatic titrator, as we have described previously.[4] In the second replication, AChE was also determined colorimetrically, and the results obtained by the two kinds of analysis were very similar.[3]

Results

First let us review briefly some of the main results obtained with younger animals, exposed to the differential environments between 25 and 105 days of age:

(a) In weight of cortex (Table 6-I), the ECT group surpasses their IC litter mates by about 5%; this is highly significant statistically ($P < 0.001$). In the rest of the brain the experimental groups do not differ. In weight of total brain, the ECT animal is heavier in two-thirds of the pairs.

(b) In total activity of acetylcholinesterase (Table 6-II), the ECT animals surpass the IC by 2 to 3% throughout the brain. Although this difference is small, it is highly consistent, and it is statistically significant. At the cortex, the increase in AChE activity with enriched experience lags behind the increase in weight; at the subcortex, the increase in AChE occurs without any accompanying increase in tissue weight.

(c) With the cortex (Table 6-III), different functional regions respond differently to the experimental conditions. The visual area shows the largest changes and the somesthetic shows the least--8% and 3.9%, respectively, in weight; 4.4 and 2.2%, respectively, in AChE activity.

It should be noted that these cerebral changes did not reflect differences in body weight. On the contrary, the ECT animals were more active than the IC group and had somewhat lower body weights, although they had heavier brains. Control experiments indicated that differences in amount of locomotion or in handling could not account for the cerebral differences between the experimental groups.

Now that we have reviewed the effects found among younger animals, let us see the main results with animals kept in the differential environments from 105 to 185 days of age (Table 6-IV).

(a) In weight of total cortex, the adult ECT animals surpass their IC litter mates by 6.0%; this effect is highly significant statistically, as it indicates the difference is real and not experimental. Again, the visual cortex shows the greatest difference in weight (10.7%) between groups, and the somesthetic area shows the least change (2.3%). Even in the rest of the brain, where the younger animals showed no difference, the adult ECT animals exceed the IC in weight, with a difference of 2.4% ($P < 0.01$).

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4. M. R. Rosenzweig, D. Krech, and E. L. Bennett, Brain Enzymes and Adaptive Behavior, in Ciba Foundation Symposium on The Neurological Basis of Behaviour (J. and A. Churchill, Ltd., London 1958), pp. 337-355.

(b) In total activity of acetylcholinesterase, the adult ECT animals surpass the IC animals by about 3% throughout the brain. Again, as in the younger animals, the increase in AChE activity at the cortex lags behind the increase in weight. At the subcortex, where weight also increases in the older ECT animals, the increase in AChE activity is somewhat greater than the increase in weight.

(c) The Social Control animals, which were kept in a condition intermediate between those of the ECT and IC groups, showed cerebral values that were intermediate between those of the other two groups. Thus it appears that environmental complexity and training can lead to increased brain weight and AChE activity, and an impoverished environment can lead to decreases in both brain weight and AChE activity; this is true even with both groups starting from usual colony conditions.

The magnitudes of the relative differences between ECT and IC groups of younger and older animals are presented in Table 6-V. For most measures, the changes induced by differential experience in adult animals were as large as,or larger than, those induced in younger animals. We feel, therefore, that we can rule out the hypothesis that the cerebral responses to differential experience can be attributed merely to alterations in the rate of early growth or development. The data are consistent with the second hypothesis, that these cerebral changes are consequences of differential opportunity to learn.

Table 6-I. Effects of environmental complexity and training (ECT) and isolation (IC) on brain weights (in mg) of 33 litter mate pairs of S_1 male rats run from 25 to 105 days of age.

		Total cortex	Rest of brain (Subcortex II)	Total brain
	ECT $\bar{X}$	682.4	925.8	1608.2
	IC $\bar{X}$	647.8	936.7	1584.5
% pairs,	ECT > IC	82	45	67
	ECT/IC	1.053	0.988	1.015
	P	< 0.001	NS	< 0.05

NS = not significant; $\bar{X}$ mean of group.

Table 6-II. Effects of environmental complexity and training (ECT) and isolation (IC) on acetylcholinesterase (AChE) activity of brains of 33 litter mate pairs of S_1 male rats run from 25 to 105 days of age. (Units = AChE activity per mg × weight of section)

		Total cortex	Rest of brain (Subcortex II)	Total brain
	ECT $\bar{X}$	6135	17306	23319
	IC $\bar{X}$	5965	16876	22740
% pairs,	ECT > IC	73	69	76
	ECT/IC	1.028	1.025	1.025
	P	< 0.10	< 0.01	< 0.001

Table 6-III. Effects of ECT and IC on AChE activity and weights of young S_1 rats in several regions of cerebral cortex.

		Visual area	Somesthetic area	Remaining dorsal cortex	Ventral cortex
Total AChE activity[a]	ECT $\bar{X}$	328	304	2073	3468
	IC $\bar{X}$	314	297	1999	3374
% pairs,	ECT > IC	75	58	72	66
	ECT/IC	1.044	1.022	1.037	1.028
	P	< 0.01	NS	< 0.01	NS
Weight (mg)	ECT $\bar{X}$	55.6	42.3	279.2	305.3
	IC $\bar{X}$	51.5	40.7	264.2	291.3
% pairs,	ECT > IC	85	58	73	70
	ECT/IC	1.080	1.039	1.057	1.048
	P	< 0.001	NS	< 0.001	< 0.05

[a] AChE activity in terms of mμmoles ACh hydrolyzed per minute
NS = not significant

Table 6-IV. Effects of ECT and IC on AChE activity and brain weights of 24 litter-mate pairs of S_1 male rats run from 105 to 185 days of age.

		Visual area	Somesthetic area	Remaining dorsal cortex	Ventral cortex	Total cortex	Rest of brain (Subcortex II)	Total brain
Total AChE activity[a]	ECT $\bar{X}$	422	371	1948	3608	6348	18286	24634
	IC $\bar{X}$	385	370	1892	3489	6139	17786	23923
% pairs,	ECT > IC	67	50	62	79	79	79	79
	ECT/IC	1.096	1.003	1.029	1.034	1.034	1.028	1.030
Weight (mg)	ECT $\bar{X}$	72.6	52.9	280.4	338.0	744.0	1019.0	1762.9
	IC $\bar{X}$	65.6	51.7	266.0	318.9	702.2	994.6	1696.9
% pairs,	ECT > IC	85	50	71	67	92	71	71
	ECT/IC	1.107	1.023	1.054	1.060	1.060	1.024	1.039

[a] AChE activity in terms of mμmoles ACh hydrolyzed per minute

Table 6-V. Comparison of effects of environmental complexity and training (ECT) and isolation control (IC) on brains of young and adult S_1 male rats (ECT mean/IC mean).

	Days run	Visual area	Somesthetic area	Remaining dorsal cortex	Ventral cortex	Total cortex	Rest of brain (Subcortex II)	Total brain
Weight (mg)	25-105	1.080	1.039	1.057	1.048	1.053	0.988	1.015
	105-185	1.107	1.023	1.054	1.060	1.060	1.024	1.039
Total AChE activity	25-105	1.044	1.022	1.037	1.028	1.028	1.025	1.025
	105-185	1.096	1.003	1.029	1.034	1.034	1.028	1.030

7. IMPROVEMENTS IN PAPER CHROMATOGRAPHIC TECHNIQUES FOR LABELED CELL EXTRACTS

V. Moses and Julia J. Chang

For more than 10 years the principal paper chromatographic methods used in this Laboratory for the analysis of labeled cell extracts from metabolic experiments have been those developed by Benson et al.[1] The paper used has been Whatman No. 4, a fast-running paper, prewashed by immersion in 1% (w/v) oxalic acid, followed by extensive rinsing with distilled water to remove superfluous oxalic acid. The purpose of this treatment with oxalic acid was to bind Ca^{++} and Mg^{++} present in the paper and thereby prevent sticking of phosphate esters on the paper as a result of precipitation of their calcium and magnesium salts.

The solvent used for development in the first direction was distilled phenol (100 g) plus distilled water (39 ml). In the second dimension, the chromatograms were irrigated with a mixture of butan-1-ol:propionic acid:water (46.8:22.5:30.7 v/v/v). This technique had the following advantages: (a) development was rapid (12 h for the first dimension and 7 h for the second), and (b) the compounds in which we were interested were distributed over the whole area of the chromatogram fairly satisfactorily, though some interesting compounds (e.g., glucose, seriene, and glycine) were not adequately resolved. On the other hand, the system also suffered from a number of disadvantages: (a) a tendency of the compounds to streak, particularly, but not exclusively, in the first dimension; this seriously impaired the degree of resolution that might be achieved in practice; (b) the resolution achieved varied greatly from one chromatogram to another, even though solvents from one batch were used, and sheets of paper were removed consecutively from one packet; (c) the use of phenol in chromatography results in the decomposition of some compounds, e.g., amino acids;[2,3] (d) removal of residual phenol from the paper requires a long period of drying (24 h); (e) phenol is corrosive to the human skin.

Improvements have been sought in modifications of the solvents and in the use of papers other than Whatman No. 4.

A. Solvents

A series of attempts to improve the phenol-water solvent resulted in the development of a mixture designated "semistench";[4] many of the disadvantages resulting from the use of phenol were largely avoided with this mixture (streaking, and variability of resolution), though the problem of overlapping remained. Glucose, serine, and glycine were still inadequately separated, and glutamine, well separated from this group in the phenol-water

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1. A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas, and W. Stepka, J. Am. Chem. Soc. 72, 1710 (1950).
2. A. K. Huggins and V. Moses, Nature 191, 668 (1961).
3. V. Moses, J. Chromatog. 9, 241 (1962).
4. G. J. Crowley, V. Moses, and J. Ullrich, J. Chromatog., in press.

system, now ran together with these substances. There were other less important instances of closer coincidence of compounds in semistench than in phenol. These disadvantages have been, in our opinion, adequately counterbalanced by the more reproducible separations, lessened streaking, and minimized decomposition of amino acids in semistench than in phenol.

Simplification in the methods involving phenol, however, has also been made. Pretreatment of the paper with oxalic acid was found to be unnecessary, and distillation of the phenol can be avoided if a mixture with the following composition is used:

Liquid phenol (88% in water) (Mallinckrodt "chromatography" grade)	840 ml
Distilled water	160 ml
Glacial acetic acid	10 ml
M-Ethylenediaminetetraacetic acid (potassium salt), pH 7	1 ml

Improvement of the second solvent would be desirable mainly because most of the compounds of interest to us are confined to that half of the chromatogram nearest the origin, the remaining area being largely empty. It would be desirable if these relatively crowded compounds were to be distributed over a larger area of the paper, without losing altogether the faster running substances migrating to positions near the solvent front. Experiments to attain this end are still under way, and consist at the moment of partial or complete replacement of the butan-1-ol in the mixture either by propan-1-ol, propan-2-ol, or mixtures of these, combined with similar partial or complete replacements of the propionic acid with acetic and formic acids. It seems at this stage that manipulation of this sort will ultimately achieve the desired result.

B. Paper

We systematically examined a number of different filter papers for chromatography to see how resolution of the various compounds is affected by differences in the characteristics of the paper. A standard mixture of labeled substances extracted from baker's yeast supplied for 2.5 min with [^{14}C] glucose was chromatographed in the standard semistench and butan-1-ol:propionic acid:water mixtures on a variety of papers (Table 7-I). From this experiment, it appeared that Ederol No. 202 paper was most satisfactory for our purpose, as the spots were small, with a minimum of streaking and with the highest resolution. Unfortunately, this paper is made in West Germany and there is no United States distributor. We are therefore obliged to obtain supplies directly from the manufacturer, with consequent long delays in delivery. It appears, nevertheless, that once sufficient supplies become available, this will become the standard paper in this Laboratory for analysis of the complex mixtures obtained in metabolic tracer experiments.

Table 7-I. Characteristics of different papers.

Paper	Size (cm)	Density (mg/cm^2)	Development times at 23°, h		Quality of separation
			Semi-stench	Butan-1-ol: propionic acid: water	
Whatman No. 4	57×46	9.12	12	7	Poorest
Whatman No. 1	57×46	8.92	32	17	Better than No. 4
Whatman No. 20	57×46	9.31	72	44	Better than No. 1
Whatman No. 20	57×46	9.84	32	17	Better than No. 20
Ederol No. 208[a]	60×48.2	11.83	32	17	Better than No. 2[b]
Ederol No. 202[a]	60×48.2	11.20	32	17	Best[b]

[a]Manufactured by: J. C. Binzer, Vertriebs - G. M. B-H, 3559 Hatzfeld/Eder, West Germany

[b]The quality of separation on these two papers was almost indistinguishable.

C. Resolution of the Glucose-Glycine-Serine-Glutamine Complex

No efforts are now being made to resolve these compounds on the original chromatograms of the cell extracts. We are thus forced to a secondary separation after elution of the complex from the original paper. Although no one-dimensional development has been evolved that will separate all these substances, they are resolved two-dimensionally and with no difficulty on Whatman No. 1 paper, using pyridine:water (80:20 v/v) as the first solvent and the standard phenol-water solvent as the second (Table 7-II).

Table 7-II. Secondary resolution of some potentially overlapping substances.

Run on Whatman No. 1 paper, developed first with pyridine:water (80:20 v/v) and second with the standard phenol-water mixture

Compound	Rf	
	Pyridine:water	Phenol:water
Asparagine	0.20	0.34
Aspartic acid	0.13	0.13
Fructose	0.87	0.51
Glucose	0.86	0.34
Glutamic acid	0.21	0.26
Glutamine	0.33	0.55
Glycine	0.29	0.37
Serine	0.43	0.30

8. MEASUREMENT AND ADJUSTMENT OF pH IN SMALL VOLUMES OF SOLUTIONS

V. Moses and Julia J. Chang

Metabolic tracer experiments, particularly with substrates with high specific radioactivity, are likely to involve performing reactions in very small volumes of liquid. For example, in current preliminary studies on the metabolism of a variety of substrates by chick fibroblasts grown in tissue culture, a suspension of the chick cells in 30 µl of an appropriate buffer is mixed and incubated with a solution of the substrate in 10 µl of the same buffer. Since the buffer contains a number of inorganic salts that are detrimental to the later chromatographic separation of metabolic products formed from the substrate, not more than about 40 µl of buffer may be applied to the chromatogram. It is not practicable in these experiments to separate the cells from the buffer before chromatographic analysis; thus, any increase in volume of the incubation mixture beyond about 40 µl represents a waste of both tissue (which is complicated to grow) and labeled substrate (which is expensive to buy). See reference 1 for further discussion of these considerations. [1]

Some of the substrates are made by chemical or biochemical methods in minute amounts in our laboratory; these are usually purified on paper chromatographs before use. Other materials, purchased commercially, are also subjected to chromatographic purification. Thus, the final substrate preparations that contain a very small mass of labeled material are frequently contaminated with solvent residues from the paper chromatogram. When the substrate is redissolved in buffer before being supplied to living cells, there may be sufficient residual acidity to seriously reduce the pH of the final solution from that desired. Many substrates, such as phosphate esters, are themselves strong acids, and this, too, contributes to a fall of pH.

In such instances it becomes imperative to measure, and if necessary to adjust, the pH of volumes of solution as small as about 20 µl. We are not familiar with any form of microelectrode that would permit this to be done with a pH meter, and have therefore made use of the dependence on pH of the absorption spectra of certain indicators. Phenol red indicator is not toxic to chicken fibroblasts, and the cells are even grown in the presence of this substance. Phenol red exhibits absorption peaks at 471 mµ and 533 mµ, and the ratio of the heights of these two peaks is characteristic of the pH of the solution.

Measurement and adjustment of pH is performed in spectrophotometer cuvettes having internal measurements of 3 mm × 3 mm, and a height of 48 mm. In such cuvettes, 20 µl of liquid forms a layer 2.2 mm deep, apart from meniscus effects. The cells are masked on the surface facing the light source with black Scotch tape in which is cut a slit 2.3 mm wide and 0.5 mm high. As the top of the slit is 0.9 mm above the bottom of the inside of the cuvette, 8.1 µl of liquid must be placed in the cuvette to bring the level of

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1. V. Moses, Preparation of Biological Material for Radioautographic Studies of Intermediary Metabolism, UCRL-9688, May 1961.

liquid above the top of the cuvette. In practice, an additional volume of perhaps 7 µl must be added to compensate for the effect of the meniscus. The liquid to be measured contains phenol red at a concentration of 1:10,000 (w/v). The optical density of the solution is measured at the two wavelengths mentioned above, and the ratio of the two readings compared with ratios obtained with phenol red in a series of standard buffers. The pH of the solution being measured may then be obtained directly from the calibration curve. At pH 7.2, the accuracy is about ± 0.02 pH units.

If adjustment of pH is necessary, small volumes (as low as 0.05 µl, if appropriate) of KOH or HCl solution are added until the desired pH is reached. The alkali or acid is dispensed from a micrometer or other microsyringe onto an inside wall near the top of the cuvette rather than into the liquid directly. The cuvette is then briefly centrifuged, the solution thoroughly mixed with a Vortex mixer, and the optical densities measured. When the desired pH has been obtained, the solution is made up to a standard volume indicated by a calibration mark on the cuvette itself and is mixed, and accurately measured aliquots are removed as required.

When colored solutions, in which an absorption peak from the colored substance interferes with one of the phenol-red peaks, are used, the pH may be determined from the specific absorption at one wavelength only. Although we have not critically examined the accuracy using one peak only, we feel that this is probably subject to greater error than using the ratios between two peaks.

It is obvious that the choice of indicator will be governed by toxicity to the biological system, the pH range to be covered, the presence of other colored materials, and other factors; however, by selection of an appropriate indicator, the method may be used with general application as a microtechnique for pH measurement.

9. CARBON-14 AND NITROGEN-15 TRACER STUDIES OF AMINO ACID SYNTHESIS DURING PHOTOSYNTHESIS BY CHLORELLA PYRENOIDOSA

J. A. Bassham and Martha R. Kirk

In a previous Quarterly Report, we described results of dual tracer studies with $^{15}NH_4^+$ and $^{14}CO_2$ of amino acid synthesis by photosynthesizing Chlorella.[1]

Although a number of tentative conclusions could be drawn at that time regarding rates and pools in amino acid biosynthesis in Chlorella, certain experimental difficulties made further interpretation impossible. One of the difficulties was the limited accuracy and reproducibility of the determination of the total pools of given amino acids. The other problem was that we were obliged to starve the algae during about 30 min of photosynthesis without a supply of $^{14}NH_4^+$, in order that $^{15}NH_4^+$ could be introduced without excessive dilution of the label. From the results of the experiments that we reported, it was seen that this period of starvation apparently caused a build-up of α-keto acids which led, upon the addition of $^{15}NH_4^+$, to a temporary abnormally rapid synthesis of glutamic acid, and possibly of aspartic acid. This transitory rapid synthesis of these amino acids tended to obscure the kinetics of the steady-state synthesis.

In order to minimize this transitory effect, we have altered the nutrient solution slightly so that the steady-state concentration of ammonium ion was decreased. This has been accomplished by preparing a nutrient solution with the ratio of K_2HPO_4/KH_2PO_4 adjusted to give a buffer at pH = 6, when saturated with 2% CO_2. In all other respects the nutrient solution was the same as reported before. After the algae were suspended in this medium and had commenced to photosynthesize, about 0.3 ml of 0.1 N HCl was injected into the suspension, and the pH control turned on, causing about 0.3 ml of the 0.1 N NH_4OH solution to be added automatically. The algae then commenced using the NH_4^+ ion. The pH of the medium was automatically controlled at 6, but the NH_4^+ ion was kept at a low concentration. In this way we hoped to avoid a long period of NH_4^+ depletion prior to the addition of $^{15}NH_4^+$. The accuracy of the determination has been somewhat improved, through the use of larger samples in some cases, and by our using more standard samples to check the reagents.

Two steady-state experiments with ^{15}N and ^{14}C have now been performed under these new conditions. Analyses of these experiments are not yet complete, but the amounts of total amino acid, ^{14}C, and ^{15}N in alanine and glutamic acid are shown in Figs. 9-1 and 9-2. In order to permit direct comparison of labeled atoms with total atoms, we have expressed the ^{15}N and the ^{14}C as μmoles of compound (μmoles of ^{14}C divided by the number of carbon atoms per molecule). Thus the degree (%) of labeling can be found by dividing the quantity indicated by the ^{14}C or ^{15}N curve by the quantity indicated by the total amino acid curve.

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1. J. A. Bassham, Martha Kirk, and Gerald Crowley, in Bio-Organic Chemistry Quarterly Report, UCRL-10634, Jan. 1963, p. 1.

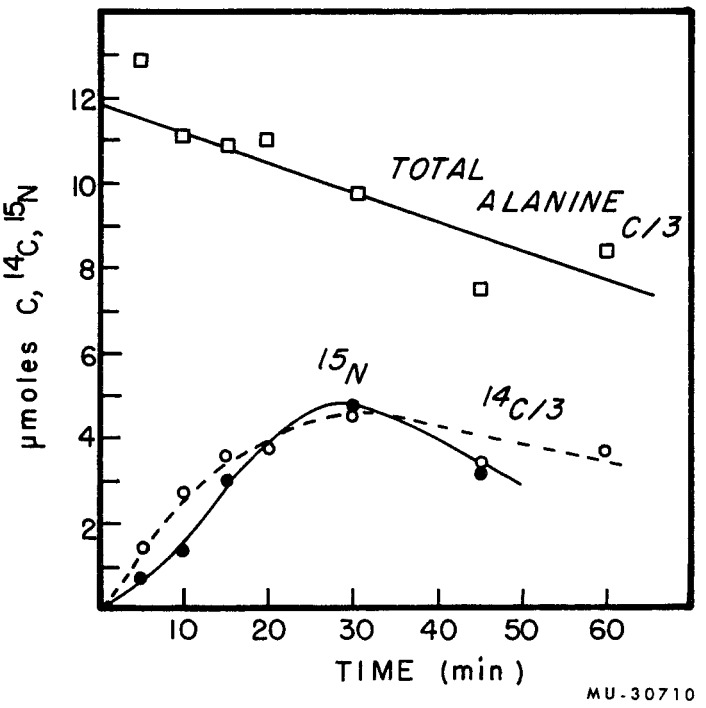

Fig. 9-1. Labeling of alanine.

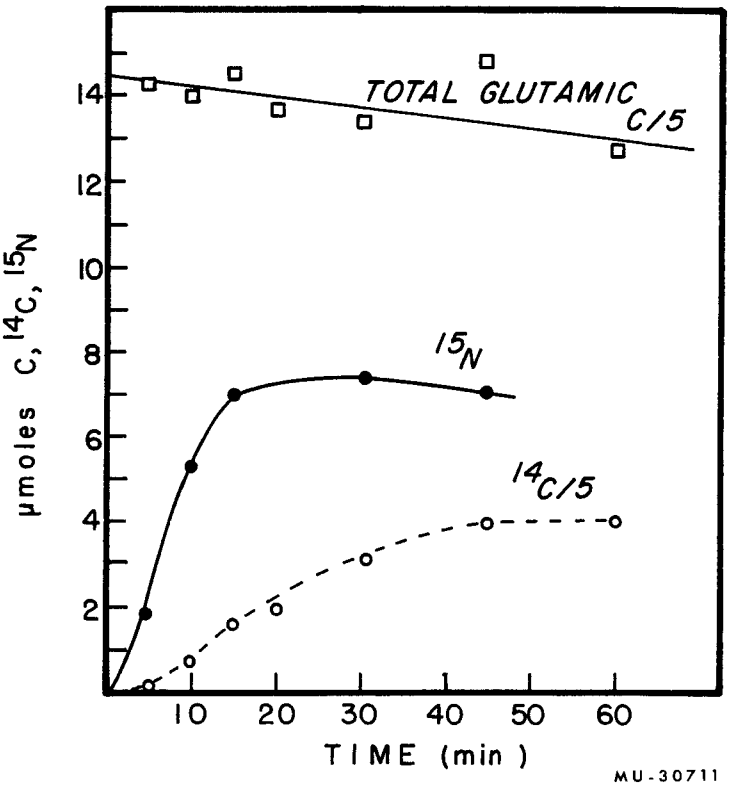

Fig. 9-2. Labeling of glutamic acid.

The transitory rapid synthesis encountered under the earlier studies was decreased. However, there appears to be a steady decrease in the levels of these two amino acids during the course of these new experiments. Possibly this decrease is a function of the physiological state of the algae. Prior to the administration of tracers, the algae had been in the steady-state apparatus in the nutrient solution mentioned above for 24 h. However, they had a "resting" period of 8 h of darkness ending 10 h prior to the administration of the tracers. Thus it is possible that a large number of the algae was in some phase in which the sizes of free amino acid pools were decreasing.

The changing pool size notwithstanding, it is possible to draw some conclusions from these results:

(a) The existence of more than one pool of a given amino acid is clearly illustrated. In the case of glutamic acid, it appears that there must be three pools: One which becomes saturated with ^{14}C at about 4 μmoles, one which becomes saturated with ^{15}N (and may include the ^{14}C pool) at about 7 μmoles, and one which has only a very slow rate of labeling with either tracer and is about 7 μmoles. In the case of alanine, the pools that become saturated with ^{15}N and ^{14}C seem to be about the same size, and they could be one and the same.

(b) From consideration of pool sizes and rates of labeling with ^{15}N, it appears that the incorporation of nitrogen into alanine can be accounted for in terms of transamination by glutamic acid, and that there is no evidence for a direct incorporation of $^{15}NH_4^+$ into alanine by reductive amination.

The rapid labeling of glutamic acid to a level higher than the level to which it becomes saturated with ^{14}C is consistent with all our earlier observations on the formation of glutamic acid independently of the primary carbon-fixing reactions, its formation from added acetate, etc.[2]

A plausible scheme for the formation of glutamic acid is that it is synthesized at appreciable rates at two sites. At one of these, near the site of CO_2 reduction in the chloroplast, it is made from a carbon skeleton derived from the carbon-reduction cycle. Some intermediate compounds of appreciable concentration lie between the cycle and glutamic acid, so that the rate of carbon labeling is slower with glutamic acid than with alanine. However, glutamic acid synthesis is the principal path for incorporation of ammonium ion, and glutamic acid transaminates other carbon skeletons to make other amino acids such as alanine.

At the other site of glutamate synthesis, its carbon skeleton must be formed from unlabeled intermediates, such as acetate formed by respiration of storage substances in the cytoplasm (i.e., fats, protein, polysaccharides). At both sites, one may suppose that there is a cyclic turnover of glutamate to α-keto-glutarate to glutamate, which incorporates $^{15}NH_4^+$ at a rate greater than the net synthesis of the carbon skeleton.

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2. D. C. Smith, J. A. Bassham, and Martha Kirk, Biochim. Biophys. Acta 48, 299 (1961).

The approximate maximum rate of labeling of the glutamic carbon skeleton in this experiment was 0.17 μmole/min (0.85 μmole of carbon per min), while the ^{15}N labeling rate is 0.63 μmole/min. The corresponding rates for alanine are: ^{14}C, 0.3 μmole/min (0.9 μmole of C per min); and ^{15}N, 0.3 μmole.

The fact that the maximum rates for the alanine carbon skeleton and amino groups are the same further supports the idea that the actively turning over pools of alanine are one and the same for ^{14}C and ^{15}N labeling.

10. PHOTOSYNTHESIS OF ^{14}C-LABELED PROTEIN FROM $^{14}CO_2$ BY CHLORELLA

Bronislawa Morawiecka and J. A. Bassham

Previous studies at this Laboratory showed that there is a very rapid light-stimulated synthesis of labeled amino acids from $^{14}CO_2$ by Chlorella.[1] Actively turning over pools of certain amino acids, such as alanine and glutamic acid, were determined by the technique of saturating them with radioactivity during conditions of steady-state photosynthesis with $^{14}CO_2$ of constant, measured specific radioactivity,[2] and analyzing resultant plant material by paper chromatography, radioautography, and measurement of ^{14}C in individual compounds. The results of those experiments suggested the strong possibility that the photosynthesizing Chlorella form "active" pools of amino acids in the chloroplasts for use in the photosynthesis of protein.

In order to learn more about the photosynthesis of amino acids, we have been allowing Chlorella to photosynthesize in the presence of $^{14}CO_2$ and $^{15}NH_4^+$. A preliminary report of the results of incorporation of these tracer elements into the free amino acid pools appeared in a previous quarterly report.[3]

Parallel to the studies of photosynthesis of free amino acids, we have begun a study of the incorporation of ^{14}C into the amino acids bound in protein during photosynthesis in Chlorella. The preliminary results of this study are reported here.

A. Experimental Procedure

The procedures for the photosynthetic experiment, amino acid determination, chromatography, and radioautography are as previously reported.[2,3]

The procedure for protein extraction from Chlorella pyrenoidosa is as follows (see also Fig. 10-1). Approximately 2-ml samples (2.3 to 2.5% of algae suspension), containing 46 to 50 µl of wet packed algae, were taken into weighed tubes containing 8 ml of methanol, at different times following introduction of $^{14}CO_2$ to the algae photosynthesizing under steady-state conditions.

After 20 min the killed algae suspension was centrifuged at 3600 rpm (2000 g) for 20 min at 5°. The supernatant solution was set aside for determination of protein by use of the microtannic method.[4] The solid material (sediment) was mechanically mixed for 15 min with 400 µl of 1.0 N NaOH

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1. D. C. Smith, J. A. Bassham, and M. Kirk, Biochim. Biophys. Acta 48, 299 (1961).

2. J. A. Bassham and M. Kirk, Biochim. Biophys. Acta 43, 447 (1960).

3. J. A. Bassham, M. Kirk, and G. J. Crowley, in Bio-Organic Chemistry Quarterly Report, UCRL-10634, Jan. 1963, p. 1.

4. W. Mejbaum-Katzenellenbogen, Acta Biochim. Polon. 2, 279 (1955).

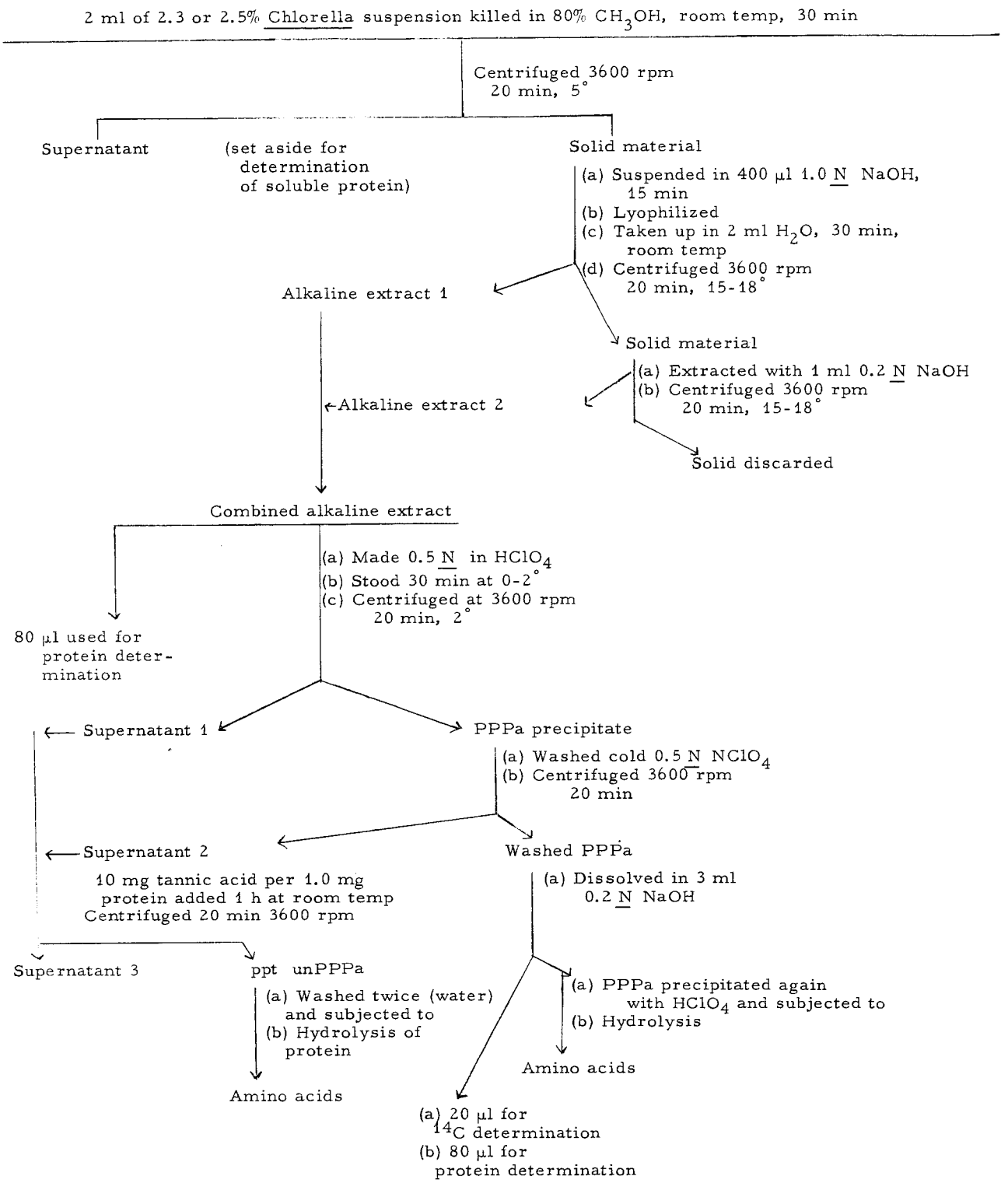

Fig. 10-1. Protein extraction and determination.

in a tube which was then put into liquid nitrogen until the mixture was frozen. The frozen material was lyophilized overnight in a desiccator over H_2SO_4. Water (2 ml) was added to the dried sample, to give a solution that was 0.2 N in NaOH. After 30 min of occasional mechanical agitation, the protein was extracted at room temperature. The sample was then centrifuged at 3600 rpm for 20 min at 15 to 18°. The solid material was washed with 1 ml of 0.2 N NaOH solution and centrifuged again at 3600 rpm for 20 min.

The supernatants were combined for the determination of the amount of protein and for the precipitation of the protein by perchloric acid. The protein from 3 ml of the alkaline extract was precipitated with 5 N $HClO_4$; this left a final concentration of 0.5 N $HClO_4$. After standing for 30 min at 0 to 2°, the protein was centrifuged at 3600 rpm for 20 min at the same temperature. The precipitate was washed once with cold 0.5 N $HClO_4$ and centrifuged again. The washed precipitate of protein which precipitates with perchloric acid (PPPa) was dissolved in 3 ml of 0.2 N NaOH. The level of the PPPa and the total radioactivity of this alkaline solution were determined.

The PPPa was precipitated again by $HClO_4$ in the manner described above. The washed precipitate of PPPa was subjected to acid hydrolysis for 22 h by 6 N HCl at 110°. A small black precipitate formed during the acid hydrolysis was washed and separated by centrifuging. The HCl was then removed from the clear bright yellow supernatant solution by twice adding water and distilling in vacuum in a desiccator over P_2O_5 and KOH.

After nearly complete removal of HCl, the hydrolyzed PPPa was taken up in 0.5 ml H_2O, and the solution was passed through an ion-exchange column (cation-exchange resin AG 5 OW-X8, H^+ form). Approximately 1 ml alkaline effluent (4 N NH_4OH) from the column was evaporated in a desiccator over H_2SO_4 overnight.

The dried residue was dissolved in 60 or 80 µl of water. An aliquot (20 µl) of solution was diluted, and its ^{14}C total radioactivity and total amino acids were determined.

The supernatant solutions from the perchlorate precipitations were collected for determination of their proteins (UnPPPa). UnPPPa were precipitated by tannic acid--by means of 10 mg tannic acid per 1 mg of UnPPPa.[5] The precipitate was washed twice with water and hydrolyzed in the same manner as the PPPa fraction.

The residue of hydrolyzed UnPPPa was dissolved in 40 µl of water, and the entire solution was placed on the chromatogram. A portion (20 µl) of the hydrolyzed protein was placed on the Whatman No. 1 or 2 paper, previously washed by oxalic acid, as reported earlier. The solvent system was based on the use of Semistench[6] in the first direction and n-butanol: propionic acid:water in the second direction. The paper was developed in

5. B. Morawiecka and W. Mejbaum-Katzenellenbogen, Clin. Chim. Acta 7, 722 (1962).

6. G. J. Crowley, V. Moses, and J. Ullrich, J. Chromatog., in press.

the first (long) direction with semistench for 26 to 28 h, and 22 to 24 h in the second direction. After chromatography was completed, the paper was placed in contact with a sheet of single-emulsion x-ray film to determine the position of the radioactive compounds, as previously reported.[2,3] In this way, we obtained the radioautograph of amino acids from algae protein, as shown in Fig. 10-2.

B. Results

The results of the protein extraction and precipitation scheme are shown in Table 10-I. The recovery of protein in the precipitated and unprecipitated fractions is reasonably reproducible from one sample to another. The total protein found is about 46 mg/g of wet packed algae (estimated dry weight 110 mg). The total ^{14}C labeling of the PPPa protein is shown in Fig. 10-3. After 90 min, about 70 μmoles of ^{14}C was incorporated into protein; the amount incorporated was based on the measured specific radioactivity of the $^{14}CO_2$ used. (See Refs. 1 and 2 for the method of calculation.) The maximum rate of net increase in ^{14}C in the protein (between 60 and 90 min) was 1.25 μmoles/min/g of wet packed algae. If the UnPPPa protein labeling is proportional to the PPPa labeling, the total rate of labeling would be $45.6/31.5 \times 1.25 = 1.75$ μmoles ^{14}C per min. If correct, this rate of labeling would account for an appreciable fraction of the utilization of the ^{14}C coming from the free-amino-acid pools. Current experiments will permit direct comparison between the labeling of the free-amino-acid pools and that of the bound amino acids in protein. As seen in the radioautograph (Fig. 10-2), the pattern of labeling of bound amino acids is considerably different from that of the free-amino-acid pools. This difference is to be expected, since most free-amino-acid pools are very small. Only those free pools of "primary" amino acids such as alanine, glutamic acid, aspartic acid, etc., are large enough to appear prominent on a radioautograph of the free amino acids.

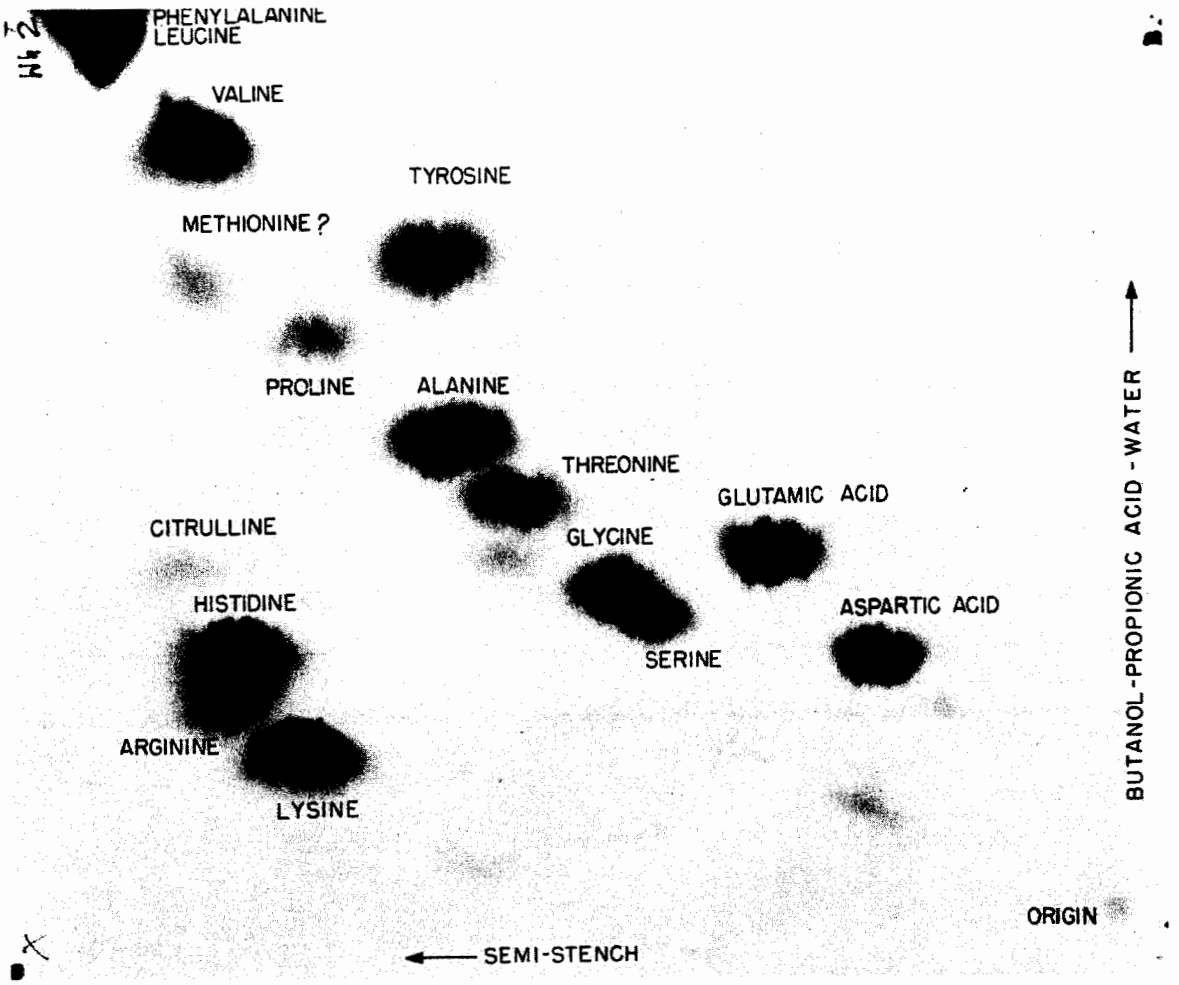

Fig. 10-2. Radioautograph of ^{14}C-labeled amino acids obtained from hydrolysis of protein PPPa.

Table 10-I. Level of the protein precipitated from Chlorella pyrenoidosa solution and not precipitated in perchloric acid.

Sample no.	Time of PS (min)	Algae (mg)	Protein			
			Extracted by 0.2 N NaOH (mg)	Extracted by 0.2 N NaOH mg per g per algae	Precipitated by 0.5 N $HClO_4$ PPPa per g algae	Not precipitated by $HClO_4$ UnPPPa per g algae
1	5	49.3	2.46	49.9	31.6	18.3
2	10	48.5	2.34	48.2	32.8	15.4
3	15	52.8	2.37	44.9	32.9	12.0
4	20	49.9	2.31	46.3	31.3	15.0
5	30	50.2	2.31	46.0	31.1	14.9
6	45	51.2	2.28	44.5	31.1	13.4
7	60	47.4	2.01	42.4	29.1	13.3
8	90	50.2	2.22	44.2	32.3	11.9
9	120	55.3	2.43	43.9	31.5	12.4
Mean value	-	50.5	2.29	45.6	31.5	14.1

PS = Photosynthesis

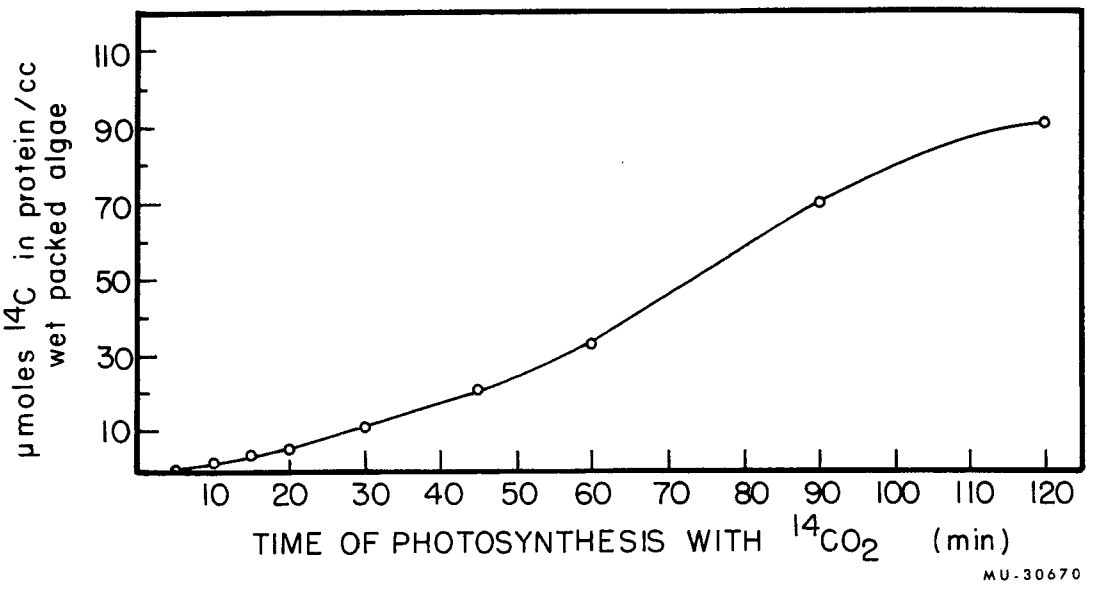

Fig. 10-3. Total ^{14}C radioactivity of the protein during steady-state photosynthesis by algae.

11. FURTHER STUDIES ON CARBOXYDISMUTASE

P. W. Trown

I. Introduction

Attempts by various workers[1-7] to isolate the enzyme carboxydismutase from plant tissues have not so far resulted in a preparation with a specific activity greater than 20 times that of the crude extract. Furthermore, although samples of the enzyme have been obtained which, by some criteria, appear to be pure, none has been shown, by every technique available, to be completely homogeneous. Consequently, relatively few studies of the physical, chemical, and biochemical properties of the enzyme have been undertaken.

The work reported here consists of further attempts to isolate the enzyme in a pure and highly active form, by means of some of the more modern techniques, such as gel filtration, which have recently become available to the enzymologist. In addition, studies were made of the long-term effects of such classes of compounds as salts, mild reducing agents, and detergents upon the stability of the enzyme.

II. Experimental

Chloroplasts were isolated from spinach leaves (as described by Park and Pon[8]) and lysed by use of 0.02 M tris, pH 8.0, or 0.02 M potassium phosphate buffer, pH 7.4. Removal of the lysed chloroplasts by centrifugation gave a clear supernatant solution, the "chloroplast extract," which was used as the starting material in all the purifications of carboxydismutase described.

Protein concentrations were determined by using the relationship determined for carboxydismutase by Pon,[7] i.e., (optical density at 280 mμ) × 0.6 = conc of protein (mg/ml). Phosphorus determinations were made by the method of Chen et al.[9] Carboxydismutase activity was

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1. J. Mayaudon, A. A. Benson, and M. Calvin, Biochim. et Biophys. Acta 23, 342 (1957).

2. E. Racher, Arch. Biochem. Biophys. 69, 300 (1957).

3. A. Weissbach, B. L. Horecker, and J. Hurwitz, J. Biol. Chem. 218, 795 (1956).

4. W. B. Jakoby, D. O. Brummond, and S. Ochoa, J. Biol. Chem. 218, 811 (1956).

5. J. W. Lyttleton and P. O. P. T'so, Arch. Biochem. Biophys. 73, 120 (1958).

6. D. W. Kupke, J. Biol. Chem. 237, 3287 (1962).

7. Ning G. Pon, Studies on the Carboxydismutase System and Related Materials, (Ph. D. Thesis) Lawrence Radiation Laboratory Report UCRL-9373, August 1960.

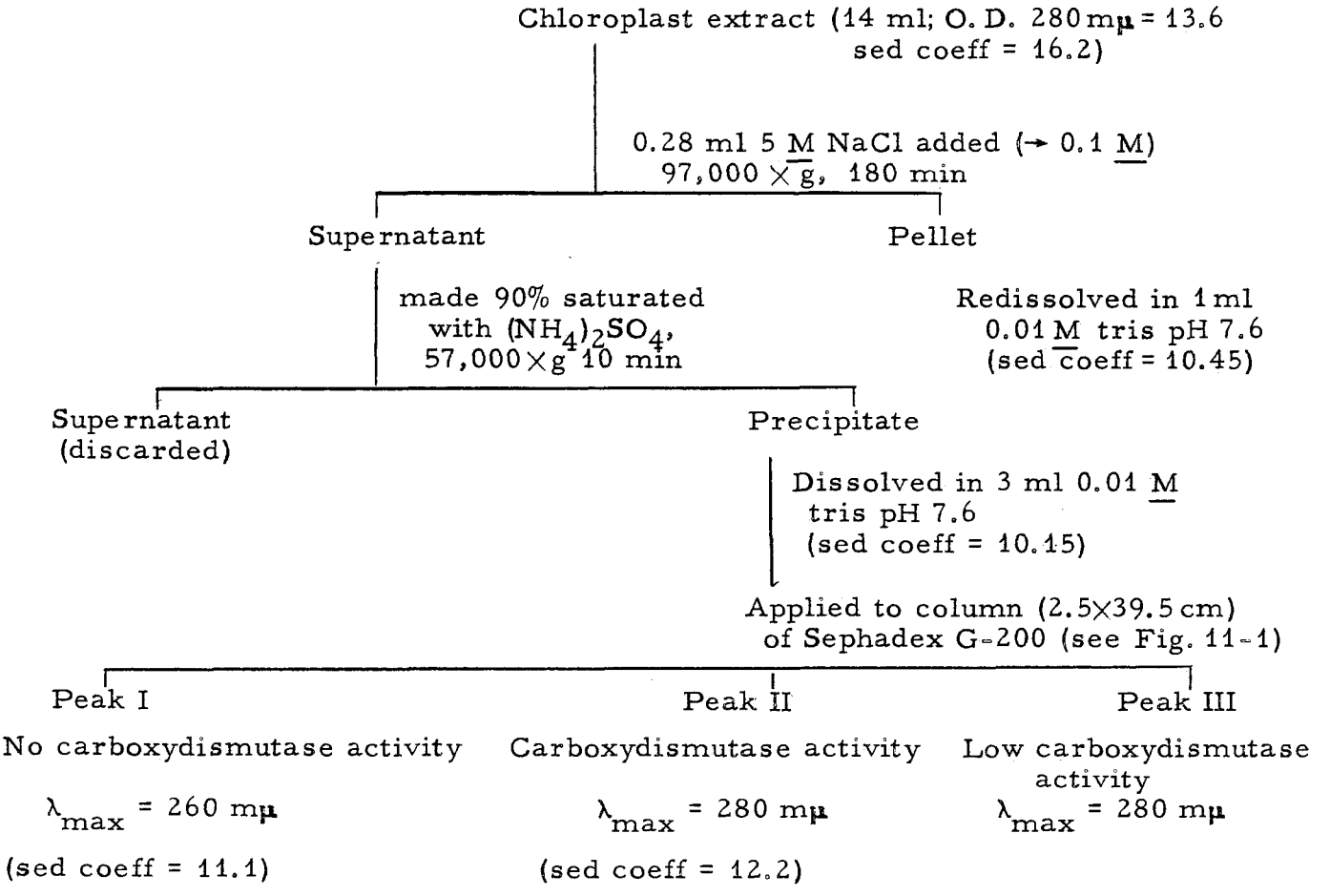
8. R. B. Park and N. G. Pon, J. Mol. Biol. 3, 1 (1961).

9. P. S. Chen, Jr., T. Y. Toribaba, and H. Warner, Anal. Chem. 28, 1756 (1956).

measured by a standard assay procedure described by Pon.[7] Sedimentation coefficients were determined by using a Spinco Model E analytical ultracentrifuge. The protein solutions were made 0.1 M in sodium chloride, and measurements were taken at 4°.

A. Gel Filtration on Sephadex G-200

The crude chloroplast extract resulting from two bunches of spinach (450 g leaves) was treated according to the flow sheet:



It was found difficult to reproduce the values for the sedimentation coefficient of carboxydismutase (ranging from 16 to 21, depending on isolation procedure and conditions of measurement) observed by other workers.[3, 5, 6, 7] In only two experiments during this work did the sedimentation coefficient observed fall in this range. In all other experiments, values between 10 and 12 were obtained, even for the highly purified enzyme. Indeed, during the experiment quoted above, the sedimentation coefficient changed from 16.2 for the crude extract to approx 10 for both the supernatant and pellet, which were obtained by centrifugation of the extract at 97,000 × g for 3 h in the presence of 0.1 M NaCl (see flow sheet).

By using Sephadex G-200 it was possible to effect a partial purification of carboxydismutase as shown in Fig. 11-1. High-molecular-weight material, which was assumed to be unable to enter the Sephadex grains, was nevertheless partially resolved into two fractions (Peaks I and II, Fig. 11-1). The first fraction (Peak I) appeared to contain nucleic-acid-like material and to have no carboxydismutase activity, while the second (Peak II) contained mostly protein which had a high carboxydismutase activity. A third fraction (Peak III), of lower molecular weight, also contained protein having a specific enzymic activity which in some experiments was comparable with that in Peak II.

B. Large-Scale Isolation of Chloroplasts and Fractional Ammonium Sulfate Precipitation of Chloroplast Extract

Chloroplasts from 50 bunches of spinach were isolated by John Biggins and Paul A. Loach, using the Servall Model RC2 centrifuge with a continuous-flow attachment. Four liters of phosphate buffer was used to lyse the chloroplasts which were separated by centrifugation, again by the continuous-flow method. The resulting chloroplast extract was made 90% saturated with respect to ammonium sulfate (the pH being maintained at approx 7.5 by addition of concentrated ammonium solution) and left for 2 days at 4°. The precipitate was collected by centrifugation, redissolved in 0.01 M tris, pH 7.6, and the solution clarified by centrifugation and made up to 100 ml. The concentration of ammonium sulfate at this stage, as determined by Kjeldahl estimation, was 27.5% saturated.

Weighed aliquots of solid ammonium sulfate were then consecutively added to the solution, which was stirred for at least 1 h at 4° after each addition. Any precipitate that formed after each addition was collected by centrifugation, redissolved in 0.01 M tris, pH 7.6, and dialyzed against the same buffer overnight at 4°. Aliquots of each fraction were diluted for measurement of optical density and carboxydismutase activity. The results of the fractionation are given in Table 11-I.

As shown in Table 11-I, more than 60% of the total recovered protein precipitated in the range 30 to 35% saturated ammonium sulfate. This fraction also had the highest specific carboxydismutase activity. It was divided into seven aliquots (each containing approx 160 mg protein), to each of which was added solid ammonium sulfate to bring the concentration to 50% saturation. The precipitated enzyme was stored at 4° until further use.

C. Purification of Carboxydismutase by Zone Precipitation on Sephadex G-100

Carboxydismutase, partially purified by fractional ammonium sulfate precipitation (see above), was subjected to zone precipitation as described by Porath.[10]

10. J. Porath, Nature 196, 47 (1962).

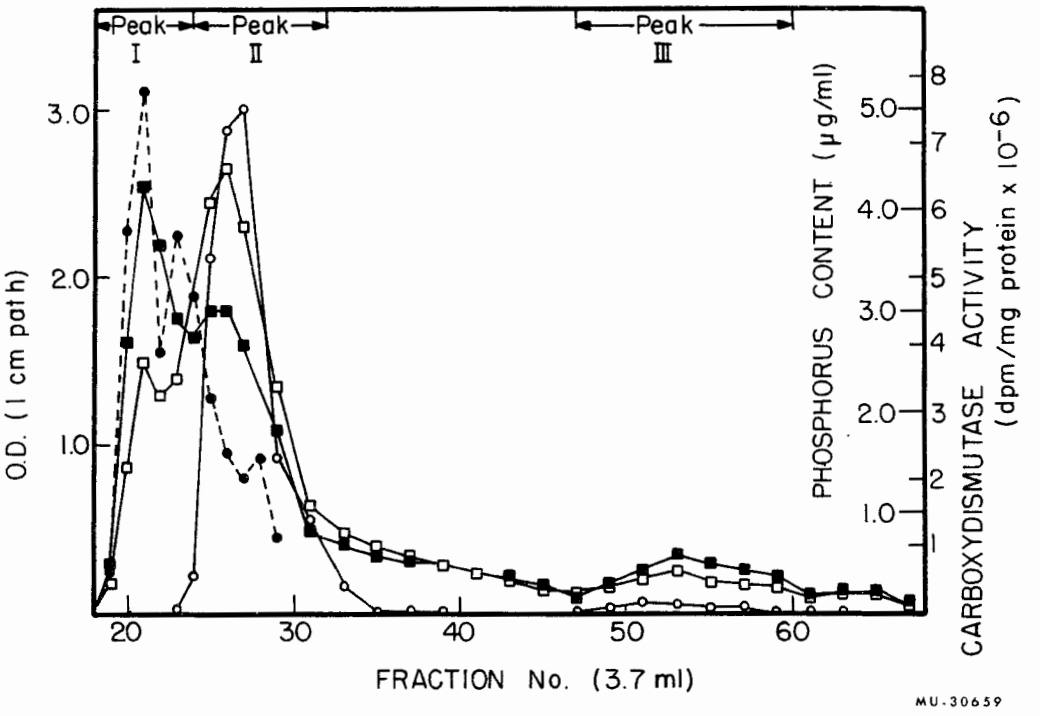


Fig. 11-1. Fractionation of carboxydismutase on Sephadex G-200: Closed squares, O. D. at 260 mμ; open squares, O. D. at 280 mμ; closed circles, phosphorus content, μg/ml; open circles, carboxydismutase activity, dis/min per mg protein $\times 10^{-6}$. (10 min incubation at 25°C, specific activity of $H^{14}CO_3^-$ used in assay, 25.1 μC/μM; these assay conditions were used throughout the work.)

Table 11-I. Ammonium sulfate fractionation of crude chloroplast extract.

Wt of ammonium sulfate added (g)	Ammonium sulfate saturation (%)	Protein recovered (%)	λ_{max} (mμ)	Specific carboxydismutase activity (dis/min per protein) $\times 10^{-6}$
0	27.5	9.5	258	1.84
1.7	30	1.8	277	25.84
3.5	35	62.2	278	51.13
3.5	40	12.4	276	31.81
4.0	46	5.2	268	1.41
3.5	51	5.5	268	0.07
3.5	56	2.1	266	0.03
7.0	66	2.3	264	0.01

Sephadex G-100 was suspended in 0.01 M tris, pH 7.6, 50% saturated with ammonium sulfate, and allowed to equilibrate overnight. It was then poured, with continuous stirring, into a glass column, and allowed to settle to give a gel bed 28×2.1 cm. Liquid was allowed to run from the column until the top of bed was just exposed, when 2-ml portions of 45, 40, 35, 30, 25, and 20% saturated ammonium sulfate, in 0.01 M tris, pH 7.6, were consecutively added. Each portion was allowed to enter completely into the top of the bed before addition of the next; this produced a stepwise gradient from 50 to 20% saturated ammonium sulfate solution at the upper end of the column. Carboxydismutase (60 mg, specific activity = 40×10^6 dis/min per mg protein), dissolved in a solution containing 20% saturated ammonium sulfate, 0.01 M tris, pH 7.6, 10^{-3} M glutathione (reduced), and 10^{-3} M Versenol (1.3 ml), was then applied to the top of the column and allowed to enter the gel bed. The column was eluted by using a linear concentration gradient of ammonium sulfate ranging from 20 to 10% saturated and containing also tris, glutathione, and Versenol, as above (total volume 500 ml).

As elution of the column proceeded, material precipitated in an opaque white band which slowly moved (by continuous solution and reprecipitation) to the bottom of the column, finally redissolving and being collected in solution. Aliquots of the fractions were diluted for measurements of optical density and carboxydismutase activity, the results of which are shown in Fig. 11-2. Fractions 38 to 52 were combined, dialyzed (during which process considerable deactivation of the enzyme occurred), and concentrated to 1.5 ml. Ammonium sulfate was added to bring the concentration to 20% saturated, and the protein solution was added to a second column of Sephadex G-100, identical with that described above; this latter column was again eluted in the same way. Figure 11-3 shows the elution curve for this second zone-precipitation experiment.

The specific activity of the most active fraction from the first zone-precipitation experiment was approx 50% greater than that of the material applied to the column, indicating that some purification had been achieved.

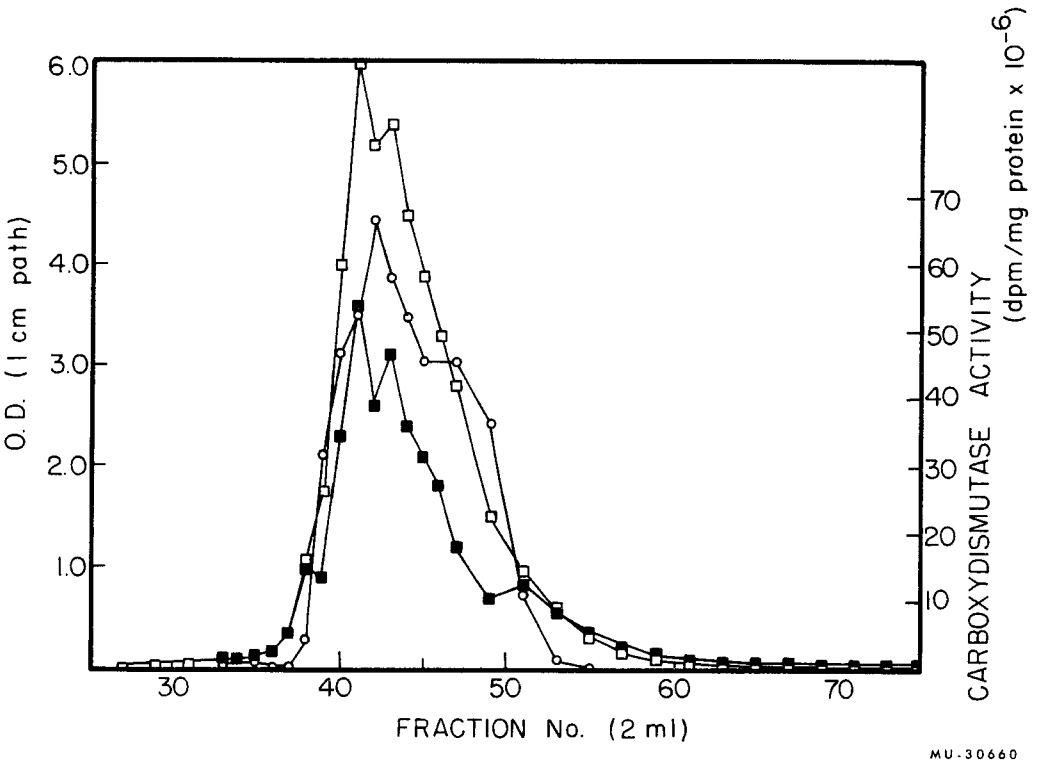


Fig. 11-2. Zone precipitation of carboxydismutase with an ammonium sulfate concentration gradient on a column of Sephadex G-100: closed squares, O. D. at 260 mμ; open squares, O. D. at 280 mμ; open circles, carboxydismutase activity, dis/min per mg protein $\times 10^{-6}$.

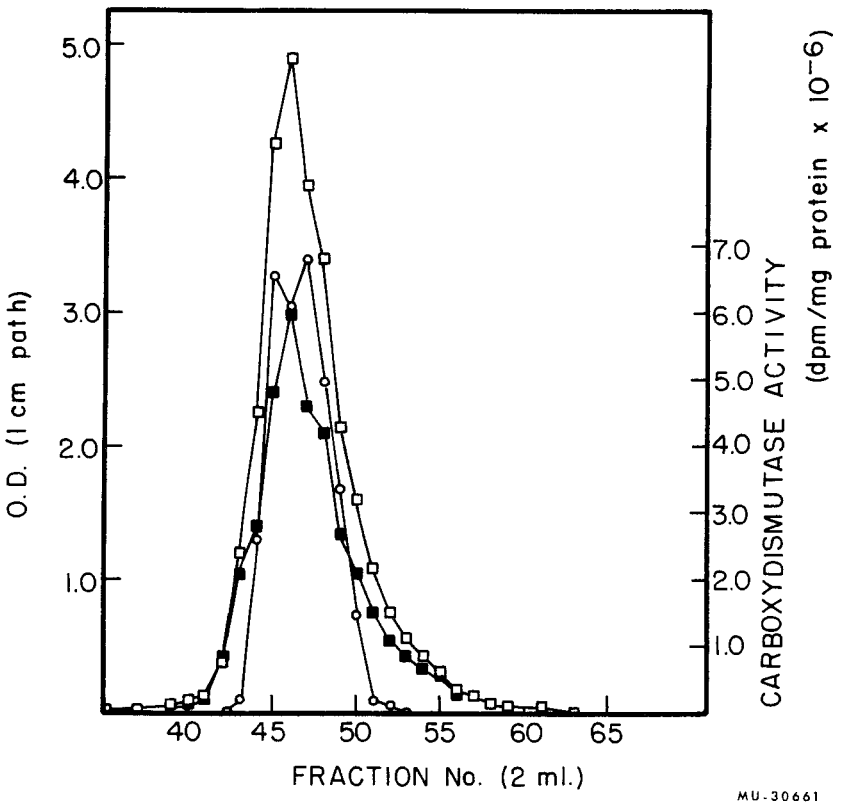


Fig. 11-3. Repeat zone precipitation of carboxydismutase from first zone-precipitation experiment (Fig. 11-2) under identical conditions: closed squares, O. D. at 260 mμ: open squares, O. D. at 280 mμ: open circles carboxydismutase activity, dis/min per mg protein $\times 10^{-6}$.

In addition, there was a long "tail" (not shown in Fig. 11-2), which was found to contain relatively large amounts of nucleic-acid-like material which had thus been separated from the enzyme. The second elution curve showed no such "tail," and the curves for optical density and specific activity were quite closely parallel, indicating homogeneity. However, the enzyme was still not homogeneous in the analytical ultracentrifuge, containing approx 5 to 10% of material with a lower sedimentation coefficient than the main band. The specific activity of the most active fraction from the second zone-precipitation experiment was the same as that of the material applied to the second column. Although this activity was only one-tenth of that of the best material obtained in the first experiment, it was increased by a factor of 2.5 on dialyzing away the ammonium sulfate. This latter phenomenon was thought to be worthy of further study.

D. Effect of Various Compounds on the Stability and Activity of Carboxydismutase

The compounds listed below were added at the stated concentrations to solutions of carboxydismutase in 0.01 M tris, pH 7.6, containing Versenol 10^{-3} M, which were then stored at 0°. Aliquots were withdrawn and either diluted or used undiluted for assay of carboxydismutase activity by the standard assay procedure (Pon[7]).

a. Glutathione, Sodium Ascorbate, Cysteine

Carboxydismutase (0.4 mg/ml) was stored in the presence of reduced glutathione (10^{-3} M), sodium ascorbate (10^{-3} M), and cysteine (10^{-3} M), respectively. Over a period of 160 h the enzyme lost only 5% of its initial activity in the presence of reduced glutathione, whereas the control solution and those containing sodium ascorbate and cysteine lost approx 30% of their respective initial activities over the same period.

b. Sucrose

Carboxydismutase (0.8 mg/ml) was stored in the presence of 1.0 M, 0.5 M, and 0.25 M sucrose, respectively. Aliquots were withdrawn at various times, diluted 10× with 0.01 M tris, pH 7.6, containing 10^{-3} M Versenol, and assayed. In 160 h the control enzyme solution lost 60% of its initial activity, the solutions containing 0.5 M and 0.25 M sucrose lost 55% of their respective initial activities, and that containing 1.0 M sucrose lost 40% of its initial activity.

c. Triton X-100 and Sodium Dodecyl Sulfate

Carboxydismutase (0.08 mg/ml) was stored in the presence of 0.05, 0.01, and 0.002% Triton X-100 (Rohme and Haas), and also of 10^{-3} M sodium dodecyl sulfate. The control enzyme solution and those solutions containing 0.01 and 0.002% Triton X-100 all lost 60% of their initial activities over a period of 160 h. However, the solution containing 0.05% Triton X-100 lost only 25% of its initial activity in the same period. This same solution did not change in activity during the first 48 h of the experiment. Sodium dodecyl sulfate completely destroyed the enzymic activity in less than 1 h.

d. Ammonium Sulfate and Sodium Chloride

Carboxydismutase (0.3 mg/ml) was stored in solutions containing each of the following: (a) 0.25 M NaCl, (b) 0.083 M NaCl, (c) 0.25 M $(NH_4)_2SO_4$, (d) 0.083 M $(NH_4)_2SO_4$. Each solution also contained 0.01 M tris, pH 7.6, reduced glutathione, 10^{-3} M, and Versenol, 10^{-3} M. A control solution of the enzyme containing only tris, glutathione, and Versenol was also stored under the same conditions. After 48 h, 1-ml samples of each of the five solutions were withdrawn and dialyzed overnight against 0.01 M tris, pH 7.6, containing reduced glutathione, 10^{-3} M, and Versenol, 10^{-3} M at 4°. Samples of each of the five dialyzed and five undialyzed solutions were assayed 74 h after the experiment had begun. Figures 11-4 and 11-5 show the variation of carboxydismutase activity with time, and following dialysis, for enzyme solutions containing sodium chloride and ammonium sulfate, respectively. Clearly, although the presence of ammonium sulfate decreases the activity of the enzyme, the latter is completely restored following removal of the ammonium sulfate by dialysis, indicating that this salt stabilizes the enzyme against irreversible deactivation. The presence of sodium chloride also decreases the activity of the enzyme, although not as rapidly as does ammonium sulfate; with the latter the maximum effect was evident only 1 min after mixing. However, when the sodium chloride was removed by dialysis, the enzyme did not return to full activity. Sodium chloride, then, does not stabilize the enzyme against irreversible deactivation. It remains to be determined whether the anions or the cations, or their particular combinations, are responsible for these salt effects.

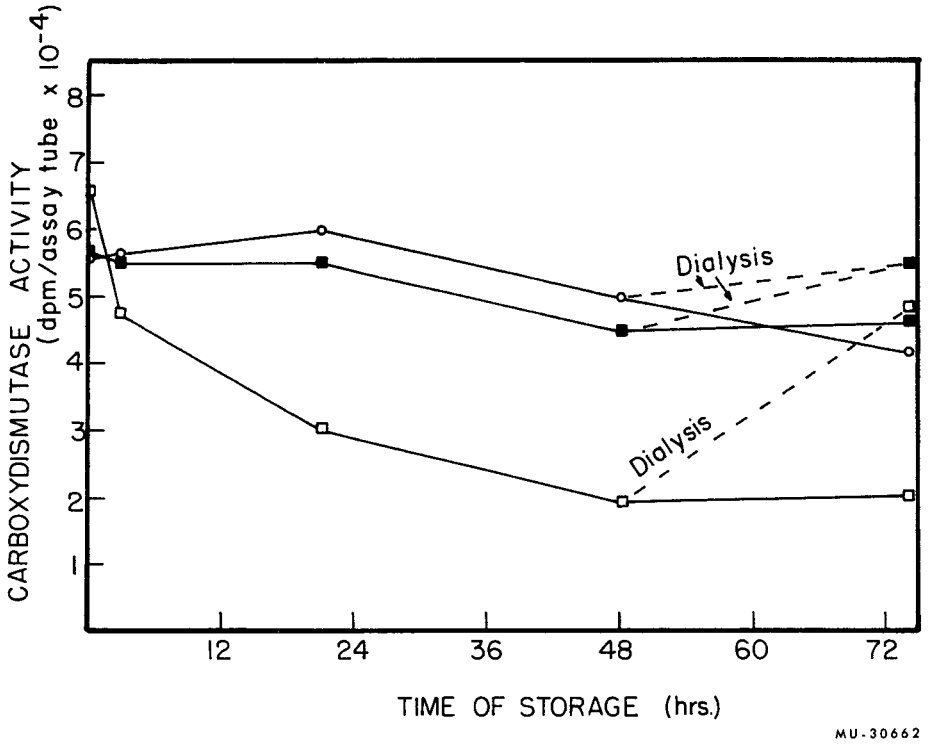


Fig. 11-4. Effect of sodium chloride on the stability and activity of carboxydismutase: open squares, 0.25 M NaCl; closed squares, 0.083 M NaCl; open circles, control.

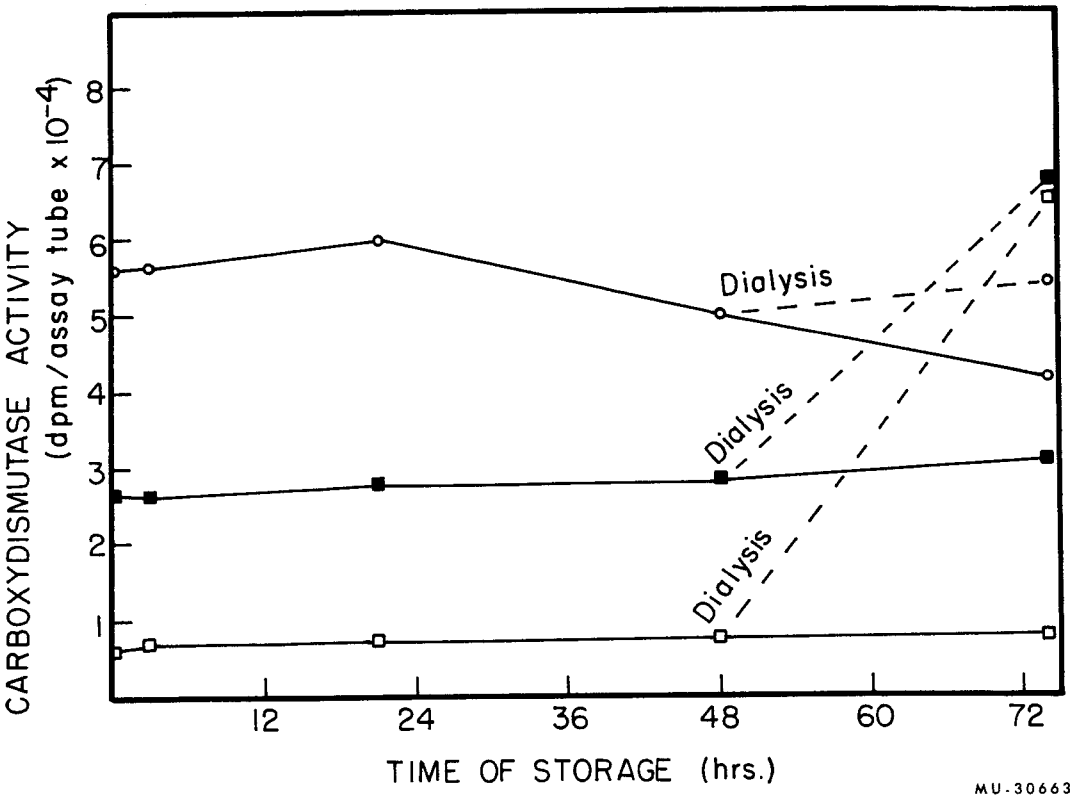


Fig. 11-5. Effect of ammonium sulfate on the stability and activity of carboxydismutase: open squares, 0.25 M $(NH_4)_2SO_4$; closed squares, 0.083 M $(NH_4)_2SO_4$; open circles, control.

12. ELECTRON MICROSCOPY OF CHLOROPHYLL A CRYSTALS

Terry Trosper and Roderic B. Park

Microcrystals of chlorophyll a in 100% isooctane were obtained from A. F. H. Anderson.[1] The isooctane suspension was centrifuged for 5 min at 160 × g. A drop of the supernatant suspension was placed on carbon-covered grids and air dried. The grids were shadowed with uranium metal by means of a Mikros VE-12 vacuum evaporator and a shadowing angle given by $\tan\theta = 1/4$. Micrographs were taken on an Akashi electron microscope TRS-50 at magnifications varying from 8,000 to 16,000 diameters.

The micrograph (Fig. 12-1) is a representative example of the type of microcrystal observed. The scale of the enlargement is 100 Å = 0.8 mm. Measurement of the shadow length indicates a specimen thickness of 20 to 30 Å. The fact that the uneven carbon film shows through the microcrystal is a further indication of the thinness and flexibility of the specimen.

The dimensions of the chlorophyll molecule[2] and those observed for the microcrystal suggest that a double-layer model be used for a description of crystal structure.[3]

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1. A. F. H. Anderson and Melvin Calvin, Nature 194, 285 (1962).

2. E. I. Rabinowitch, Photosynthesis. I. (Interscience Publishers, New York, N. Y., 1945).

3. A. F. H. Anderson, to be published in Nature.

ZN-3900

Fig. 12-1. Electron micrograph of chlorophyll a microcrystal.

13. THE POSSIBLE ROLE OF CHROMANYL PHOSPHATES IN OXIDATIVE AND PHOTOSYNTHETIC PHOSPHORYLATION

Peter M. Scott

Introduction

Plastoquinone,[1] vitamin K_1,[2,3] and possibly coenzyme Q[4,5] are lipophilic quinones believed to be associated with phosphorylation coupled to electron transport.[6,7] (These compounds are designated, respectively, Ia, II, and Ib in Fig. 13-1.)

In the case of vitamin K_1, anaerobic incubation of the quinone with an electron donor and a Mycobacterium phlei preparation yielded a reduced phosphorylated derivative,[2] which underwent enzymatic auto-oxidation to vitamin K with release of inorganic phosphate; this was stated to be a naphthochromanyl phosphate. The 6-chromanyl phosphate (III) of vitamin $K_{1(20)}$ has been synthesized,[8] and shown to undergo enzymatic oxidation by cytochrome c (with ATP formation) in a M. phlei extract. Mono-ethyl menadiol-1-phosphate can also serve as substrate for oxidative phosphorylation in the bacterial system.[9]

Phosphorylated chromanols of coenzyme Q and plastoquinone have neither been isolated from nor applied to an extract of their respective systems; in fact, they have not been synthesized. However, the chemical relationship of these quinones to vitamin K, especially the 2, 3-unsaturation in the isoprenoid side chain which permits reductive cyclization to the chromanol, suggests the possible existence of such an intermediate.

A mechanism whereby a chromanyl phosphate might be oxidized, with accompanying phosphorylation of ADP, has been proposed.[10] This involves ring-opening of the chromanyl phosphate (IV) (Fig. 13-2) to a hydroquinone phosphate (V); the latter is then oxidized to a quinone (VI or VIII). The oxidation of hydroquinone phosphates has been realized chemically[11] and is very facile with such oxidizing agents as bromine and ceric sulphate. It seemed desirable, therefore, to examine the chemistry of chromanyl phosphates, especially their oxidation, and some preliminary results (mainly negative) are reported here.

1. D. W. Krogmann and E. Olivero, J. Biol. Chem. 237, 3292 (1962).
2. P. J. Russell and A. F. Brodie, Biochim. Biophys. Acta 50, 76 (1961).
3. R. D. Dallam and W. W. Anderson, Biochim. Biophys. Acta 25, 439 (1957).
4. H. Rudney, J. Biol. Chem. 236, PC39 (1961).
5. Y. Hatefi, Biochim. Biophys. Acta 31, 502 (1959).
6. A. F. Brodie, Fed. Proc. 20, 995 (1961).
7. D. I. Arnon, Fed. Proc. 20, 1012 (1961).
8. A. Asano, A. F. Brodie, A. F. Wagner, P. E. Wittreich, and K. Folkers, J. Biol. Chem. 237, PC2411 (1962).
9. A. F. Brodie and B. R. Davies, Fed. Proc. 18, 198 (1959).
10. M. Vilkas and E. Lederer, Experientia 18, 546 (1962).
11. V. M. Clark, D. W. Hutchinson, G. W. Kirby, and A. Todd, J. Chem. Soc (1961), p. 715.

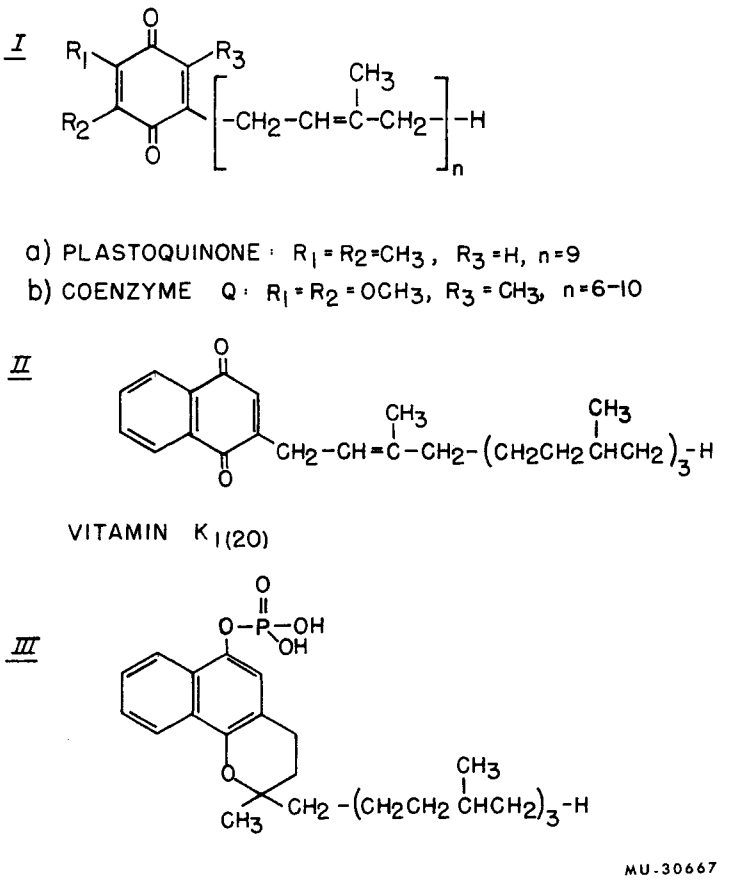

Fig. 13-1. Formulae of plastoquinone, coenzyme Q, and vitamin $K_{1(20)}$.

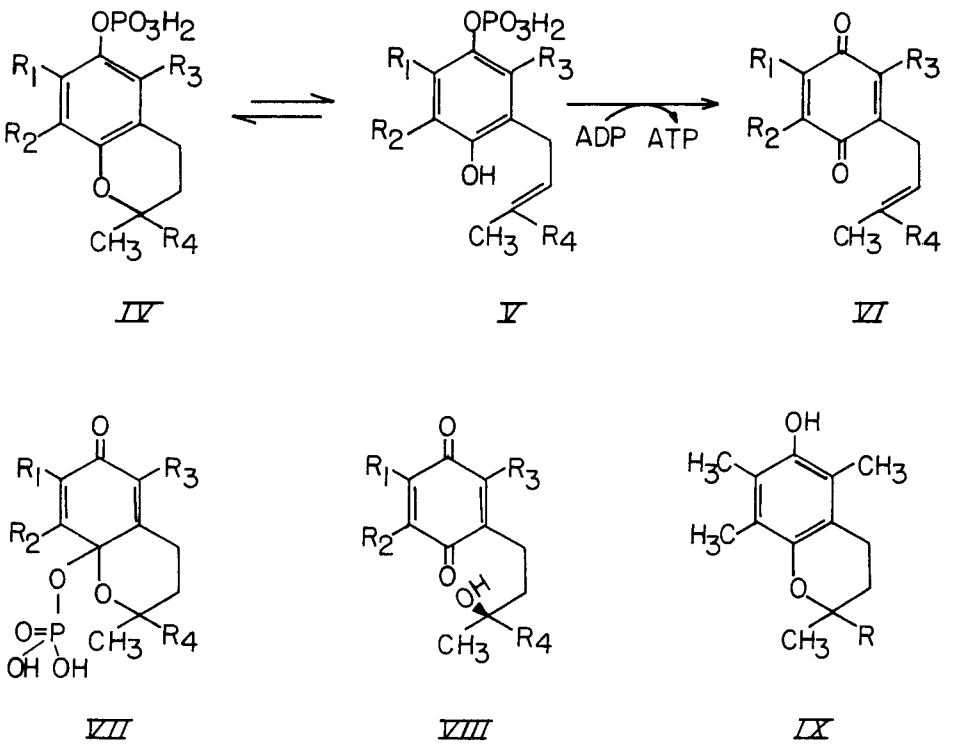

Fig. 13-2. Formulae of chromanyl phosphate (IV) hydroquinine phosphate (V), quinone (VI and VIII), diene-one phosphate (VII), and 2, 2, 5, 7, 8-pentachroman-6-ol (IX).

The diene-one phosphate (VII) (which has not yet been prepared, and would be expected to be very labile) has also been postulated as a possible quinone-phosphate derivative.[12, 13]

Results

Two chromanyl phosphates were used: d, 1-α-tocopheryl phosphate (IV: $R_1=R_2=R_3=CH_3$, $R_4=C_{16}H_{33}$) and 2, 2, 5, 7, 8-pentamethylchromanyl-6-phosphate (IV: $R_1=R_2=R_3=R_4=CH_3$). Both compounds were stable under acidic and alkaline conditions, but were rapidly oxidized by acidic ceric sulphate to the quinone (VIII: $R_1=R_2=R_3=CH_3$, $R_4=CH_3$ or $C_{16}H_{33}$) also obtained by oxidation under milder conditions of the corresponding chromanol (IX: $R=CH_3$ or $C_{16}H_{33}$). Since this would not appear to be an oxidation following a hydrolysis, it is possible that the equilibrium IV ⇌ V is established with immediate oxidation of the hydroquinone phosphate. Such an equilibrium would favor the chromanol form, at least at low pH, since 2, 2, 5, 7, 8-pentamethylchroman-6-ol (IX: $R=CH_3$) (Fig. 13-2) can be obtained in good yield from the hydroquinone of the quinone VIII ($R_1=R_2=R_3=R_4=CH_3$) under mildly acidic conditions.[14] Deuterium exchange studies should detect this equilibrium if it exists; observations so far show no incorporation of deuterium from heavy water into the heterocyclic ring of the chromanyl phosphate (IV: $R_1=R_2=R_3=R_4=CH_3$).

Other oxidizing agents, bromine in organic solvents (including acetic acid), ferric chloride, and auric chloride, do not react with the chromanyl phosphates, at least not under conditions in which they oxidize hydroquinone phosphates.[11] Unless naphthochromanyl phosphates (III) are more readily oxidized chemically, the statement by Brodie[6] that the bacterial naphthochromanyl phosphate could be oxidatively dephosphorylated by bromine in acetic acid is in disagreement with this.

With bromine in water, a rapid reaction occurs, the nature of which is not yet clear (for details, see Experimental section).

Experimental Procedure

Infrared, ultraviolet, and NMR (nuclear magnetic resonance) spectra were recorded on Beckman IR-7, Carey Model 14, and Varian A-60 spectrophotometers, respectively.

d, 1-α-Tocopheryl phosphate (IV: $R_1=R_2=R_3=CH_3$, $R_4=C_{16}H_{33}$) disodium salt

This compound is supplied by Calbiochem and has the following properties:

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12. V. M. Clark and A. Todd, Ciba Foundation Symposium on Quinones in Electron Transport (1961), p. 190.

13. R. D. Dallam, Biochem. Biophys. Res. Comm. 4, 106 (1961).

14. L. I. Smith, P. M. Ruoff, and S. Wawzonek, J. Org. Chem. 6, 236 (1941).

uv λ_{max} (H_2O): 286 ($E^{1\%}_{1\,cm}$ 39.4), 280 (shoulder) mμ;
ir ν_{max} (Nujol mull): 3300 cm^{-1} (OH, water probably present).
NMR (D_2O): broad peaks at τ = 7.8, 8.7, 9.0 (H_2O internal reference).
Molecular weight (titration with 0.01 N HCl): mean of four determinations = 614 (mean deviation 14) (calc for $C_{29}H_{49}O_5PNa_2$, 554.7).
Paper chromatography (free phosphate): With Whatman No. 1 paper and pyridine: isobutanol: acetic acid: water (20:30:5:50), Rf = 0.96;[15] with alumina-impregnated paper, Rf = 0 (benzene or chloroform). Detection was with ferric chloride-sulphosalicylic acid.[16]
Stability: The compound had quantitatively identical uv spectra at several pH's ranging from 5.4 to 11.3. No change was observed (uv paper chromatography) after heating (100°) for 1 h in buffer pH 11.3, and similar treatment with 10% KOH solution also produced no change in absorption spectrum. An aqueous alcoholic HCl solution (pH 1.1, λ_{max} 284 mμ) showed no change after refluxing for 40 min.

Oxidation of d, 1-α-tocopheryl phosphate

(a) Bromine

Optical scale: Treatment of d, 1-α-tocopheryl phosphate disodium salt in buffer, pH 7.0, with one drop of saturated bromine water produced a rapid change; the peak at 286 mμ was replaced by a peak at 260 mμ (approx six-fold increase in optical density (O. D.).
Large scale: The phosphate salt (145 mg) in phosphate buffer, pH 7.0 (30 ml), was treated dropwise with saturated bromine water (6 ml). After 5 min the solution was acidified and extracted with ether, the ether layer was washed well with water, dried (with Na_2SO_4), and the solvent evaporated to give a pale-yellow oil (135 mg) (ν_{max}: 1765, 1700, 1650 cm^{-1}) which did not contain α-tocopherylquinone (paper chromatography) but did contain phosphate (Wade spray[16]). It had initial λ_{max} at 259 mμ (ether), but was very unstable, and after a few hours the spectrum of the oil was ill-defined.

The same reaction took place at pH 5.4, but not at pH 11.3.

With the free phosphate in acetic acid, ether, or chloroform, no reaction was observed spectrally upon the gradual addition of a solution of bromine in the organic solvent.

(b) Silver oxide

d, 1-α-tocopheryl phosphate in ether was not oxidized by dry silver oxide (no spectral change after 1.5 h shaking).

(c) Auric chloride

d, 1-α-tocopheryl phosphate disodium salt (7.3 mg) in buffer, pH 7.0, (3 ml) was treated with auric chloride solution (0.1 N, 0.26 ml). After 20 min the

15. F. Navazio, Boll. Soc. Ital. Biol. Sper. 31, 550 (1955).
16. H. E. Wade and D. M. Morgan, Nature 171, 529 (1953).

solution was acidified and extracted with cyclohexane. The spectrum was the same as that of the starting material.

(d) Ceric sulphate

d, l-α-tocopheryl phosphate (from 62 mg disodium salt) was shaken with 95% ethanol (20 ml), 0.1 M ceric sulfate in 0.1 N H_2SO_4 (12 ml) and ether (peroxide-free, 100 ml) for 3 min, according to Kofler.[17] The ether layer was washed with water, filtered, diluted to 1 liter with 95% ethanol, and the α-tocopherylquinone estimated spectrally by use of the published extinction value at 268 mμ[18] (approx 47% conversion). The α-tocopheryl-quinone was identified (after purification on an alumina column) by paper chromatography, uv spectrum, and ir spectrum (ν OH 3500 cm^{-1}).

2, 2, 5, 7, 8-Pentamethylchroman-6-ol (IX: R=CH_3)

Trimethylhydroquinone, prepared by oxidation of 2, 3, 5-trimethyl-phenol with Fremy's salt[19] followed by reduction (sodium dithionite), was treated with isoprene under acidic conditions according to Smith et al.[20] The crude chromanol was purified either by chromatography on alumina (Merck) in petrol-ether (bp 30-60°) and elution with ether-methanol (8:1), or by extraction of the chromanol from a solution in petrol-ether (bp 30-60°) with 10% NaOH in methanol-water (1:). It had mp 95 to 96.5° (lit.[21] 95.5-97°); λ_{max} (95% ethanol) 290 mμ (ϵ = 3170).

2, 3, 5-Trimethyl-6-(3'-methyl-3'-hydroxybutyl)-1, 4-benzoquinone (VIII: $R_1=R_2=R_3=R_4=CH_3$)

A solution of 2, 2, 5, 7, 8-pentamethylchroman-6-ol in ethanol was oxidized by ferric chloride[22] to give the quinone as an orange oil. It had λ_{max} (95% ethanol): 263, 268 ($E^{1\%}_{1cm}$ 765, lit[21] 783), 342 ($E^{1\%}_{1cm}$ 11) mμ; λ_{max} (cyclohexane): 260, 268, 332 mμ; ν_{max} 3550 (OH), 1650 (C=O) cm^{-1}; Rf (paper chromatography on alumina paper, Schleicher and Schüll 288) = 0.47 (benzene/chloroform 1:1) and 0.05 (benzene). Intensive uv irradiation in 95% ethanol (quartz cuvette) destroys the quinone in 5 min, but in a glass vessel (cyclohexane as solvent) it is quite stable (uv spectrum) for at least 30 min.

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17. M. Kofler, Helv. Chim. Acta 30, 1053 (1947).

18. M. Tishler and N. L. Wendler, J. Am. Chem. Soc. 63, 1532 (1941).

19. H. J. Teuber and W. Rau, Chem. Ber. 86, 1036 (1953).

20. L. I. Smith, H. E. Ungnade, H. H. Hoehn, and S. Wawzonek, J. Org. Chem. 4, 311 (1939).

21. P. Schudel, H. Mayer, J. Metzger, R. Ruegg, and O. Isler, Helv. Chim. Acta 46, 636 (1963).

22. W. John, E. Dietzel, and W. Emte, Z. Physiol. Chem. 257, 173 (1939).

2, 2, 5, 7, 8-Pentamethylchromanyl-6-phosphate (IV: $R_1=R_2=R_3=R_4=CH_3$)

A solution of 2, 2, 5, 7, 8-pentamethylchroman-6-ol (1.0 g) in dry pyridine (5 ml) was cooled and added to a solution of phosphorus oxychloride (1.2 ml) in dry pyridine (5 ml). The mixture was left overnight at room temperature, then poured into water, acidified to pH 1, and the precipitate (1.0 g) collected; if a gummy solid formed, this was extracted into ether. Recrystallization from ethyl acetate (twice), and then dioxan gave needles, mp 150-152° (softening from 130°) (found on a sample dried at 100°/2 mm: C, 58.4; H, 7.0; P, 9.1; N, 2.2 $C_{14}H_{21}O_5P.(1/2)C_5H_5N$ requires C, 58.3; H, 7.0; P, 9.3; N, 2.1%), λ_{max} (95% ethanol): 245, 252, 258, 264 (pyridine) and 280 (shoulder), 286 mμ.

An ether solution of the crude chromanyl phosphate was washed with dilute hydrochloric acid and water, then dried, and the ether removed. Treatment of an ethanolic solution of the residue with the calculated quantity of aqueous NaOH, then cooling, gave the monosodium salt, which crystallized from aqueous ethanol as needles, mp > 300° (found on a sample dried at 100°/10^{-3} mm: C, 51.9; H, 6.2; P, 9.9. $C_{14}H_{20}O_5PNa$ requires C, 52.2; H, 6.3; P, 9.6%), λ_{max} (water): 284 (ϵ = 1770) mμ, ν_{max} (Nujol): 3400 cm^{-1} (broad, OH). The salt could not be titrated by either 0.01 N HCl or 0.01 N NaOH, and gave a solution with pH 6.3 (17.8 mg/6 ml water). No change in the ultraviolet spectrum was seen on heating (100°, 1 h) in buffers at pH 5.4 or 11.3, or in 5% KOH solution.

Treatment of an ethanolic solution of the crude phosphate with excess ethanolic NaOH solution yielded the disodium salt [recrystallized from aqueous ethanol, it formed glistening plates which lost weight (approx 30%) on being dried at 100°/0.6 mm. Found: C, 46.2; H, 6.2; P, 8.7%; M, by titration with 0.01 N HCl, 360. $C_{14}H_{19}O_5PNa_2 \cdot H_2O$ requires C, 46.4; H, 5.8; P, 8.6%; $\bar{M}$, 362.3].

λ_{max}(water): 284 (ϵ = 2300) mμ. ν_{max}: 3000 cm^{-1} (broad, OH). NMR spectrum (D_2O), τ = 7.28 - 7.62(2), 7.81(6), 7.98(3), 8.11 - 8.45(2), 8.78(6), using H_2O as internal reference (τ = 5.28); the number of protons involved is shown in parentheses. No incorporation of deuterium was observed even after 50 h.

A solution of 16.5 mg of the disodium salt in 5 ml of water had pH 8.5.

The free phosphate was obtained by acidification of an aqueous solution of the disodium salt, and after recrystallization from ethyl acetate/petrol ether (bp 30 to 60°) had mp 200-205° (found on a sample dried at 100°/1 mm: C, 55.9; H, 7.0; P, 10.2. $C_{14}H_{21}O_5P$ requires C, 56.0; H, 7.0; P, 10.3%), Rf 0.78 (Whatman No. 1 paper, ethanol/water 2:9).

Oxidation of 2, 2, 5, 7, 8-pentamethylchromanyl-6-phosphate

(a) Bromine water (Fig. 13-3)

An aqueous solution of the monosodium salt was treated with one drop of saturated bromine water that was freshly prepared. The spectrum

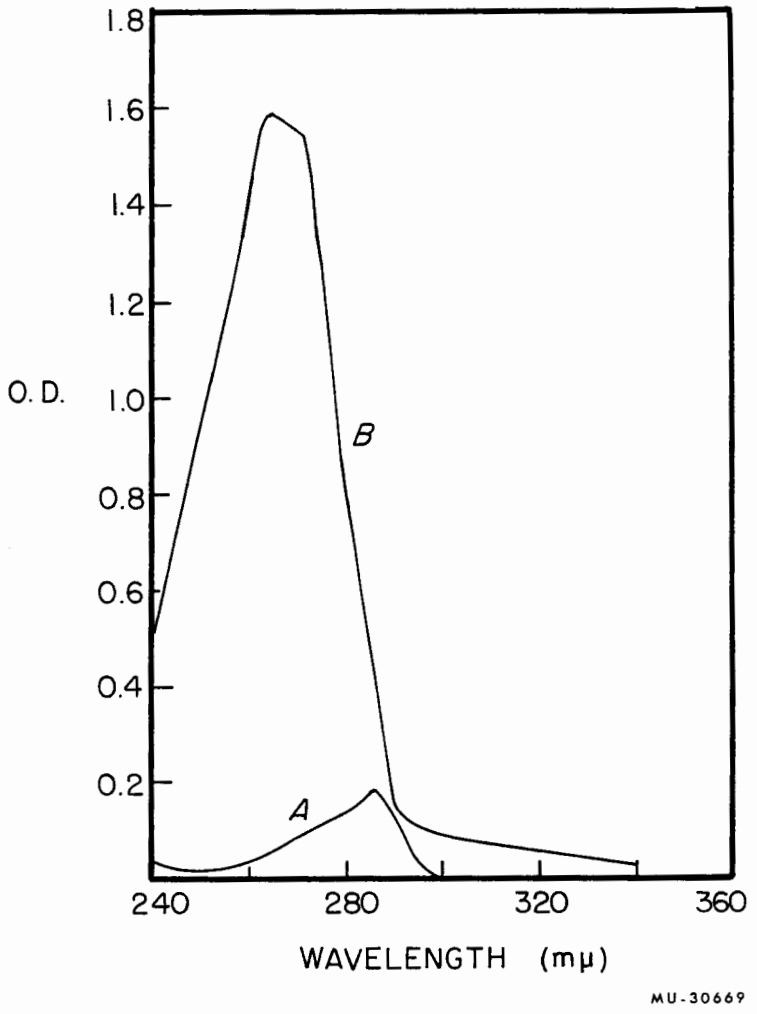

Fig. 13-3. Absorption spectrum of 2, 2, 5, 7, 8-pentamethylchromanyl-6-phosphate (di-sodium salt, in water).
A: pure salt.
B: After addition of 1 drop of bromine water (saturated).

changed at once to λ_{max} = 265 mμ (8-10 fold increase in O.D.). Addition of $NaBH_4$ gave λ_{max} = 281 mμ (same O.D. as original solution), and the change to λ_{max} = 265 mμ was repeated with more bromine water. Precisely the same changes were observed with the disodium salt. The product contains phosphate, and no free orthophosphate is formed (Hanes-Isherwood spray[23]).

(b) Bromine in acetic acid

A solution of the disodium salt in glacial acetic acid (λ_{max} 285 mμ, O.D. 0.19) was treated dropwise with bromine in acetic acid (0.6%). No change in the spectrum was observed other than the small increase in absorption due to bromine.

(c) Ferric chloride

A solution of the monosodium salt in water, treated with dilute aqueous ferric chloride solution, showed no spectral change after allowance for $FeCl_3$ absorption.

(d) Ceric sulphate

2, 2, 5, 7, 8-Pentamethylchromanyl-6-phosphate (containing 0.5 molecule pyridine) (7.28 mg) in 95% ethanol (3 ml), was shaken with peroxide-free ether (15 ml) and ceric sulphate solution (approx M in N H_2SO_4, 2 ml) for 4 min. The ether layer was separated, washed twice with water (no phosphate could be detected in the organic phase), and diluted to 250 ml with 95% ethanol [λ_{max} 263 mμ, 268 mμ (O.D. 1.24)]. The product was identified as 2, 3, 5-trimethyl-6-(3'-methyl-3'-hydroxybutyl)-1, 4-benzoquinone (VIII: $R_1=R_2=R_3=R_4=CH_3$), formed in approximately 78% yield.

Similar experiments using the monosodium salt and the free phosphate gave the same quinone (yields 39% and 69% respectively).

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23. C. S. Hanes and F. A. Isherwood, Nature 164, 1107 (1949).

14. OXIDATION-REDUCTIONS OF SOME COENZYMES

G. K. Radda

The work presented here is part of a program aimed at elucidating the mechanism of the photoreduction of flavin adenine mononucleotide (FMN) and of its analogs. A kinetic study of the intramolecular photorearrangement described earlier[1] is not very likely to yield enough information about the mechanism of the reaction, as it is impossible to vary the concentration of the reducing agent (the side chain of FMN), and because it results in the destruction of the flavin nucleotide. It is desirable, therefore, to use an external reducing agent in these studies. Ethylenediaminetetraacetic acid has been shown to be an effective photochemical reducing agent,[1] but our lack of knowledge of its oxidation products is again a complicating factor. Recent qualitative observations[2, 3] that reduced diphosphopyridine nucleotide (DPNH) is a good donor towards photochemically excited FMN offered a promising approach to kinetic studies.

When a solution of FMN (0.3×10^{-4} M) is mixed with approximately 10-fold excess DPNH under anaerobic conditions, a slow decrease is observable in the 447 mμ absorption of FMN and in the 340 mμ absorption of DPNH. This decrease is considerably enhanced by white light (curves 1 and 2 in Fig. 14-1). This indicates that DPNH reacts with FMN to give dihydroflavin and oxidized pyridine nucleotide (DPN). That the "dark" reaction is not in fact a photoreduction induced by the monochromatic light of the spectrophotometer is shown on curve 2 in Fig. 14-1, as the reaction proceeds at the same rate when the spectrophotometer is turned off. Previous reports stated that no such dark reaction takes place under anaerobic conditions,[3, 4] while the reaction was shown to go in air.[5, 6] This report deals only with this dark reaction.

Kinetics of the Dark Reaction

The cell compartment of the apparatus used previously[1] was modified for this work so as to allow accurate temperature control during the reaction. This was achieved by circulating water from a thermostat through glass jackets placed at two sides of the photometric cell. The temperature was monitored inside the cell by a thermocouple (Fig. 14-2). It was shown that the presence of the thermocouple in the cell did not affect the reaction rates.

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1. G. K. Radda, in Bio-Organic Chemistry Quarterly Report, UCRL-10743, March 1963, p. 37.

2. L. P. Vernon, Biochim. Biophys. Acta 36, 177 (1959).

3. W. R. Frisell and C. G. Mackenzie, Proc. Natl. Acad. Sci. U. S. 45, 1568 (1959).

4. K. Uehara, I. Muramatsu, and M. Makita, Vitamins (Kyoto) 13, 261 (1957); Chem. Zentr. 129, 9520 (1958).

5. T. P. Singer and E. B. Kearny, J. Biol. Chem. 183, 409 (1950).

6. C. H. Suelter and D. E. Metzler, Biochim. Biophys. Acta 44, 23 (1960).

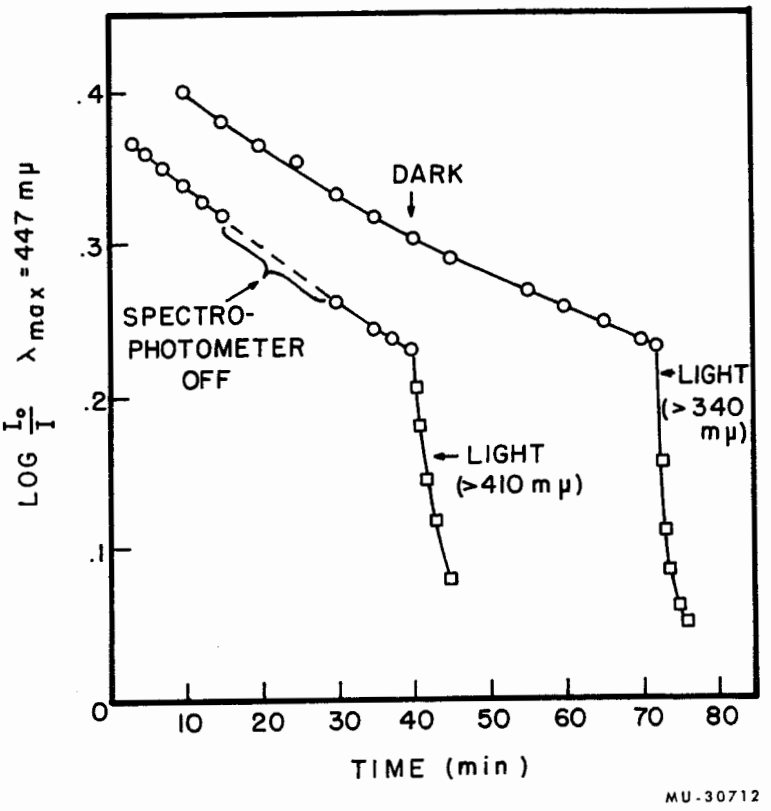

Fig. 14-1. Reaction of DPNH with FMN (in dark, and light-catalyzed). pH = 7, phosphate buffer.

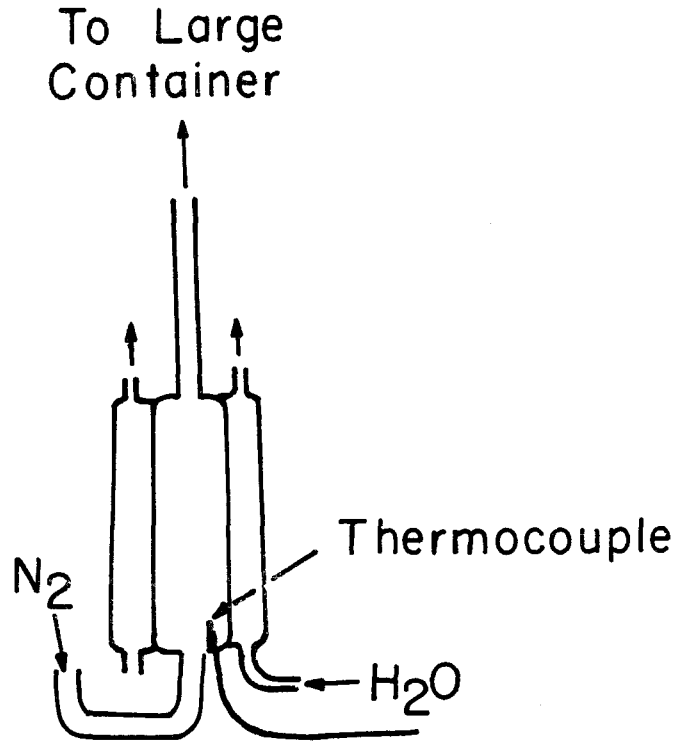

Fig. 14-2. Design for cuvette for anaerobic reaction at controlled temperature.

The solution was brought to the required temperature through a similar arrangement in the prereaction container, designed for deoxygenation, which was in a closed circuit with the cuvette. The temperature was kept with ± 1°. Concentrated solutions of DPNH were added after 1 h, during which time nitrogen was bubbled through the solution of FMN. Excess of DPNH was used in all runs, and the results gave good pseudo first-order plots in FMN. When the concentration of DPNH (still in excess) was varied, a plot of the pseudo-first-order rate constants vs DPNH concentration gave a good straight line, showing the reaction to be second order (Fig. 14-3). The results (calculated by the method of least squares) are tabulated in Table 14-I, together with the second-order rate constant at 37°. The temperature dependence of the reaction was measured similarly (Table 14-II). The Arrhenius activation energy (calculated from Fig. 14-4) is 8.3 ± 0.5 kcal/mole. The activation entropy can be obtained indirectly from the same graph from the intercept at $1/T = 0$. It is about -7 eu, although it is subject to large errors because of the necessary extent of extrapolation.

Table 14-I. Pseudo-first-order rate constants for FMN + DPNH reaction at pH = 7, 0.1 ionic strength, 37°

Concentration of DPNH (mole/liter)	Rate constant (min^{-1})
1.085×10^{-3}	0.0116
0.868×10^{-3}	0.0089
0.651×10^{-3}	0.0072
0.434×10^{-3}	0.0053
0.217×10^{-3}	0.0014

From these, k_2 (second-order rate constant) = 0.159 liter/mole·sec

Table 14-II. Temperature dependence of the reaction. Other conditions as in Table 14-I.

Temperature (°C)	k_2 $\left(\frac{\text{liter}}{\text{mole}\cdot\text{sec}}\right)$
44.0	0.360
36.0	0.149
30.0	0.084
17.0	0.020

Comparison of the Results with Some Enzyme-Catalyzed Reactions

The results show that there is a clean second-order reaction between DPNH and FMN that yields DPN and $FMNH_2$. This is in agreement with a recent study on the reaction of DPN models with riboflavin.[6] Another recent observation that a mixture of DPNH and FMN gives an electron spin resonance (ESR) signal is probably also a result of this reaction and not due to a charge-transfer complex as suggested.[7] Absorption and fluorescence

7. I. Isenberg, S. L. Baird, and A. Szent-Gyorgyi, Proc. Natl. Acad. Scil U. S. 47,245 (1961).

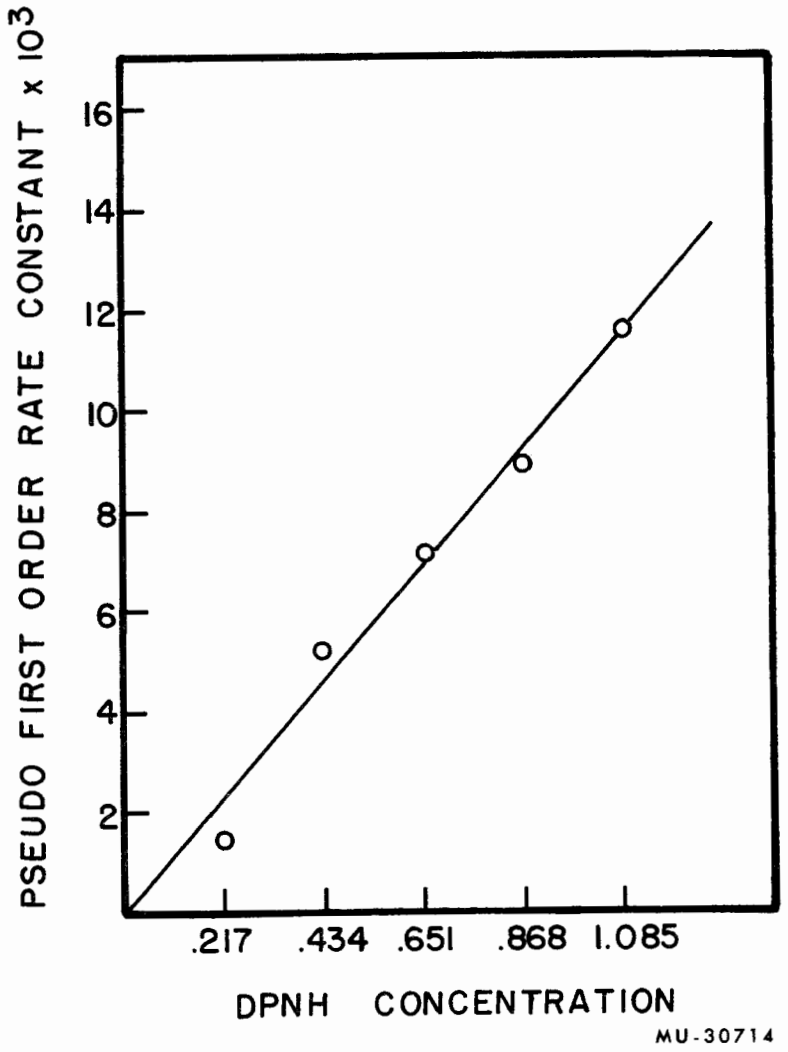

Fig. 14-3. Plot of pseudo-first-order rate constants vs DPNH concentration.

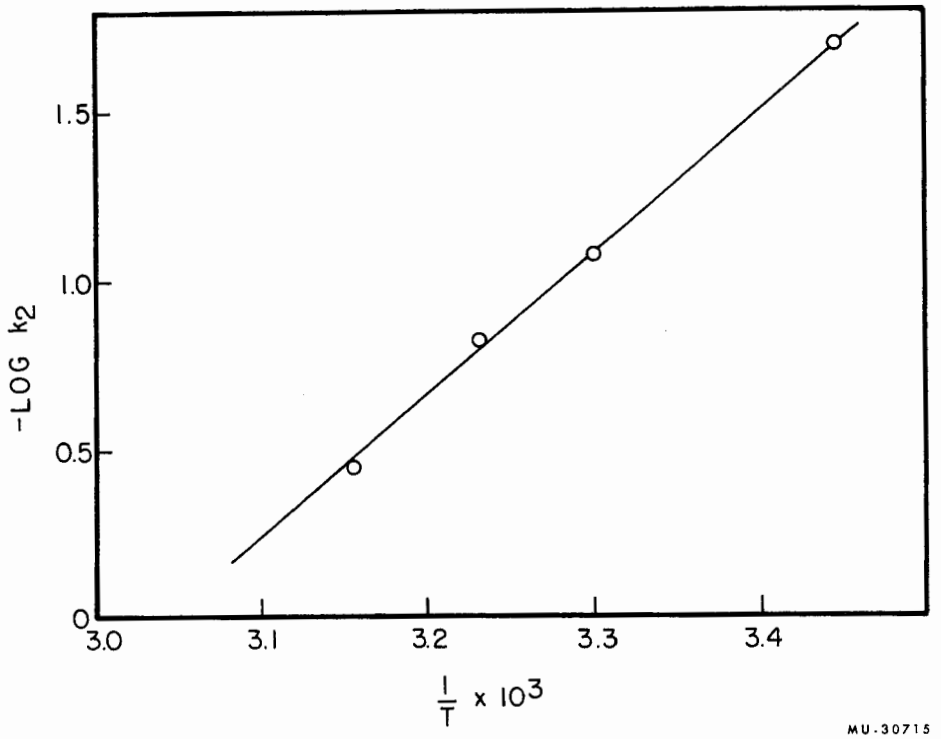

Fig. 14-4. Arrhenius plot for FMN + DPNH reaction.

spectral measurements show no evidence for complexing between DPNH and FMN.

The reaction of these two coenzymes represents a useful "model" for some of the enzyme processes of the electron-transport system, particularly since $FMNH_2$ is a good reducing agent towards Fe^{3+} and Cu^{2+} ions or their complexes (e.g., cytochrome c and hemoglobin in the +3 oxidation states). (These observations will be discussed in detail, together with the light-catalyzed reactions, in a later report.) In this respect it is interesting to compare the model reaction with a recent study on the microsomal cytochrome b_5 reductase system.[8] This enzyme (a flavoprotein) mediates the reduction of cytochrome b_5 by DPNH. The rate-determining step has been shown to be the reduction of the flavoprotein by DPNH.[8] The rate of the nonenzymic reduction of FMN by DPNH under the same conditions as the enzymic process can be computed from the rate data described above. If this rate constant is expressed in the same units as those given for the enzymic reaction (as a pseudo-first-order rate constant), the value is $k = 10^{-6}$ sec^{-1}. The reaction between DPNH and cytochrome b_5 reductase is faster by a factor of about 10^8 or 10^9. In addition, from the temperature dependence of the enzymic process measured by Strittmatter,[8] one can calculate the Arrhenius activation energy of 4.0 kcal/mole. The difference between the activation energy of the model reaction (8.3 kcal/mole) and that for the enzymic reaction accounts for about a 100-fold rate difference only. This leaves a factor of 10^6 to 10^7 to be understood. Koshland pointed out that two other possible factors have to be considered when comparing enzyme reactions with their nonenzymic analogs, namely, the proximity and orientation effects.[9] The former represents an effective increase in concentration due to the accommodation of the reactants on the enzyme surface, while the latter is a stereochemical factor resulting from the alignment of the two reactants in a way that results in reaction on each collision. Both these effects can be calculated from an expression of the form

$$\frac{v_{enzymic}}{v_{model}} = 55.5\,\theta_1 \cdot \theta_2 \frac{[Enzyme]}{[A]\ [B]} ,$$

where θ_1 and θ_2 are assumed to be 10, and [A] and [B] are the concentrations of the two reactants. (For a detailed discussion of the meaning of these factors see Ref. 9.) For our two systems the calculated rate ratios are 10^6 to 10^8. (In this case the calculations are helped by the fact that the binding constant of the enzyme towards DPNH is known.[10]) Hence the proximity and orientation effects can easily account for the remaining difference in reactivities of the enzymic and nonenzymic reactions. These two factors do not affect the activation energy of the reaction, but should contribute almost entirely to the pre-exponential factor of the Arrhenius equation, that is, the entropy of activation. Unfortunately, Strittmatter's data are not sufficient to calculate this entropy.[8]

8. P. Strittmatter, J. Biol. Chem. 237, 3250 (1962).

9. D. E. Koshland, in Advances in Enzymology, F. F. Nord, ed., 22 (Interscience, New York, 1960), p. 45.

10. P. Strittmatter, J. Biol. Chem. 236, 2336 (1961).

Finally, the 4-kcal difference in activation energies for the two systems can be rationalized in terms of electronic factors as follows. It is very likely that the reaction involves a hydride-ion transfer from DPNH to FMN. This is supported by studies of reductions of thioketones by DPNH analogs[11] and by the observation that reduction of riboflavin by DPNH analogs proceeds faster at higher ionic strengths.[6] We would therefore expect that the transition state is of a polar nature (Fig. 14-5), in which the partial negative charge is distributed over the aromatic system of FMN and the positive charge on the ring of the DPN molecule (residing mostly on the N atom, giving a relatively stable electronic arrangement). This positive charge could be further stabilized by groups on the enzyme carrying a negative charge or highly polarizable electrons. It has been shown that an -SH group is involved in the binding of DPNH to cytochrome b_5 reductase,[10] and this group fulfills the above requirements. The effect of an -SH group, when fully bonded to the olefinic system of the reduced DPN, can be calculated from the Hammett equation as modified by Brown[12] for reactions involving positive centers in conjugation with the substituent. (In these calculations a σ^+ value of -0.7 was used for the -SH group and a ρ value of -3.0 was assumed, this being reasonable on the basis of analogous reactions.[13, 14]) From these values the logarithm of the rate ratios is $\log k_{enzyme}/k_{model} = 2.1$, hence the reaction in the presence of an -SH group would be 10^2 times as fast as in its absence. Although on the enzyme the $-S^-$ (or SH) group may not be completely bonded to the DPNH ring, one will lose only a small effect, since the sulfur atom is highly polarizable and is therefore capable of delocalizing its electrons over a large distance. The stabilization of the transition state by an $-S^-$ group is represented in Fig. 14-6. Further studies on the enzymic process, and in particular the evaluation of the entropy of activation, may contribute to a quantitative understanding of the reaction when compared to the model system.

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11. R. H. Abeles, R. F. Hutton, and F. H. Westheimer, J. Am. Chem. Soc. 79, 712 (1957).

12. H. C. Brown and Y. Okamoto, J. Am. Chem. Soc. 80, 4979 (1958).

13. P. B. D. De la Mare, J. Chem. Soc. 3823 (1960).

14. L. M. Jackman and D. T. Thompson, J. Chem. Soc. 4794 (1961).

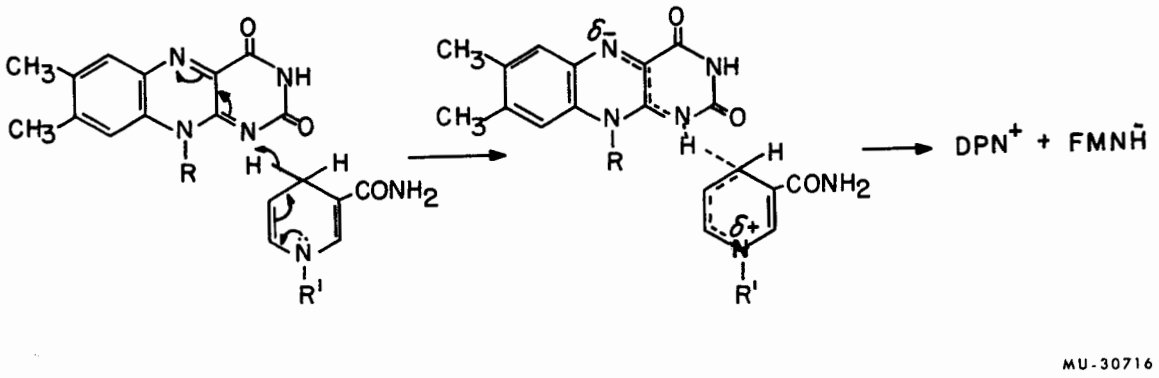

Fig. 14-5. Mechanism of FMN + DPNH reaction.

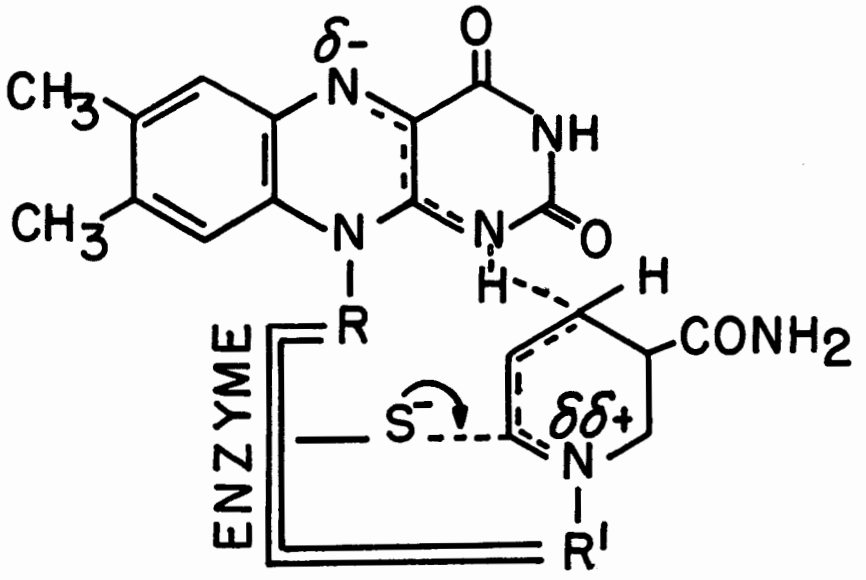

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Fig. 14-6. Stabilization of the transition state by an $-S^-$ group on enzyme surface.

15. PREPARATION OF SOME [^{14}C] LABELED SUBSTANCES: GLUCOSE-β-PHOSPHATE, FRUCTOSE-6-PHOSPHATE, 6-PHOSPHOGLUCONIC ACID, PYRUVIC ACID, AND SUCCINIC ACID

V. Moses and Julia Chang

For use in metabolic experiments with chick fibroblasts, certain [^{14}C] substrates were required which either were entirely unavailable commercially, available only at low specific radioactivity, or not available with the desired labeling pattern. Some of these substances have now been prepared on a microscale in the laboratory, and instructions for their preparation are presented in this report. The methods used are adaptations of procedures previously published, and are reported here as a record of their successful employment in our hands.

[^{14}C]Glucose-6-Phosphate

Starting material: [^{14}C]glucose, specific activity 107.1 μC/μmole.

[^{14}C]glucose (112 μC) was purified chromatographically on Whatman No. 4 paper using ethyl acetate:pyridine:water (10:4:3 v/v/v) and butan-1-ol: ethanol:water (26:16:9 v/v/v). After location by radioautography, the [^{14}C] glucose was eluted from the paper with water and lyophilized.

The [^{14}C] glucose was dissolved in 200 μl of the following solution:

0.1 M ATP, Na_2		0.5 ml
0.006% cresol red + 1.6% $MgCl_2 \cdot 6\ H_2O$		2.3 ml
0.1 N NaOH added until solution reddish purple in color		
0.1 M glycylglycine buffer, pH 9		1.0 ml
water	to	30.0 ml

To the solution of [^{14}C] glucose in this mixture was added 10 μl of a solution of crystallized yeast hexokinase. This hexokinase, obtained originally from Calbiochem and suspended in 3 M $(NH_4)_2SO_4$ solution, was dialyzed by C. Prevost against 0.1 M tris buffer, pH 8, and after dialysis contained 2.9 mg enzyme protein per ml. The specific activity of the enzyme was reported by the manufacturers to be 140 enzyme units per mg, as defined below.

The mixture was incubated for 75 min at 37°, then chromatographed in toto for 20 h as a band on oxalic acid-washed Whatman No. 4 paper by use of butan-1-ol:propionic acid:water (46.8:32.5:30.7 v/v/v). Two products were obtained, which were shown by hydrolysis with prostatic acid phosphatase and further chromatography with authentic markers to be [^{14}C] glucose-6-phosphate (about 80% of total) and [^{14}C]fructose-6-phosphate (20%). Very little of the original [^{14}C]glucose remained unchanged. The presence of [^{14}C]fructose-6-phosphate is presumably due to phosphohexose isomerase which was known to be a contaminant in the hexokinase preparation.

[^{14}C]Fructose-6-Phosphate

Starting material: [^{14}C]fructose, specific activity 107.1 μC/μmole.

[^{14}C]Fructose (107 μC) was purified in the same way as [^{14}C]glucose (v. sup.). The reaction conditions were identical with those used above, except that 30 μl of hexokinase solution was used instead of 10 μl. Again there were two products due to phosphohexose isomerase: [^{14}C]fructose-6-phosphate (20% of total) and [^{14}C]glucose-6-phosphate (80% of total).

6-Phospho[^{14}C]gluconic Acid

Starting material: [^{14}C]glucose-6-phosphate, specific activity, 107.1 μC/μmole.

[^{14}C]Glucose-6-phosphate (52 μC), prepared as above, was lyophilized and redissolved in 150 μl of the following solution, followed by 0.1 N NaOH until the solution was reddish purple:

TPN (Na salt) (87 mg/ml)	30 μl
0.006% cresol red + 1.6% $MgCl_2 \cdot 6\ H_2O$	75 μl
0.1 M glycylglycine buffer, pH 9	30 μl
0.1 N NaOH added until solution reddish purple in color	
water	to 250 μl

To this solution was added 25 μl of yeast glucose-6-phosphate dehydrogenase solution (1 mg/ml in 3.3 M $(NH_4)_2SO_4$, pH approximately 6; from Calbiochem; the specific activity of this enzyme was reported by the manufacturers to be 70 units/mg, defined as μmole conversion of substrate per min per mg enzyme protein). The pH was again adjusted with 0.1 N NaOH, to give a reddish-purple solution. The mixture was incubated at 37° for 3 h and chromatographed in toto on unwashed Whatman No. 4 paper in one dimension for 96 h with semistench as solvent. Two slowly running distinct bands and some diffuse bands migrating further were obtained. The slowest-moving band (1.41 μC) did not yield gluconic acid on treatment with prostatic acid phosphatase and was not identified. The more rapidly moving discrete band (33.6 μC) yielded only [^{14}C]gluconic acid on hydrolysis.

[^{14}C]Pyruvic Acid

This material was prepared according to methods described by Rowsell.[1]

Starting material: L-[^{14}C]alanine, specific activity 5.14 μC/μmole.

The reaction mixture was constituted as follows:

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1. E. V. Rowsell, Methods in Enzymology 5, 685 (1962).

L-[^{14}C]Alanine (1 μC/μl)	5 μl
0.16 M Na α-ketoglutarate in 0.1 M potassium phosphate buffer, pH 7.4	250 μl
Pyridoxal phosphate (0.5 mg/ml) in 0.1 M potassium phosphate buffer, pH 7.4	5 μl
Glutamic-pyruvic transaminase (Calbiochem) (2 mg/ml; specific activity 32.7 units/mg, defined as mole substrate conversion per min per mg enzyme protein)	5 μl

The mixture was incubated for 1 h at 37° and chromatographed in toto in 10 aliquots on unwashed Whatman No. 4 paper; semistench was used for the one-dimensional development. Six spots were obtained: residual alanine (29.2% of total ^{14}C), pyruvate (65.1%), and four unidentified substances together constituting 5.7%. There was probably some loss of [^{14}C]pyruvate from the chromatogram due to volatility.

[^{14}C]Succinic Acid

This material was prepared according to methods described by Strassman and Weinhouse.[2]

Starting material: L-[^{14}C]glutamic acid, mono NH_4^+ salt, specific activity 3.85 μC/μmole.

Reaction mixture:

L-[^{14}C]glutamic acid (187.5 μC/ml)	80 μl
10.8 N H_2SO_4	10 μl
1.5 N $KMnO_4$ (added slowly)	25 μl

The mixture was incubated for 2 days at 37° and chromatographed in toto on Whatman No. 4 paper, by use of semistench followed by butan-1-ol: propionic acid:water. Four spots were obtained. Three of these were not identified and together contained about 3.24 μC of ^{14}C. The fourth was identified by cochromatography as succinic acid, and contained 4.64 μC of ^{14}C.

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2. M. Strassman and S. Weinhouse, J. Am. Chem. Soc. 75, 1680 (1953).

16. ATTEMPT TO SYNTHESIZE HIGH-MOLECULAR-WEIGHT POLYNUCLEOTIDES BY SCHRAMM'S PURELY CHEMICAL METHOD

Gilbert Weill

Research into the purely chemical synthesis of high-molecular-weight polynucleotides starting from nucleotide, or, even better, starting from purines, pyrimidines, sugars, and a phosphorylating agent, has two principal goals:

(a) to find a way of preparing model molecules with known composition and sequence for the purpose of studying their physicochemical as well as their biological properties;

(b) to find a possible mechanism for the formation of these chemical components of the living cell during the course of terrestrial evolution.

A paper by Schramm, Grötsch, and Pollmann describes a synthesizing procedure which represents an important step in both directions.[1] They used the phosphoric ester obtained by the reaction of ethyl ether on P_2O_5, a reactant used previously for the polycondensation of peptides,[2] to perform the following series of reactions:

(a) condensation of sugars to form polysaccharides with highly specific bonds;

(b) preparation of nucleosides from sugar and the bases;

(c) incorporation of new bases in apurinic acid;

(d) synthesis of natural polynucleotides from nucleotides or nucleosides.

The first three reactions correspond to activation of a hydroxyl group followed by condensation. The degree of specificity claimed by the authors for the reaction (b) has been recently questioned by Canbon.[3]

We have attempted to repeat the preparation of polyadenylic acid from AMP as we are primarily interested in the preparative possibilities.

Experimental Results

The so-called polyphosphate ester (PPE) has been prepared by following Schramm's procedure without further purification. Analyses of P content, density, and index of refraction agree with previous measurements.[4] The several polymerization experiments we ran with slight variations in time, temperature, and pyridine content showed almost identical results. We now describe a typical preparation.

A sample of PPE (9 g) is mixed with 360 mg of 5'-adenylic acid and 0.3 ml of pyridine; the heterogeneous mixture is heated at 55° for 24 h in a rotating flask. The product is homogeneous and slightly colored. It is

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1. G. Schramm, H. Grötsch, and W. Pollmann, Angew. Chem. Inter. Ed. Engl. 1, 1 (1962).

2. G. Schramm and H. Wissmann, Chem. Ber. 91, 1073 (1958).

3. J. A. Canbon, Chem. and Ind. 529 (1963).

4. G. M. Kosolapoff, Organophosphorus Compounds (John Wiley and Sons, N. Y. 1950).

dissolved in 50 ml of water and dialyzed at 4° with continuous stirring against 3 liters of solutions of sodium bicarbonate in decreasing concentrations. The external bath is controlled by its O. D. at 2600 Å, where both pyridine and AMP absorb strongly. Table 16-I gives the conditions of dialysis.

Table 16-I. Conditions of dialysis.

External bath	Time of dialysis (h)	Composition of bath	O. D. at replacement time
1	1.5	$NaHCO_3$ 0.2 M	3
2	1.5	$NaHCO_3$ 0.2 M	2
3	3	$NaHCO_3$ 0.1 M	0.7
4	4	$NaHCO_3$ 0.1 M	0.3
5	11	$NaHCO_3$ 0.1 M	0.3
6	4	$NaHCO_3$ 0.05 M	0.02
7	6	Water	0.02
8	16	Water	0.01

At the end of the dialysis period, the bag contains about 100 ml of solution, with an optical density of 2600 Å of 8. On the basis of a molar absorbance of 10^4 for the monomeric unit, the yield is about 8%. The product is freeze-dried (total wt 60 mg), and part of it is dissolved in 0.1 M NaCl for the following experiments.

(a) Ultracentrifugation. One ml of solution is examined by means of absorption optics in a Spinco Model E centrifuge at 56,100 rpm. Densitometry of the plates reveals a very broad distribution of sedimentation constants. The mean value is therefore difficult to determine; it is estimated to be 2 ± 0.5 Svedberg.

(b) Determination of the molar absorbancy per P atom. The absorption spectrum at neutral pH is determined on a Cary 14 spectrophotometer. The phosphorus analysis is performed according to the method of Chen et al.[5] The spectrum closely resembles that of polydenylic acid, with the maximum absorbancy at 2590 Å, but a value of $\epsilon p = 860$ demonstrates the presence of a large excess of phosphate. (Typical values for different preparations range between 600 and 1200.)

(c) Hypochromicity. The value of the hypochromicity defined as $H = 1 - \epsilon p(\text{polymer})/\epsilon(\text{monomer})$ is a reliable criterion for the characterization of the polymeric structure of the polynucleotides. In our case its value can be determined from hydrolysis experiments. Acid hydrolysis (N HCl for 1 h at 100°) produces a slight increase in the maximum absorbancy, with a shift in wavelength, which reveals the release of the free base (Fig. 16-1). The results must be compared with the change of absorption upon hydrolysis of AMP. In these conditions $H = 1 - [\epsilon(\text{native polymer})/\epsilon(\text{hydrolyzed polymer})]^2 \times [\epsilon(\text{AMP hydrolyzed})/\epsilon(\text{AMP})] = 1 - 0.94 \times 0.85 = 0.20$.

5. P. S. Chen, T. Y. Toribara, and H. Warner, Anal. Chem. 28, 1756 (1956).

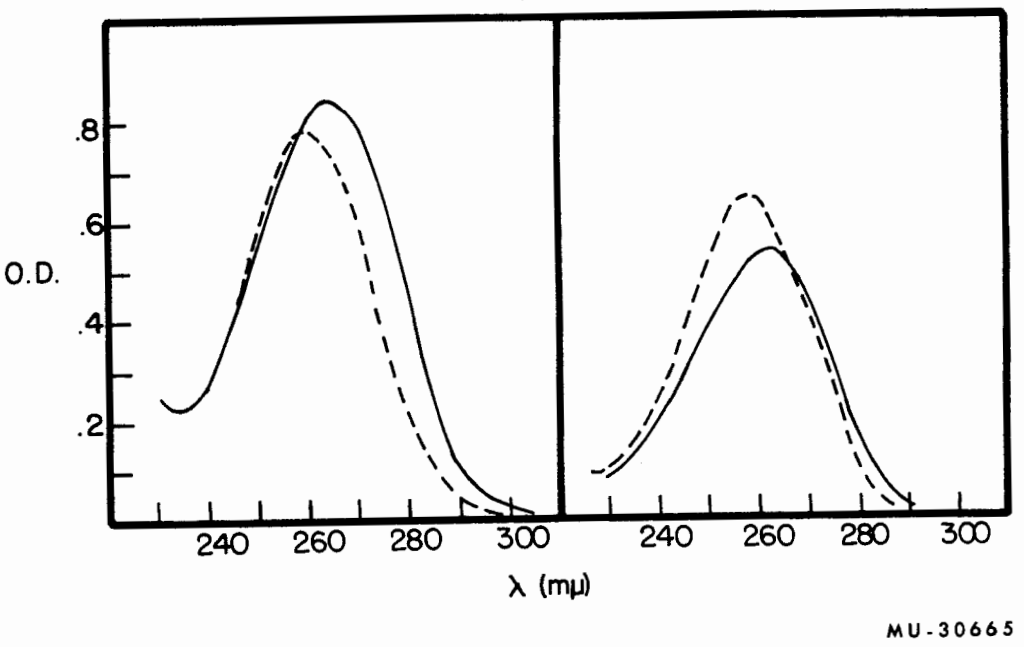

Fig. 16-1. Change in O. D. at Ph 5 upon acid hydrolysis. Left: Reaction product (dashed line, native; solid line, hydrolyzed). Right: AMP.

Alkaline hydrolysis (3 N KOH at 37° for 2 h) leads directly to an increase of 20% in optical density with no shift in the spectrum.

The sedimentation constant and hypochromicity are used by Schramm as proof of the success of the polymerization; no data on P content are available in his paper. It is necessary to investigate whether the excess phosphorus mentioned above corresponds to the presence of polyphosphates apart from the polynucleotide, or to the incorporation of nucleotides and excess phosphorus simultaneously into nondialyzable molecules.

The formation of polyphosphates in the reaction mixture can be demonstrated by heating the phosphate ester with pyridine in the absence of nucleotides. The product is submitted to equilibrium dialysis. After 100 h the concentration of P inside the bag is 2.16 times that outside. A subsequent equilibrium dialyses against a new external bath shows practically no release of small molecules; after 16 h the ratio of the concentrations inside and outside is greater than 200.

The presence of pure polyadenylic acid in the reaction product could be demonstrated in principle through absorption changes as a function of the pH.[6] No significant change has been observed between pH 2 and pH 10. The presence of polyphosphates, however, makes this observation equivocal. A separation of the constituents has therefore been attempted in two ways:

(a) By passing the product through a Sephadex G-25 coarse column. Figure 16-2 gives the elution pattern with respect to optical density and P content. Calculation of molar absorbancy per P atoms show that the first fraction has values very close to those for pure polyadenylic acid.

(b) By chromatography of the product with the solvent system described by Ebel et al.[7] for the separation of polyphosphates and RNA. This procedure is quite effective on a mixture of enzymatically prepared polyadenylic acid and polyphosphates; the polynucleotide stays at the origin where it can be detected by uv absorption or a standard phosphate spray. The reaction product shows no such components at the origin; the chromatogram consists of three poorly resolved spots, one consisting of orthophosphate, and the others corresponding closely but not exactly with the polyphosphates (Kuroll salt).

The foregoing experiment shows that nucleotides are actually incorporated into a polymeric structure which is not identical with a natural polynucleotide. It is reasonable to believe that the PPE activates several OH groups of the nucleotide, or even the amino group of the adenine. The method does not seem valuable for preparative purposes and its interest lies eventually in the more general problem of activation by phosphoric esters. A few remarks on the structure of the polyphosphate ester must be made in this respect.

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6. R. F. Steiner and R. F. Beers, Jr., Polynucleotides, (Elsevier, New York City, 1961).

7. J. P. Ebel, G. Dirheimer, M. Yacoub, and S. Muller-Felter, Bull. Soc. Chim. Biol. 44, 12 (1962).

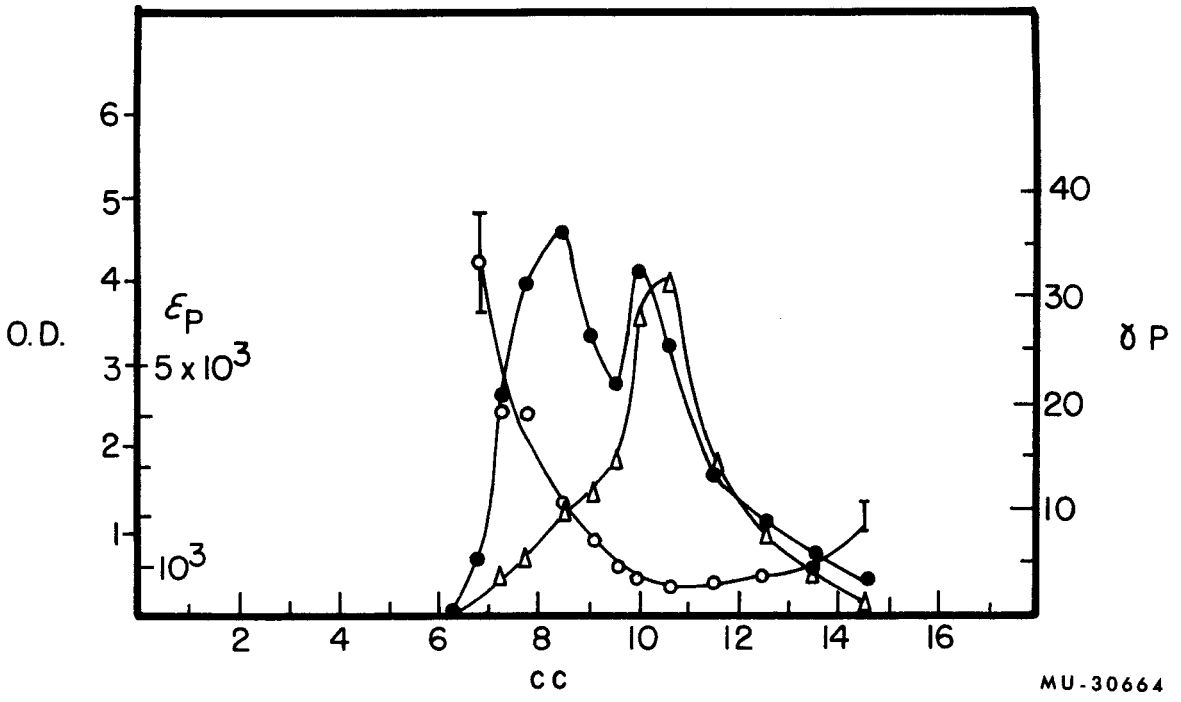
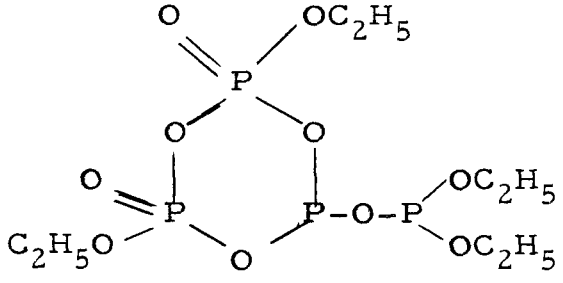


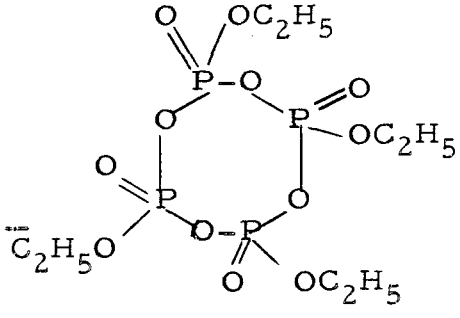
Fig. 16-2. Elution pattern of the nondialyzable material on Sephadex G-25.
Open circles: Molar absorbancy per P atom (ϵ p).
Closed circles: O. D.
Triangles: P content.

Observations on the polyphosphate ester

The structure of the product of the reaction of ether with P_2O_5 is still controversial. The analysis corresponds to a metaphosphate; molecular weight measurements by cryoscopy and ebullioscopy give values between those of a dimer and a hexamer. We have taken an ir spectrum of a solution in chloroform and found the bands attributed to P-O-P and P-O-C vibrations. A polymetaphosphate must have a cyclic structure. On the basis of the analysis of the individual barium salts of the products of hydrolysis (1 h at 100°), and starting with the structure of P_4O_{10}, Rãtz and Thilo have proposed the structure 1 with a small amount of structure 2:[8]



(1)



(2)

Nuclear magnetic resonance measurements on the phosphorus by Van Wazer[9] show only two different peaks, in the ratio 1 to 3, which are interpreted from the value of the chemical shift as arising from "middle" and "end" phosphorus atoms. This contradicts the presence of a "branching group" as in structure 1.

Knowledge of the exact structure of this active compound would be very important from two points of view. The first is the recent demonstration by Khorana[10] that a trimetaphosphate is the initial phosphorylating

8. R. Rãtz and E. Thilo, Ann. Ber. Chem. 272, 173 (1951).

9. J. Van Wazer, C. F. Callis, J. N. Shoolery, and R. C. Jones, J. Am. Chem. Soc. 74, 5715 (1956).

10. G. Weinmann and H. G. Khorana, J. Am. Chem. Soc. 84, 4329 (1962).

agent in the reaction involving the action of carbodiimide on phosphoric esters; the second is the activity of the PPE in aqueous solution for incorporation of bases in apurinic acid. The structure 1 would allow a partial hydrolysis with conservation of the cyclic structure.

Titration curves of the PPE after solution in water (Fig. 16-3) show the instantaneous appearance of about two strong acid functions for four P atoms (accompanied by liberation of heat), followed by a slow hydrolysis of the remaining bonds. Total hydrolysis, under the conditions of Rätz and Thilo, releases four strong and 2.6 weaker acid groups for four P atoms; this, too, is in favor of structure 1.

Taking advantage of the slow hydrolysis after the appearance of the first acid groups, we have tried with Dr. W. Rohr a high-voltage electrophoresis of the products of complete and partial hydrolysis. We hoped to be able to identify in the products of partial hydrolysis only two spots, corresponding to the cyclic trimetaphosphate plus the diethylester. Unfortunately, we have obtained six well-defined spots and some spreading. None of them corresponds to inorganic phosphate. The completely hydrolyzed product shows three spots, one of which is inorganic phosphate; one of the other spots migrates faster than inorganic phosphate, and the third more slowly.

This problem does not appear solvable by qualitative analysis. The following program of experiment should permit the definitive establishment of the structure and mechanism of hydrolysis:

(a) NMR of phosphorus of the pure polymetaphosphate ester and of solutions, in carefully dried $CHCl_3$ to decrease the viscosity. Study of aqueous solutions as a function of time.

(b) Preparation of the compound with [^{14}C]ether. The spots obtained by electrophoresis should then be characterized by the ratio C_2H_5 (radioactive counting) to phosphorus (direct analysis).

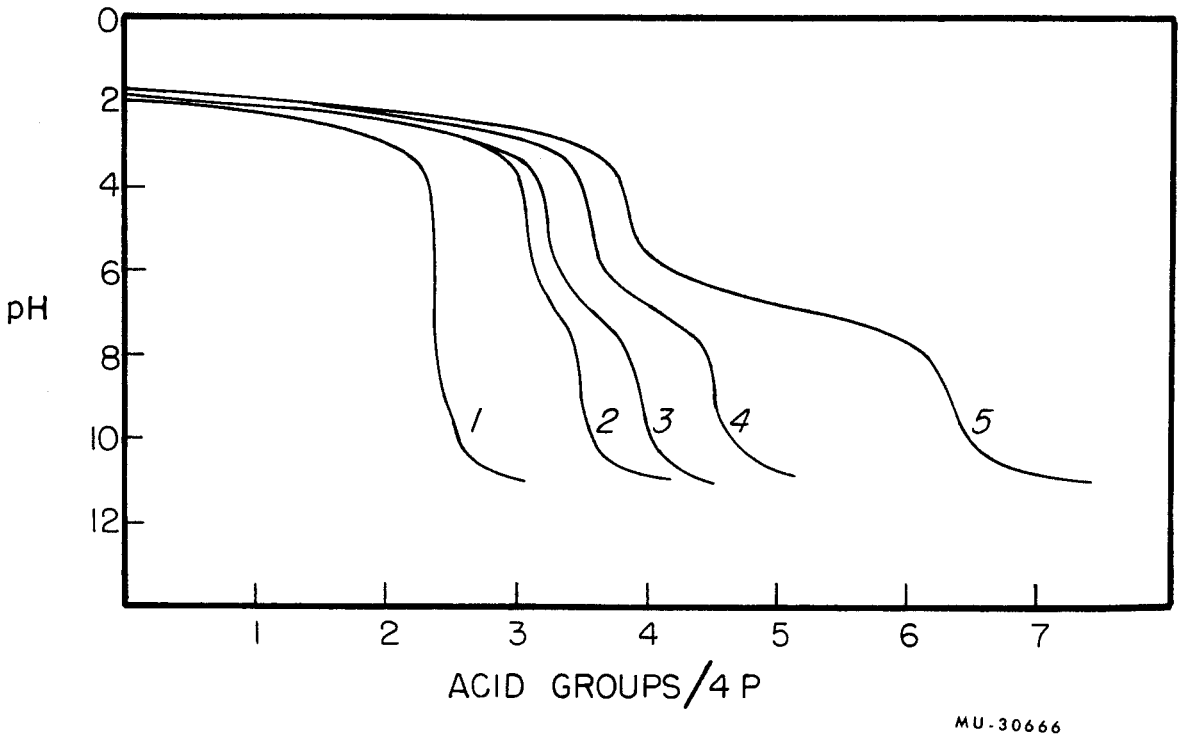


Fig. 16-3. Titration of PPE in water solution: 5, after solubilization; 4, after 6 h; 3, after 24 h; 2, after 52 h; 1 totally hydrolyzed.

17. OPTICAL PROPERTIES OF SOME DYE-POLYANION COMPLEXES

Gilbert Weill

Chromophore-macromolecule complexes represent an important class of compounds of biological interest. The regular ordering of chromophores on a polymeric substrate is thought to be a key to the mechanism of energy transfer, both in the primary steps of photosynthesis and in the process of vision. The work reported here represents part of an effort to understand the optical properties of such an array of chromophores. The results also have significance in terms of the structure of the polymeric substrate. The results reported in this section deal with the absorption and emission spectra, relative quantum yield, and polarization of fluorescence of two cationic dyes of the acridine series with polyphosphate and DNA.

Experimental Techniques

The absorption spectra were measured on a Cary 14 spectrometer. Careful corrections have been carried out for the deviation from zero of the base line. Complexes were prepared directly in 1-cm quartz cells by mixing solutions of dye and polymer with magnetic stirring. Measurements of concentrated solutions have been made possible by reducing the optical path with quartz blocks. Checks of the relative thickness were provided by measurements on compounds obeying Beer's law over a wide range of concentrations. (Hemoglobin with the chromophores bound to a globular protein is a suitable standard.) Turbidity corrections have been calculated and found negligible up to ratios of polymer:dye of 100:1. They are, in any event, compensated by using a solution of the polymer in the reference cell.

Emission spectra were measured on an Aminco Bowmann spectrofluorometer. Parker has studied in detail the corrections needed to obtain true action and fluorescence spectra.[1] As our main interest is the relative quantum yield of free and bound dye, no systematic investigation has been made of the wavelength dependence of the number of quanta supplied by the source through the first monochromator. A rapid determination of this dependence may be obtained by comparing the apparent action spectrum with the absorption spectrum only (on the assumption that there is no wavelength dependence of the quantum yield).[2] Comparisons of quantum yield are possible if true fluorescence spectra are known. The correction of the apparent spectrum rests on factors involving the dispersion of the monochromator, the transmittivity of the whole optical system, and the sensitivity of the photomultiplier as a function of frequency. A grating monochromator provides a constant bandwidth, and the transmittivity of the monochromator can be assumed to be constant in the narrow range 5000 Å to 6000 Å. Thus, the only important factor remaining is the spectral response of the photomultiplier. Curves provided by the manufacturer are easily transformed into amperes/quantum. From corrected spectra, it is easy to deduce the ratio of the number of quanta observed at a given frequency with a given bandwidth to

1. C. A. Parker and W. T. Rees, Analyst 85, 587 (1960).
2. G. Weber and F. W. J. Teale, Trans. Faraday Soc. 58, 640 (1957).

the total number of quanta integrated over the whole range of fluorescence wavelengths. Changes in this ratio must be taken into account in the calculation of relative quantum yield. These can now be computed from the relative changes of intensity of light fluoresced at one wavelength (where absorption changes are simultaneously recorded) and at one angle, except for the case in which changes in polarization of fluorescence occur simultaneously. If observations are made at 90° to the incident beam, the relative values must be corrected by the ratio of the factors (p + 3), where p is the polarization of fluorescence in vertically polarized incident light.

This value (p) can be measured directly on the Aminco spectrofluorometer by inserting polarizing prisms in the incident and fluorescent beams. Four components (V_v, V_h, H_v, H_h) can be measured; if the capital letter indicates the state of polarization of the fluoresced light, and the subscript that of the incident light, one can write

$$p = (V_v - H_v)/(V_v + H_v) .$$

In fact, direct measurement of V_v and H_v gives results affected by a difference of transmittivity of the monochromator for vertically and horizontally polarized light. The ratio of these transmittivities is given by a measure of the components V_h and H_h, which are theoretically equal. This fact also affects the assumption of constant transmittivity of the monochromator. A correction may be made but, in a study of the change of quantum yield in natural incident light, this is negligible compared with the magnitude of the observed changes.

As the apparatus allows observation only at 90° to the incident beam, it is necessary to work at extremely low concentrations to eliminate any weakening of the incident beam before it reaches the fluorescent volume; the latter is defined by two series of slits. If the geometry of the beam is known, an "internal screen coefficient" can be used if the concentration corresponds to optical densities greater than 0.02. For a 1-cm cell with a small fluorescent volume in the middle of the cell, the fluorescent intensity will be

$$F = K\eta I \exp(0.5\epsilon c) ,$$

where F = fluoresced intensity, K = geometrical factor, η = quantum yield of fluorescence, I = incident intensity, ϵ = molar absorbency of the solution, and c = concentration of the solution in moles/liter. If the screen effect is due to a nonfluorescent molecule (DNA in the uv region), accurate results can be obtained only in the optical-density range between 0 and 1.

Accessory techniques: Phosphorus analyses were performed by using the method of Chen et al.[3] The molecular weights of the polyanions were measured by light scattering with a Brice Phoenix photometer.

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3. P. S. Chen, T. Y. Toribara, and H. Warner, Anal. Chem. 28, 1756 (1956).

Study of the Pure Dyes (Proflavin and Acridine Orange)

a. Purification

From a solution of proflavin hydrochloride (K and K Laboratories) in a 50% water-ethanol mixture, the free base was precipitated with 0.1 N KOH. The precipitate was filtrated, washed, dried, and recrystallized from a mixture of water:ethanol (90:10). The molecular weight was determined by titration with 0.02 N HCl and agrees with the predicted value to within 2%. Stock solutions were made by neutralizing exactly a given weight of proflavin base with HCl and adding the chosen buffer to a given volume.

A double chloride of acridine orange and zinc (National Aniline Division) was precipitated with 0.1 N KOH, and the dried precipitate redissolved in chloroform and passed through an alumina column. A dark band remained on the column. The eluate was rapidly evaporated and the solid residue recrystallized as before. Molecular weight was determined and the solutions were prepared as for proflavin.

b. Absorption Spectra

Deviations from Beer's law, attributed to the formation of dimers and higher aggregates, have been extensively studied in the acridine series,[4] mostly in the visible region. Our values of ϵ agree with other published data.[4,5] The extension of the measurements to the uv range shows marked differences between these two dyes (Fig. 17-1). Proflavin presents a diminution of molar absorbency with increasing concentration, and a shift in the wavelength of the uv maximum of the same value in wave numbers as the shift in the visible, while acridine orange shows an enhancement of absorption and a much smaller shift in the uv than in the visible. These observations have some interest for the theory of coupling in dimer molecules if the different electronic transitions are well identified.

c. Polarization of Fluorescence

Measurements of the polarization of fluorescence in a viscous solvent can characterize different electronic transitions. Measurements in glycerin (Figs. 17-1 and 17-2) reveal the presence of a different transition on the long-wavelength side of the uv maximum. Measurements at liquid nitrogen temperatures[6] have shown that the two uv transitions are perpendicularly polarized and that the strongest one has a transition moment parallel to the one in the visible spectrum.

d. Fluorescence Spectra

Corrected spectra are shown in Figs. 17-1 and 17-2. Taking into account the broadening due to the manner of grabbing (wavelength and not wave

4. V. Zanker, Z. Physik. Chem. 199, 225 (1952).
5. A. R. Peacoke and J. N. H. Skerret, Trans. Faraday Soc. 52, 261 (1956).
6. V. Zanker, Z. Physik. Chem. 2, 52 (1954).

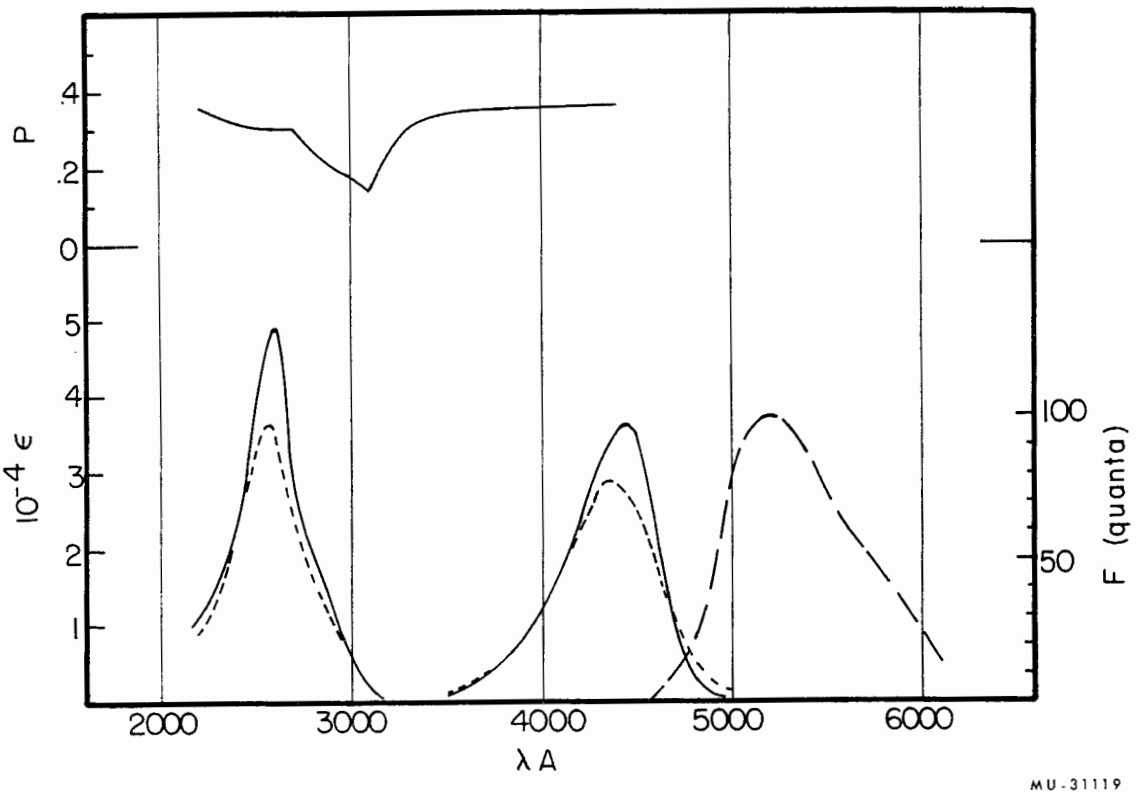

Fig. 17-1. Optical properties of proflavin^{+} in 10^{-1} M acetate buffer, pH 5.5.
——— Absorption spectrum, 5×10^{-6};
——— Absorption spectrum, 10^{-3};
- - - - - Fluorescence spectrum, 2×10^{-6};
——— Polarization of fluorescence.

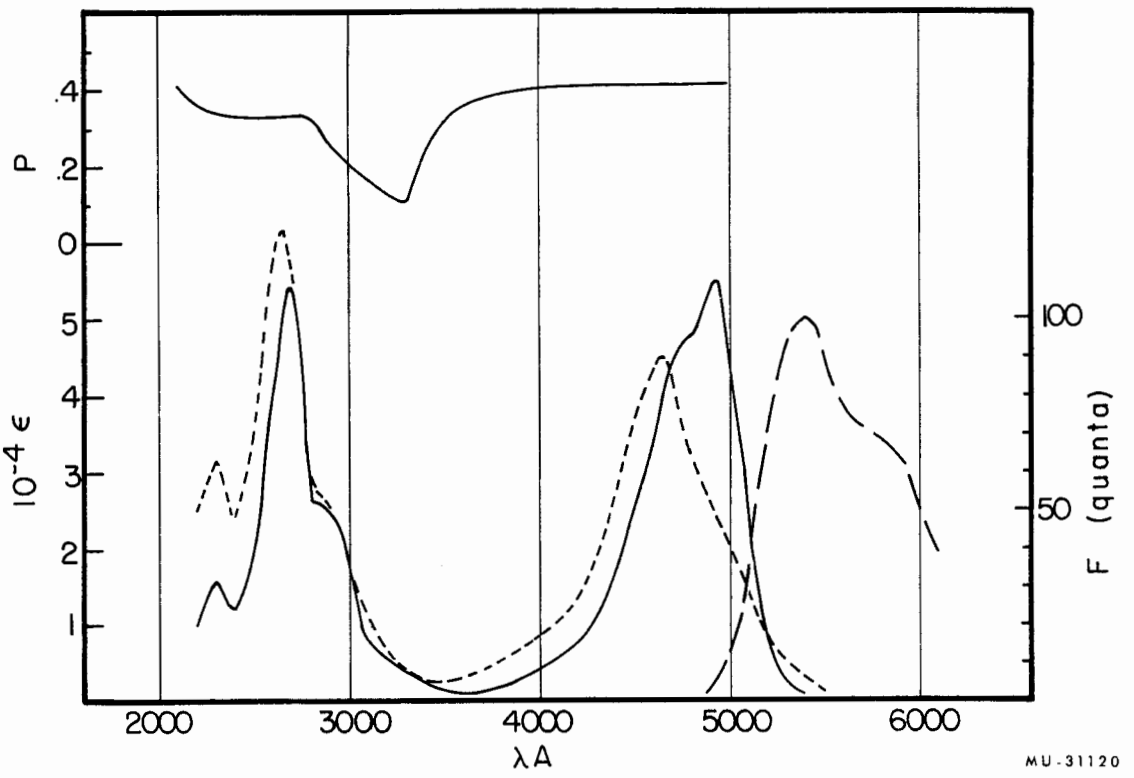

Fig. 17-2. Optical properties of acridine orange monocation in 10^{-1} M acetate buffer, pH 5.5.
_____ Absorption spectrum, 5×10^{-6}; ----- Absorption spectrum, 5×10^{-4}; — — — Fluorescence, 2×10^{-6}; _____ Polarization of fluorescence.

numbers), and to the lower resolution resulting from the increased band widths, the fluorescence spectra are satisfactory mirror images of the absorption spectrum. Data published by Oster[7] indicate that concentration quenching occurs at concentrations as low as 10^{-6} M. Table 17-I shows some results obtained between 10^{-6} and 10^{-5} M; these results do not confirm this unusual behavior.

Table 17-I. Change of fluorescence of proflavin solution as a function of concentration (10^{-3} M acetate buffer, pH 5.5).

Concentration x 10^6 (M)	Intensity of fluorescence; excitation 4400Å; emission 5100Å	O. D. at 4400Å	Intensity correction (screen effect)	10^{-6} x Int/c
1	21.5	0.036	1.04	21.3
2	40	0.072	1.08	21.8
5	91	0.180	1.23	22.4
10	140	0.360	1.52	21.4

The use of Weber's matrix method[8] to analyze the results of fluorescence show only one fluorescent species in solutions of both dyes.

Study of the Complexes with Polyphosphates

a. Characterization of the Polyphosphate

The sample was a Kuroll salt prepared at a temperature of 680 to 690°. Its molecular weight, determined by light scattering in 0.35 M NaBr, was $1.9 \pm 0.1 \times 10^6$, and its radius of gyration 610Å.

b. Absorption Spectra

The formation of a complex between the polymer and the dye in the presence of inorganic cations represents a complex equilibrium which takes into account both the degree of dissociation of the polyphosphate and the competition between organic and inorganic cations. Figure 17-3 shows the results obtained for solutions of dye (2×10^{-5} M in 10^{-3} M acetate buffer) as a function of the relative number of phosphate groups to dye molecules (P/D). The relative change of absorption in such a system presenting a well-defined isobestic point allows the calculation of the fraction of bound dye, and of the ratio (r) of occupied to unoccupied phosphate sites. The equilibrium constant for the reaction of fixation, k, is

$$k = r/c(1 - r),$$

where c is the concentration of free dye in the solution. In our conditions, r has a limiting value of 0.66 when P/D tends towards 0; k decreases with

7. F. Millich and G. Oster, J. Am Chem. Soc. 81, 1357 (1959).

8. G. Weber, Nature 190, 27 (1961).

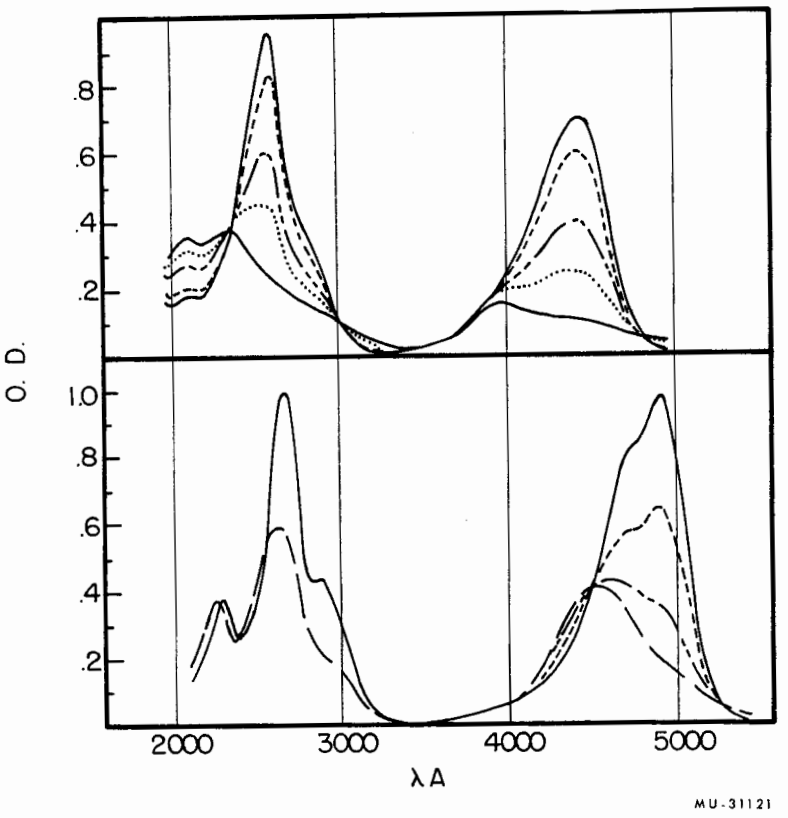

Fig. 17-3. Titration of proflavin and acridine orange cations with polyphosphates.
Top: Proflavin. _____ Pure dye; ------P/D ± 0.25; — - - — P/D = 0.75; ..... P/D = 1.25; _____ P/D between 5 and 50.
Bottom: Acridine orange. _____ Pure dye; -----P/D = 1; — - - —P/D = 2; — — —P/D between 5 and 50.

increasing P/D, having values between 1 and 5×10^5 liters/mole for both dyes. At much lower concentrations, or higher ionic strengths, complete fixation of the dye requires excess of polymers which are outside the range of solubility.

The spectrum obtained at high P/D values is, according to Bradley and Wolf, of the type corresponding to "clusters" of neighboring molecules in a linear array.[9] This leads to a large hypochromicity and a large shift towards short wavelength. Extension of the measurements to the uv region shows some peculiarities. The main uv absorption band shows hypochromicity and a shift in both cases; for proflavin, the shift in uv is comparable to that in the visible, but is considerably less in the case of acridine orange. The spectrum of the proflavin complex shows some indication that the perpendicularly polarized transition around 3100Å exhibits hyperchromism. The last band towards short wavelength exhibits a slight shift and no change or enhancement (but in the case of proflavin the separation of the two last bands is difficult). If such effects are to be explained by strong coupling between identical oscillators, the possibility of observing the effects on several electronic transitions should be very useful for a check of the quantitative treatment.

c. Fluorescence

The intensity of fluorescence decreases in direct relation to the concentration of free dye. Careful measurements of the action spectra, polarization of fluorescence, and use of the matrix method, show that the bound dye does not fluoresce (or at least has a quantum yield less than 2% of the free dye). The expected displacement towards the red of the fluorescence maximum helps to check this quenching, as the fluorescence of the free dye practically vanishes in this region.

Study of the Complexes with DNA

Changes of absorption in the visible region of dye-DNA complexes have been extensively studied with the aim of understanding the metachromatic phenomena and of finding the nature of the binding sites.[10] There are very few studies of the fluorescence of the dye, especially for high polymer-to-dye ratios.[11] The recent results of Lerman[12] and his model of intercalation for the acridines give a new interest to these studies.

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9. D. F. Bradley and M. K. Wolf, Proc. Natl. Acad. Sci. U. S. 45, 944 (1959).

10. R. F. Steiner and R. F. Beers, In., Polynucleotides (Elsevier, New York City, 1961).

11. R. E. Boyle, S. E. Nelson, F. R. Dollish, and M. J. Olsen, Arch. Biochem. Biophys. 96, 47 (1962).

12. L. S. Lerman, J. Mol. Biol. 3, 18 (1960).
V. Luzzati, E. Masson, and L. S. Lerman, J. Mol. Biol. 3, 634 (1961).
L. S. Lerman, Proc. Natl. Acad. Sci. U. S. 49, 94 (1963).

a. Characterization of the DNA

Salmon-sperm DNA was obtained from the California Corporation for Biological Research. Its molecular weight, determined by light scattering, is 5×10^6, with a radius of gyration of 2200Å. Its sedimentation constant is, however, slightly low (s = 17 Svedberg), but its molar absorbency per phosphorus ($\epsilon_P = 6700$) is characteristic of a native DNA.

b. Absorption Spectra

The results given in Fig. 17-4 have been obtained in very dilute dye solutions (2×10^{-6} M) in 10^{-3} M acetate buffer, pH 5.5. In this way the ratio of bound dye to the total number of sites is extremely low (less than 0.2 as soon as the ratio P/D is greater than 1). We are then dealing mostly with the so-called second type of binding,[5,9] and the curves present an isobestic point, very well defined for the proflavin, and less precise for acridine orange. We can use the same solutions for the fluorescence measurements. For P/D values approximately equal to 20, no more change can be detected in the absorption spectrum. The limiting spectrum in the visible is similar to those already published. Difference spectra have been measured in the uv region where DNA itself absorbs, and shows striking differences from the spectrum of the free dye: (a) a very high hypochromicity in the main peak with a very slight shift; (b) a hyperchromic effect for the weak absorption band perpendicularly polarized; (c) and hyperchromicity or no change in the absorption at short wavelength.

Do these effects represent an alteration of the electronic transitions of the dye or of the DNA? The absolute value of the change, which represents in molar absorbency the total contribution from two or three bases, as well as the spectral dependence of the changes, strongly suggests that they represent an alteration in the spectrum of the bound dye due to the interaction with the bases.

c. Fluorescence

The changes in fluorescence spectra reflect the changes in absorption in the visible. The maximum wavelength of fluorescence is shifted about 100Å towards short wavelength, and the ratio of the maximum peak height to total integrated area (k) is multiplied by 1.15 (for proflavin) and 1.20 (for acridine orange). Measurement of the change of intensity of fluorescence as a function of P/D allows calculation of the relative quantum yield of the bound dye. For the values of P/D for which a fraction (x) of the total concentration remains as free dye, the relative quantum yield is given by

$$\frac{\eta}{\eta_0} = \frac{F/F_0 - x}{1 - x} \frac{\epsilon_0}{\epsilon_1} k \frac{3 + p_1}{3 + p_0},$$

where the subscript 0 indicates the properties of the free dye, the subscript 1 those of the bound dye, and no subscript the value obtained with the mixture. Knowing ϵ_0 and ϵ_1 from the limiting spectrum, one can calculate x from absorption data; k is the factor depending on the change of shape of the fluorescence spectrum; and p is assumed to be equal to the polarization

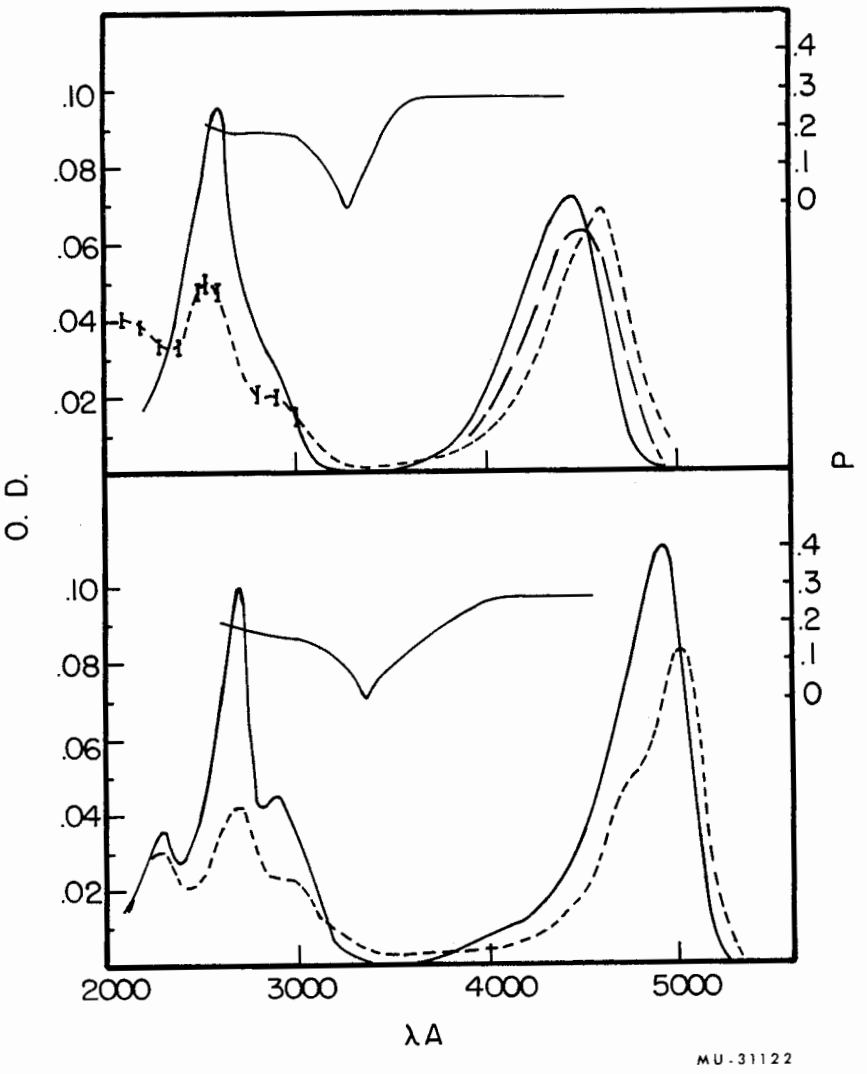

Fig. 17-4. Changes in absorption by fixation on DNA.
Top: Proflavin total concentration, 2×10^{-6} M in 10^{-3} M acetate buffer. _____ Free dye; — — — P/D = 4; ----- $\overline{P}$/D > 20.
Bottom: Acridine orange total concentration, 2×10^{-6} M in 10^{-3} M acetate buffer. _____ Free dye; -----P/D > $2\overline{0}$.

measured at high P/D ratios when no free dye is present. The precision is improved by measurements at several excitation and fluorescence wavelengths. Determination of the number of fluorescing species is also achieved with the matrix method.

The results are given in Figs. 17-5 and 17-6. The results of the excitation in the uv must be corrected for the screen effect introduced by the DNA itself. Measurements are possible up to P/D values of 100. The results show the same general behavior as for excitation in the visible, with a multiplication factor that depends on the wavelength. The upper curves of Figs. 17-5 and 17-6 correspond to the excitation at 2600Å.

d. Discussion

The change in relative quantum yield with the polymer-to-dye ratio, and the dependence of the relative quantum yield upon wavelength in the uv region, can be explained by a mechanism of energy transfer between dye molecules and from the bases to the dye (sensitized fluorescence). The theory developed by Forster[13] shows that the probability of transfer depends on three factors: (a) the overlap between the fluorescence spectrum of the donor and the absorption spectrum of the acceptor; (b) the inverse sixth power of the distance between the molecules; and (c) the relative orientation of the transition moment in the molecules.

The shape of variation of the quantum yield is very similar to that observed in concentration quenching. The value of P/D for which there is half quenching is in both cases close to 20. This corresponds to a mean distance between dye molecules of 10×3.4Å for double-stranded DNA; this value of 34Å is very similar to those obtained for free molecules in solution.[13] The shape of the curve would in this case be related to the distribution of distances between the dye molecules. This explanation implies that the transfer increases the probability of nonradiative transition to the ground state, either because the probability for the excitation to be transferred into vibrational energy increases with the number of molecules through which the energy travels, or because of the presence of quenchers in the neighborhood of one dye molecule. In this respect, the differences between proflavin and acridine orange are interesting. Bound proflavin is strongly quenched whereas bound acridine orange has a strongly enhanced fluorescence for high values of P/D where the relative quantum yield remains constant. We are now studying the quenching of the free dyes by high concentrations of mononucleotides.

The transfer from the bases to the dye implies directly that transfer from base to base is possible. More careful studies are needed to analyze the efficiency of the transfer as a function of the wavelength and of the absorption spectrum of the dye. The intercalation model provides the close geometrical relation necessary for efficient transfer. By comparing other dyes, intercalated or not, with transition moments of various orientations

13. E. Forster, Fluoreszenz Organischer Verbindungen (Vanderhoeke and Ruprecht, Gottingin, 1950).

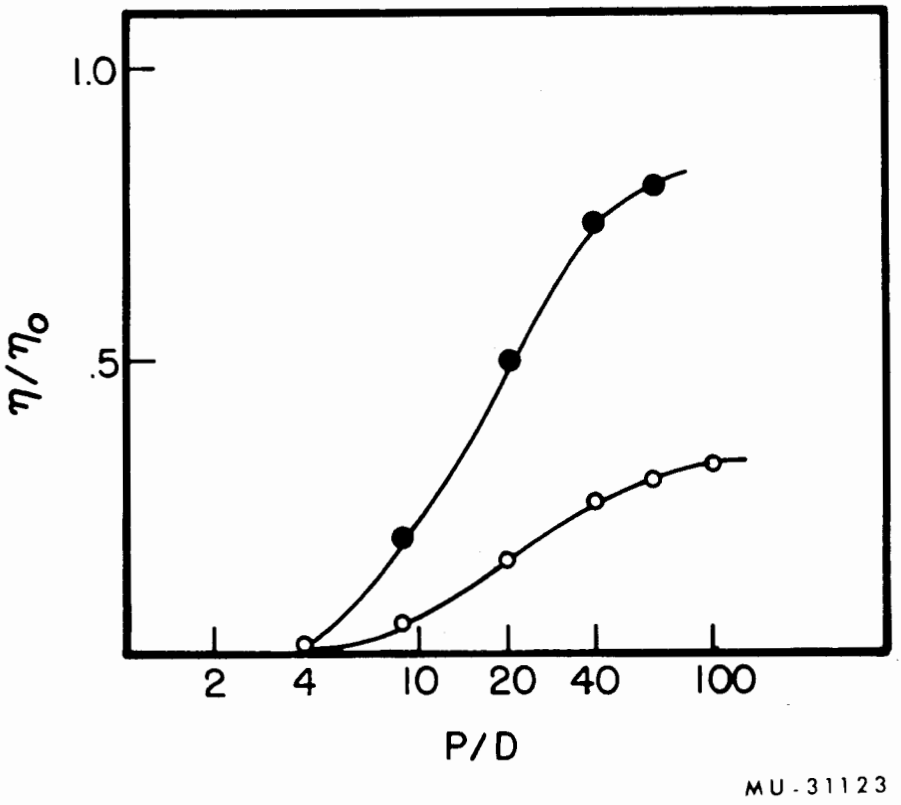

Fig. 17-5. Relative quantum yield of bound proflavin as a function of P/D. Open circles, excitation in the visible; closed circles, excitation at 2000 Å.

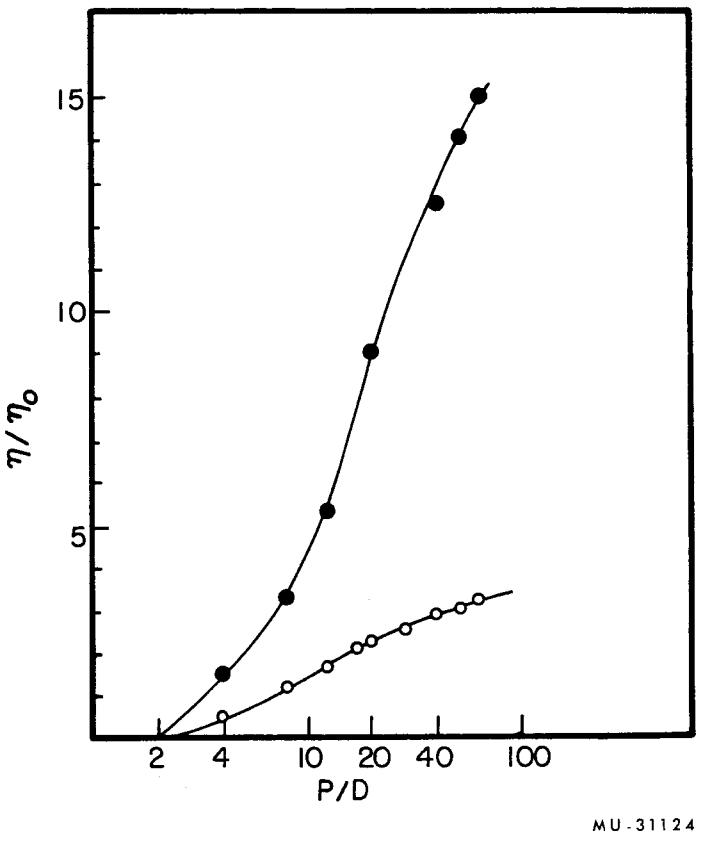

Fig. 17-6. Relative quantum yield of bound acridine orange as a function of P/D. Open circles, excitation in the visible; closed circles, excitation at 2600 Å.

with regard to the geometry of the molecule, more information should be gained on the transfer mechanism. The more efficient transfer for one specific base to one specific dye would be of help in studying the distribution of the bases along the DNA molecules.

Transfer of energy in solution is generally accompanied by a change in polarization. The dye bound to a high-molecular-weight substrate exhibits a strongly polarized fluorescence which is measured at high P/D values. The value of the polarization in the visible is very close to that measured for the free dye in glycerin. A change in this value with P/D would provide a supplementary proof of energy transfer, but no change is observed if the transferring molecules form a regular array on a rigid matrix with parallel transition moments. The experiment is complicated at low P/D ratios by the presence of free dye, and the precision is not high. On the other hand, the same argument can be applied to the transfer from the bases at very high P/D values. Only small decreases of polarization of fluorescence have been observed. The same results appear in the curves presented for DNA quinacrine complexes by Lerman.[12] Such a result is not surprising, since the probability of transfer depends strongly on the parallelism of the transition dipoles.

Taking advantage of the relative position of the absorption peaks of proflavin and acridine orange, an experiment has been designed to demonstrate energy transfer from the former to the latter. Figure 17-7 shows the fluorescence spectrum of the two bound dyes. The value of P/D chosen was 20. When the excitation wavelength is varied from 4000Å (where bound acridine orange has practically no absorption) to 4900Å (where bound proflavin is nearly transparent), and the emission wavelength is varied from 5200 to 6000Å, a comparison of the fluorescence intensity of a complex of DNA with proflavin and with proflavin + 0.2 acridine orange (molar proportions), respectively, leads to a "difference action spectrum." This spectrum, when compared with the absorption spectrum of the bound acridine orange, shows a small but well-defined phenomenon of sensitized fluorescence. More experiments are in progress to find the best conditions to demonstrate this transfer. Table 17-II gives the result of such a comparison between the fluorescence of the DNA-proflavin complex and the DNA-proflavin + 0.2 acridine orange complex.

Table 17-2. Demonstration of sensitized fluorescence of acridine orange (AO) by proflavin (Pro) in a complex with DNA (fluorescence intensity in arbitrary units).

Emission	5200 Å		5400 Å		5600 Å		5800 Å		6900 Å	
	Pro	Pro + AO	Pro	Pro + AO	Pro	Pro + AO	Pro	Pro + AO	Pro	Pro + AO
Excitation										
4000 Å	7	33	5.5	32	2.9	16	.5	6	0	4
4500 Å	27	49	21	48	11	24	4.5	11.5	0	7
4900 Å	8	37	7	47	3.6	24	1.6	11.4	0	6.5

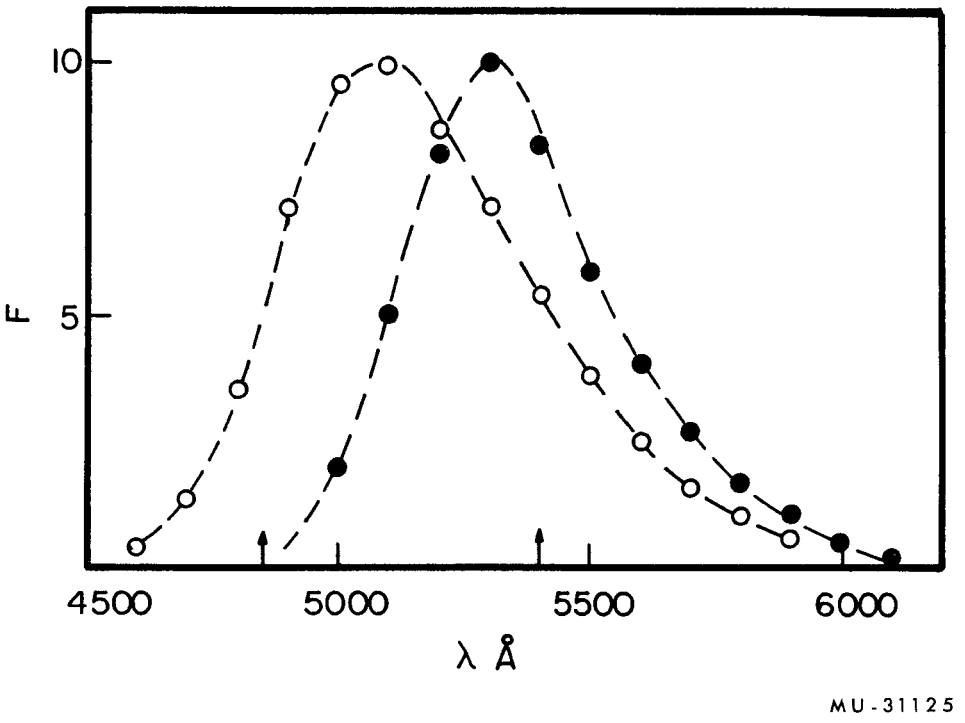

Fig. 17-7. Uncorrected fluorescence spectra of complexes of proflavin (open circles) and acridine orange (closed circles) with large excesses of DNA.

Conclusion

The change of absorption and fluorescence of dye molecules ordered along a polymeric matrix can be explained in terms of interaction and energy transfer between the transition dipoles. With the two polyanions studied, the close arrangement of the dye molecules produces a quenching of fluorescence similar to that observed for concentration quenching. In this latter case, dimer molecules are thought to be responsible for the nonradiative transition to the ground state. The mechanism of quenching in a linear array of molecules is still a problem. Experiments with more polyanions are required to understand the role played by the interaction between the dye and the polymer. Some dyes that are known to spontaneously give helical "polymers" do not exhibit this type of fluorescence quenching.[13] The energy transfer observed in complexes with native DNA suggests a number of similar experiments with denaturated single-stranded DNA and synthetic polynucleotides.

18. THE MECHANISM OF THYMINE DIMERIZATION

Joan Friedman and Richard M. Lemmon

The formation of thymine dimers in DNA has been shown to play a role in the mutation of organisms by ultraviolet light. This report is a continuation of an investigation[1] into the mechanism of this reaction in vitro, using thymine alone.

Role of Water in Dimer Formation

Thymine dimers form best when thymine in frozen aqueous solution is irradiated by uv light. In order to determine whether water plays an intimate role in the reaction or if the ice just provides an inert matrix, we irradiated the solid crystals in the absence of water. In a previous Quarterly Report, we reported that no dimers (or other products) were formed when thymine crystals were irradiated as a suspension in hexane.[1] However, Wang has reported the formation of dimers upon irradiation of thin solid films of thymine prepared by evaporating the solvent from a methanolic solution.[2] We attempted to repeat his experiment on a micro scale, using ^{14}C-labeled thymine, followed by paper chromatography for separation of products. However, we found no evidence of dimer formation. Subsequently, in a private communication, Wang acknowledged that his experiments were poorly reproducible and that formation of dimers depended on the relative humidity of the atmosphere on the days the various experiments were run, the yield increasing as the relative humidity increased. Apparently it is much more humid in Boston than in Berkeley. Our results (below) indicate that water must be present for appreciable dimerization to be observed. In addition, it should be noted that the crystal structure of thymine monohydrate has an appropriate thymine-thymine distance and orientation to allow for easy formation of one stereoisomer of the dimer.[3] It would be of interest to determine the stereochemistry of the one isomer that is produced by irradiation of thymine in ice, and see if it corresponds to the one expected from the thymine hydrate crystal structure.

Effect of Oxygen

Carrying out a reaction in the presence or absence of oxygen should indicate whether any appreciably long-lived free radicals are important intermediates, since oxygen quickly combines with free radicals and effectively quenches free-radical reactions. We tested the effect of oxygen on the dimerization reaction by preparing and irradiating thymine in ice under an atmosphere of nitrogen, and compared the results with an irradiation done in air on a solution saturated with oxygen before freezing. Our results showed that there was no significant difference in dimer formation under these two different conditions. This would seem to indicate that relatively stable free radicals are not involved. Of course, this does not rule out the possibility that short-lived radicals are intermediates. This finding fits in nicely with

1. Joan Friedman, R. M. Lemmon, and M. Calvin, in Bio-Organic Chemistry Quarterly Report, UCRL-10479, Sept. 1962, p. 40.

2. Shih Yi Wang, Nature 190, 690 (1961).

3. R. Gerdil, Acta Cryst. 14, 333 (1961).

the observation that oxygen has no effect on the rate of uv-light-induced mutation of bacteria.

Experimental Procedure

Role of Water in Dimer Formation

Two hundred μl of a 6.27 M solution of thymine-2-^{14}C (Research Specialties Corp.; 2.94×10^6 dis/min/ml) in methanol was applied dropwise to a glass dish. The solvent was allowed to evaporate, leaving a thin film (about 0.066 mg/cm^2) of thymine crystals on the bottom. Two such samples were prepared. The films were irradiated with a GE 15-W germicidal lamp at a distance of 8 in. (7700 ergs/cm^2/sec) for 3 min and 21 sec (1.54×10^6 ergs/cm^2--this is the same as the total dose reported by Wang). Each film was dissolved in 1 ml of boiling water, and 350 μl of each solution was applied to Whatman No. 4 oxalic acid—washed filter paper for chromatography. A sample (60 μl) of the original solution was run as a control. The chromatograms were developed in ethanol-water[1] and isopropanol—conc. HCl—water[1], and radioactive products were detected by radioautography. No new compounds were detected.

Oxygen Effect

1. In the absence of oxygen

One ml of thymine solution in a 10-ml beaker was deoxygenated in a glove box by bubbling nitrogen through it for about 5 min. The box was evacuated and refilled with nitrogen five times. The sample was then frozen and irradiated in the glove box for 5 min with a GE 15-W germicidal lamp at a distance of 5 in. Formation of dimer was detected by the decrease in O. D. at 265 mμ (Table 18-I).

2. In the presence of oxygen

One ml of the above solution was oxygenated by bubbling oxygen through it for 15 min. The sample was then frozen quickly (dry ice) and irradiated as above in the presence of air (Table 18-I).

Table 18-I. Changes in optical density of thymine solutions following irradiation in nitrogen, oxygen, and air.

	Optical density at 265 mμ		Dimer formed
	Before irrad	After irrad	(%)
Under nitrogen atmosphere			
Expt. 1	0.860	0.230	73.2
Expt. 2	0.890	0.220	75.3
Expt. 3	0.585	0.135	77.0
In the presence of oxygen			
Expt. 4	0.565	0.110	80.6
Expt. 5 [a]	0.625	0.145	77.0
Expt. 6 [a]	0.625	0.125	80.0
Not oxygenated, in presence of air [b]			
Expt. 7	0.840	0.200	76.1

[a] These two results were obtained from beakers irradiated simultaneously--indicates reproducibility.

[b] From previous experiment (Reference 1).

19. ULTRAVIOLET IRRADIATION OF AQUEOUS $H^{14}CN$

Rosarina Carpignano and Richard M. Lemmon

This report describes a continuation of work that was summarized in an earlier Quarterly Report.[1] The purpose of this research is to learn the kinds of processes, resulting in the production of organic molecules, that may have taken place on the "prebiotic" earth. The particular approach used here is to study the effects of ultraviolet light on aqueous solutions of HCN. We believe that such investigations are meaningful for the following reasons:

1. On the prebiotic earth (approximately 10^9 years ago) the principal source of high-energy radiation (5 eV, or higher, per quantum or particle) was ultraviolet light.[2]
2. Hydrogen cyanide has been obtained as a product of the interaction of ionizing radiation with "prebiotic earth atmospheres" (mixtures of CH_4, NH_3, H_2O, and H_2).[3,4]
3. Hydrogen cyanide occurs in comets, and it is conceivable that HCN brought to the earth by colliding comets played a subsequent major role in chemical evolution.[5]
4. Adenine, the essential constituent of nucleic acids and of many important cofactors (e.g., coenzyme A, ATP, TPN, FAD), can be synthesized "in substantial amounts by heating a solution of hydrogen cyanide (1 to 15 M) in aqueous ammonia for one or several days at moderate temperatures (27° to 100°)."[6]

Mechanisms for the formation of adenine and amino acids from HCN have been proposed by Oró[7] and by Kliss and Matthews.[8]

Experimental Procedure and Results

^{14}C-Labeled HCN was prepared by reacting $K^{14}CN$ with sulfuric acid, and carrying the evolved $H^{14}CN$ in a N_2 stream to a trap at -196°. The cyanide concentration was determined by silver-ion titration.[9]

Aliquot protions of the $H^{14}CN$ solutions (0.075 M, 63 μC/ml) were placed in quartz tubes and irradiated (for either 6 or 18 h) by a high-pressure mercury ultraviolet lamp (Hanovia AH6) at a distance of 7.5 cm. The solution

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1. T. W. Sze, in Bio-Organic Chemistry Quarterly Report, UCRL-10156, April 1962, p. 4.
2. A. J. Swallow, Radiation Chemistry of Organic Compounds (Pergamon Press, Ltd., London, 1960), p. 244.
3. S. L. Miller, J. Am. Chem. Soc. 77, 2351 (1955).
4. C. Palm and M. Calvin, J. Am. Chem. Soc. 84, 2115 (1962).
5. J. Oró, Nature 190, 389 (1961).
6. J. Oró and A. M. Kimball, Arch. Biochem. Biophys. 94, 217 (1961).
7. J. Oró, Nature 191, 1193 (1961).
8. R. M. Kliss and C. N. Matthews, Proc. Natl. Acad. Sci. U. S. 48, 1300 (1962).
9. F. L. J. Sixma, et al., Rec. Trav. Chim. 73, 161 (1954).

was then evaporated to dryness, and the total radioactivity of the residue determined. This figure was assumed to represent the nonvolatile compounds formed during the irradiation. The 6-h irradiation resulted in the fixing of 10.8% of the activity of the $H^{14}CN$ in nonvolatile form. The 18-h irradiation yielded 26.1% as nonvolatile compounds. The subsequent observations reported here apply to the 18-h irradiation.

Aqueous solutions of the residue were slightly yellow. Aliquot portions of this solution were chromatographed on oxalic acid—washed Whatman No. 4 paper. The two solvent systems used were n-butanol—propionic acid—water (75:36:49 by volume) and n-propanol—16N NH_4OH—water (6:3:1 by volume). Autoradiographs and shadowgrams[10] of the chromatograms were made.

The autoradiographs of the chromatograms (containing a total of about 1.6 μC) showed the presence of 20 to 30 distinct compounds. Of these, we focused our attention on the six major spots. The observed Rf values of these six spots are as follows:

	Rf Values	
Spot	BuOH-Prop.	Prop.-NH_4OH
1	0.29–0.33	0.44–0.48
2	0.62–0.65	0.53–0.55
3	0.63–0.65	0.64–0.66
4	0.56–0.59	0.69–0.71
5	0.67–0.69	0.80–0.82
6	0.85–0.87	0.87–0.91

To find out whether any purines or pyrimidines were formed in the irradiation, aliquot portions of the solution of the nonvolatile residue were cochromatographed with adenine, guanine, cytosine, thymine, and uracil. The corresponding uv-absorbing areas gave no measurable radioactivity above background. Calculations based on starting $H^{14}CN$ and residue-C^{14} activities show that these bases, if present, are formed in an amount less than 0.02% of the residue, or less than 0.005% of the starting $H^{14}CN$.

Cochromatography was also carried out with 4-aminoimidazole-5-carboxamide (Sigma Chemical Co.); this compound is a presumed precursor in the biosynthesis of adenine.[7] Again, no coincidence of radioactivity and uv absorption was found.

An attempt was made to prepare 4-aminoimidazole-5-carboxamidine (another presumed adenine precursor) following the procedure of Cavalieri.[11] However, we were unable to isolate the product in pure form.

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10. J. D. Smith and R. Markhan, Biochem. J. 45, 249 (1949).

11. L. F. Cavalieri, J. F. Tinker, and G. B. Brown, J. Am. Chem. Soc. 71, 3973 (1949).

Spot No. 3 on the list was identified as urea by the coincidence of the radioactivity with the lemon-yellow color developed by spraying the chromatogram with a solution of 1% dimethylaminobenzaldehyde and 2% HCl in ethanol.[12] The urea is about 5% of the residue, or 1.3% of the original $H^{14}CN$. It has already been suggested[4] that the urea appears by the reactions

$$2HCN + 2OH(H_2O_2) \rightarrow 2HOCN + H_2O ,$$

$$HOCN + NH_4OH \rightarrow (H_2N)_2CO + H_2O .$$

12. G. B. Marini-Bettolo, Farmaco (Pavia), Ed. Pract. 12, 111 (1957).

20. THE ULTRAVIOLET AND INFRARED SPECTRA OF SOME ORTHO-NITROAMIDES

Lech Skulski*

The existence of intramolecular hydrogen bonding in the molecule of o-nitroacetanilide has been supported by cryoscopic and wet melting point measurements,[1] dipole moments,[2] and polarographic,[3] infrared,[4] and ultraviolet[5] studies. This evidence is also confirmed by the relatively greater volatility[3] and solubility of this compound in naphthalene[1] and cyclohexane, when compared with the meta and para isomers. The measurements of the uv spectra of o-nitroacetanilide (I) and its N-methyl derivative (II) carried out in solutions of cyclohexane and in mixtures of cyclohexane with methanol, ethanol, and pyridine (see for example, Fig. 20-1)[6] established the existence of an equilibrium of predominantly two forms existing in solutions of (I), the position of the equilibrium between these forms depending on the amount of alcohol or pyridine added to cyclohexane solution. Similarly, the uv spectra of 2-nitro-p-acetotoluidide (III), 1-nitro-2-acetonaphthalide (V), and their N-methyl derivatives, (IV) and (VI), respectively, show that similar equilibria exist also in solutions of (III) and (V) as well as in solutions of 2,4-dimethyl-6-nitro-acetanilide (VII) (Figs. 20-2 through 20-5).

The existence of internal hydrogen bonding for (III) and (V), and the lack of chelate structure in the molecule of (VII)--a lack due to steric crowding--was suggested by Chaplin and Hunter.[1] Yet, according to Forbes et al.,[4] infrared spectra seem to indicate that neither (V) nor (VII) is intramolecularly hydrogen-bonded in (chloroform) solutions. Ultraviolet spectra presented in this paper seem, nevertheless, to point out the existence of fairly strong internal hydrogen bonding in the cyclohexane solutions of (I) and (III), less strong in (V), and weak in the molecule of (VII). Therefore, the addition of increasing amounts of an alcohol or pyridine to the dilute solutions of internally bonded compounds in an inert (hydrocarbon) solvent permits a uv study of competitive hydrogen bonding due to solute-solvent interaction.

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* Postdoctoral Fellow, Department of Chemistry, 1962-1963.

1. H. O. Chaplin, L. Hunter, J. Chem. Soc. 1938, 375.

2. A. E. Lutskii, B. P. Kondratenko, Zh. Obshch. Khim. 29, 2077 (1959).

3. M. E. Runner, E. C. Wagner, J. Am. Chem. Soc. 75, 2529 (1952).

4. E. J. Forbes, K. J. Morgan, J. Newton, J. Chem. Soc. 1963, 835.

5. H. E. Ungnade, J. Am. Chem. Soc. 76, 5133 (1954); A. E. Lutskii et al., Zh. Obshch. Khim. 30, 3782 (1960).

6. The uv absorption spectra were determined with a Cary Model 14 recording spectrophotometer using 1-cm silica cells and 0.0002 M solutions. Solutions were prepared by transferring 5 ml of 0.001 M solutions in cyclohexane into 25-ml volumetric flasks, adding an appropriate amount of methanol, ethanol, or pyridine (by volume), and filling the flasks with cyclohexane.

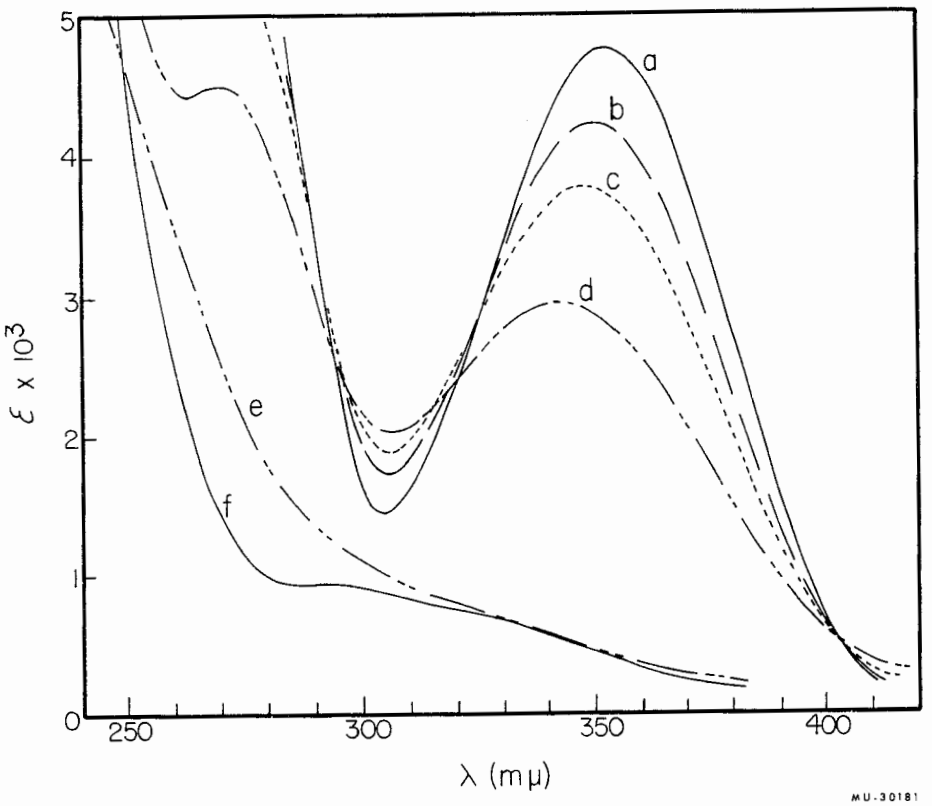


Fig. 20-1. o-Nitroacetanilide (I) and N-methyl-o-nitroacetanilide (II) (0.0002 mole/liter) in four solvents: a, f, cyclohexane; b, 92% cyclohexane–8% ethanol; c, 80% cyclohexane–20% ethanol; d, e, 20% cyclohexane–80% ethanol (by vol).

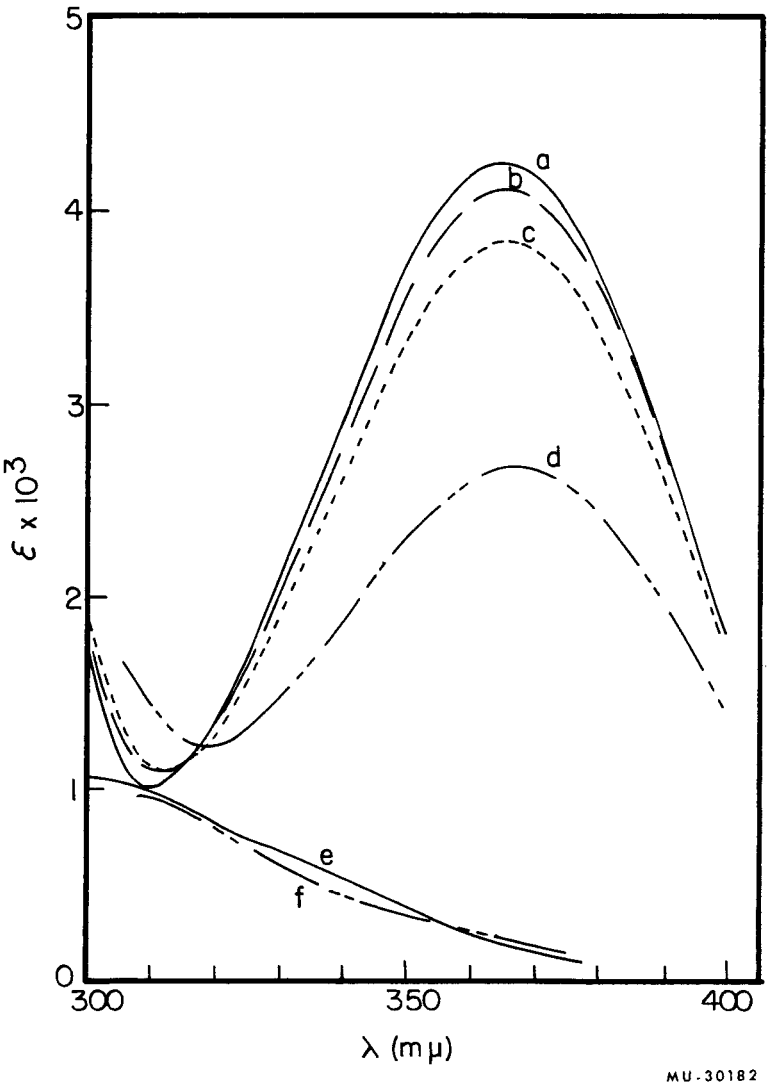


Fig. 20-2. 2-Nitro-p-acetotoluidide (III) and N-methyl-2-nitro-p-acetotoluidide (IV) (0.0002 mole/liter) in four solvents: a, e, cyclohexane; b, 96% cyclohexane-4% pyridine; c, 90% cyclohexane-10% pyridine; d, f, 20% cyclohexane-80% pyridine (by vol).

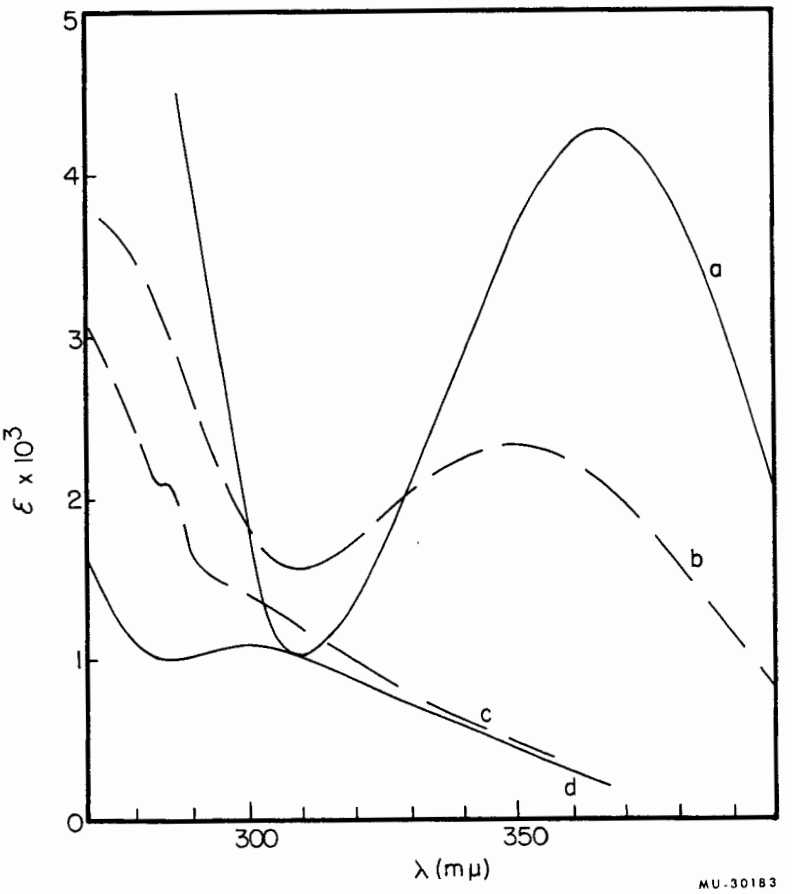


Fig. 20-3. 2-Nitro-p-acetotoluidide (III) and N-methyl-2-nitro-p-acetotoluidide (IV) (0.0002 mole/liter) in two solvents: a, d, cyclohexane; b, c, 20% cyclohexane–80% methanol (by vol).

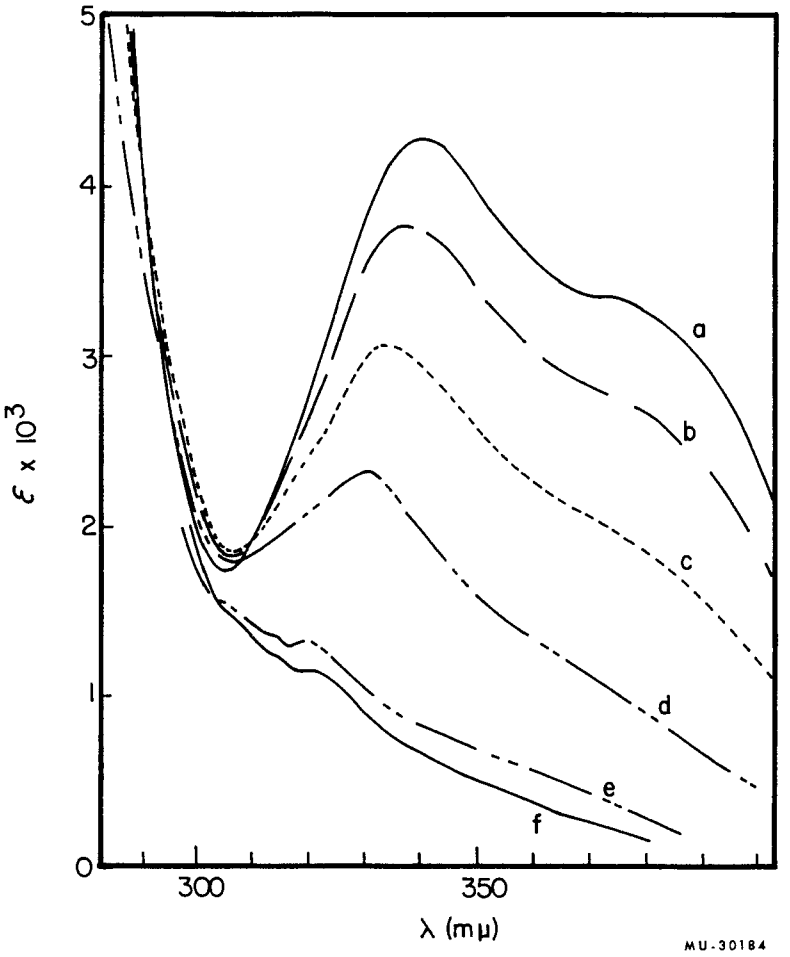


Fig. 20-4. 1-Nitro-2-acetonaphthalide (V) and N-methyl-1-nitro-2-acetonaphthalide (VI) (0.0002 mole/liter) in four solvents: a, f, cyclohexane; b, 99% cyclohexane–1% methanol; c, 96% cyclohexane–4% methanol; d, e, 20% cyclohexane–80% methanol (by vol).

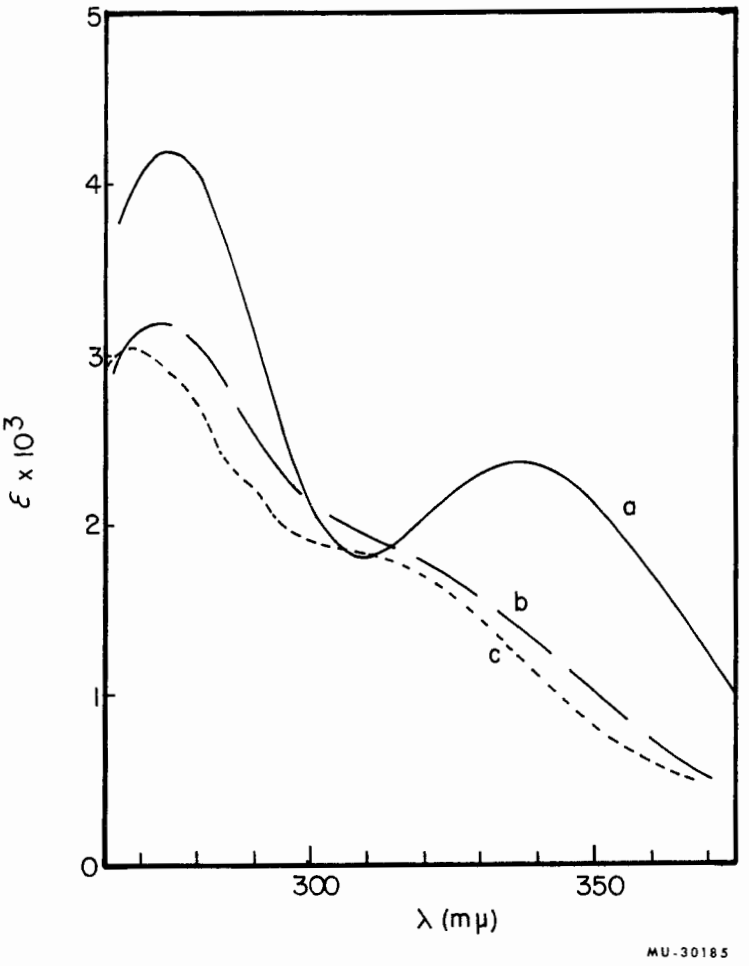
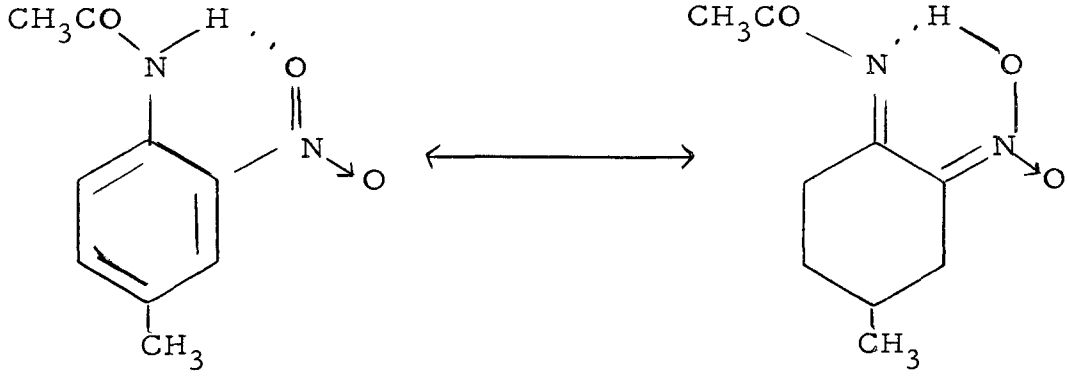


Fig. 20-5. 2,4-Dimethyl-6-nitroacetanilide (VII) (0.0002 mole/liter) in three solvents: a, cyclohexane; b, 20% aq. methanol (by vol); c, methanol.

It is known that 2-nitro-p-acetotoluidide (III) may exist in two different crystalline forms: stable white (mp 95°), and metastable yellow (mp 93.5°).[7,8] These two forms have the same uv solution spectra, but their uv and ir solid spectra differ considerably (Fig. 20-6).[9] It was also established that the yellow form of (III) may, by prolonged grinding or mixing with KBr, be partially or completely converted into the white form (Fig. 20-6).[10] These facts seem to confirm that the yellow form of (III) [closely corresponding to the only existing yellow form of (I)], exists in the solid state as strongly internally hydrogen bonded species (see below).[11]



Contrary to this, the white form of (III) probably possesses the true amide structure stabilized by intermolecular hydrogen bondings.

Further systematic studies of a number of 2-nitroacetanilides substituted in the para position are now in progress. The results of these studies will be published later.

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7. L. Gattermann, Chem. Ber. 23, 1733 (1890).

8. K. Schaum, Ann. Chem., 462, 194 (1928); the existence of the third dark yellow form (which is quickly transformed into the pale yellow one) was reported there.

9. The preliminary studies have shown a similarity between the uv spectra of the yellow form of (III) in solid state and in cyclohexane solution. There is also a resemblance between solid spectra of (IV) and the white form of (III). These measurements were carried out on suspensions of the solid compounds in Nujol and in a highly viscous perfluorocarbon.

10. For infrared studies on the influence of the time of mixing the samples with KBr, a WIG-L-BUG Amalgamator (Crescent Dental Mfg. Co., Chicago, Ill., Model 5A) was used, and the mixing time was varied within the limits of 30 to 300 sec. After 5 min of mixing the yellow form of (III) with KBr, the ir spectrum was completely identical with the spectrum of the white form. No changes due to mixing with KBr were observed in the case of (I), (V), (VII) and several other o-nitroacetanilides. Infrared spectra were determined on a Perkin-Elmer Model 237 instrument.

11. The closure of the chelate ring probably strongly stabilizes the quinonoid structure.

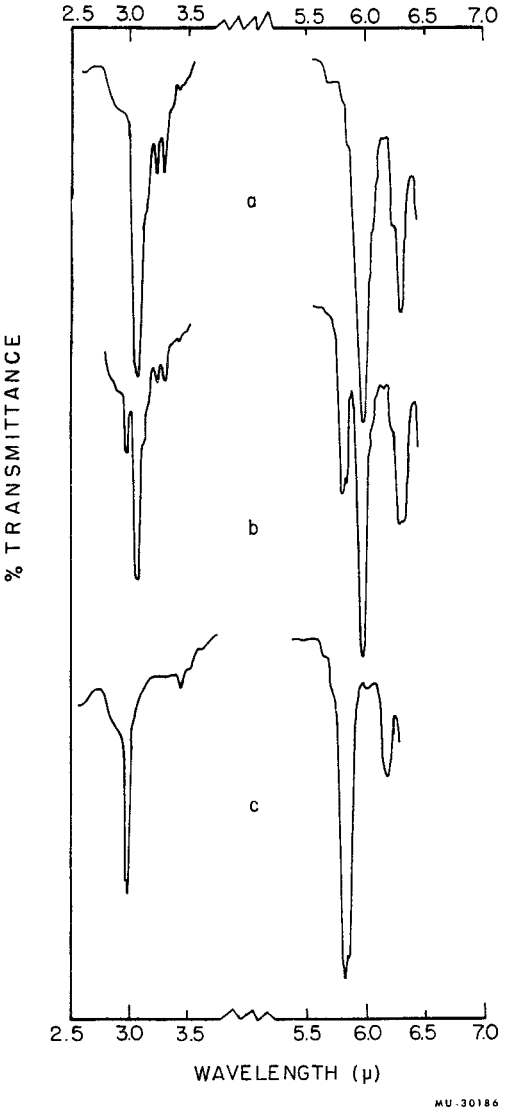

Fig. 20-6. Infrared spectra of solid 2-nitro-p-acetotoluidide (III) pressed in KBr: a, white form shaken with KBr for 30 sec; b, c, yellow form shaken with KBr for 120 and 30 sec, respectively.

Experimental Procedure

o-Nitroacetanilidide (I), 1-nitro-2-acetonaphthalide (V), and 2,4-dimethyl-6-nitroacetanilide (VII) are commercially available materials which were recrystallized before use. 2-Nitro-p-acetotoluidide (III) was obtained by acetylation of the commercially available free amine with acetic anhydride. The low-melting yellow form (mp 93.5 to 94°) and the high-melting white form (mp 95 to 96°) have been obtained, according to Gattermann.[7,12]

Good yields of N-methyl derivatives (II), (IV), (VI) of (I), (III), and (V), respectively, were prepared by the method developed by Pachter and Kloetzel.[13] The structure was confirmed by the melting points, fairly close to those described in the literature, and by the results of microanalyses and ir spectra.[14]

Analysis. Calcd for $C_9H_{10}N_2O_3$ (II), mp 70 to 71.5° (lit. mp 70°): C, 55.67; H, 5.19; N, 14.43. Found: C, 55.67; H, 5.10; N, 14.55.

Calcd for $C_{10}H_{12}N_2O_3$ (IV), mp 65 to 66° (lit. mp 64°): C, 57.68; H, 5.81; N, 13.46. Found: C, 57.88; H, 5.83; N, 13.37.

Calcd for $C_{13}H_{12}N_2O_3$ (VI), mp 114 to 115° (lit. mp 112 to 113°): C, 63.92; H, 4.96; N, 11.47. Found: C, 64.16; H, 4.98; N, 11.55.

Acknowledgment

The author wishes to thank Professor Melvin Calvin for his many helpful comments made during the course of this work.

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12. All melting points are uncorrected.

13. I. J. Pachter and M. C. Kloetzel, J. Am. Chem. Soc. 74, 1321 (1952).

14. All microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley.

21. REFLECTION SPECTRA OF BIO-ORGANIC MATERIALS IN THE REGION 2.5 TO 4 μ AND THE INTERPRETATION OF THE INFRARED SPECTRUM OF MARS*

Donald G. Rea, Theodore Belsky, and Melvin Calvin

Infrared reflection spectra have been recorded for a large number of inorganic and organic samples, including minerals and biological specimens, for the purpose of interpreting the 2.5 to 4 μ spectrum of Mars. A previous suggestion that the Martian bands indicated the presence of carbohydrates is shown not to be a required conclusion. However, no satisfactory explanation is advanced and the problem remains unsolved.

The observational evidence for the existence of life on Mars is examined. The life interpretation seems to have been unduly stressed, and alternative inorganic explanations appear at least equally probable. One prominent observation—the darkening wave that is dependent on the season—can be attributed only to a biological phenomenon when the associated temperatures are taken into consideration.

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*Abstract of paper to be published in the proceedings of the COSPAR meeting held at Warsaw, Poland, in June, 1963.

22. SOME OBSERVATIONS ON THE ESR OF CRYSTALLINE CHLOROPHYLL a AND CRUDE MIXTURES OF CHLOROPHYLL a WITH NORMALLY ASSOCIATED PIGMENTS

A. F. H. Anderson and M. Calvin

We have already described the preparation of a highly purified crystalline chlorophyll a, on which we made some preliminary electron-spin resonance (ESR) observations.[1] The electronic properties of organic solids are known to be dependent on the purity of and order in the crystals. Therefore, we compare the simple characteristics of the ESR signals (in the form of films) observed in the crystalline chlorophyll a with those obtained from crude mixtures of chlorophyll a with other pigments.

We have no detailed knowledge of the crystal structure of chlorophyll a, but two pictures are presented to show the form of the small microcrystals (Fig. 22-1) and the larger microcrystals (Fig. 22-2). The small microcrystals, which are approximately 20 Å thick, are probably two monolayers interacting by their phytyl chains or by the chlorin groups, and the larger crystals are probably well-ordered composites of the small microcrystals stacked together.

The ESR was carried out on an isooctane suspension that contained a distribution of both these crystallites, the optical properties of which will be described elsewhere.[2] About 0.25 ml of a heavy suspension of chlorophyll a crystals in isooctane was smeared on a small quartz plate to give a 1.5×0.5-cm thin film of chlorophyll a isooctane evaporated. The crystallinity of the chlorophyll a was still intact, as shown by x-ray diffractometry. The sample was illuminated by passing light from a 1000-W projection lamp through 4 cm of water to remove ir radiation, and then through a Corning 2030 filter which transmits red light from 648 to 750 mμ. The chlorophyll a crystals were at the same temperature as the cavity of the spectrometer. The chlorophyll a crystals showed a small symmetrical dark signal in the region of $g \approx 2$, with a line width of 15 gauss. Shining red light on the sample induced a symmetrical signal of $g \approx 2$ and similar line width to the dark signal, which took about 15 min to reach a steady-state intensity of about four times that of the dark signal. When the light was turned off there was a slow decay of the signal, which after 10 h had not returned to the intensity of the dark signal. The shapes of the ESR signals in dark and light are compared in Fig. 22-3, and the kinetics of the production and decay of the signal in Fig. 22-4. Two different preparations of chlorophyll a showed similar behavior.

Sogo, Jost, and Calvin made preliminary experiments on methanol extracts of Chlorella algae, which showed that adsorbed vapors (wet air vs dry nitrogen) on the surface of the extract were required for the production of light-induced ESR signals.[3] We have extended these observations with

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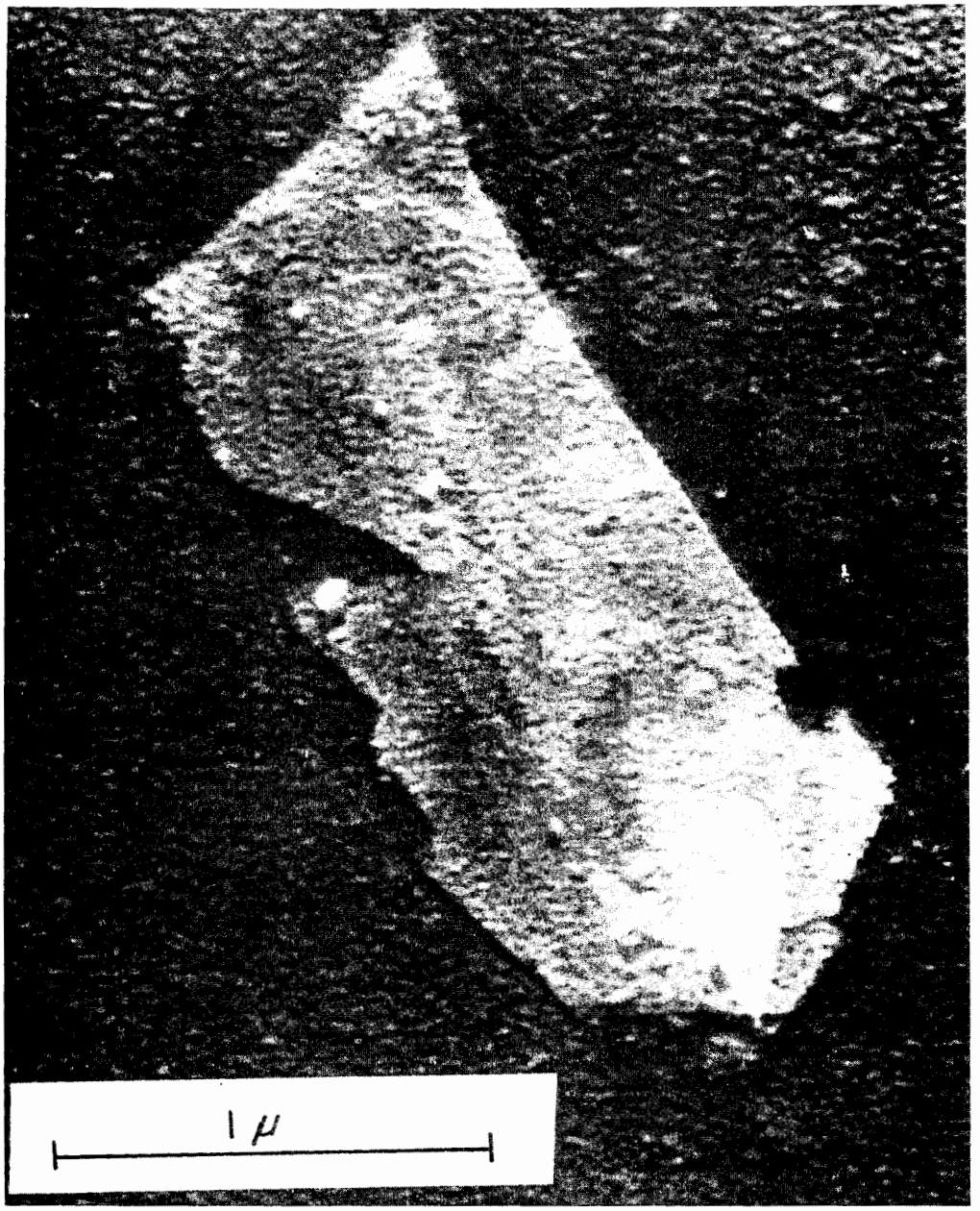
1. A. F. H. Anderson and M. Calvin, Nature 194, 285 (1962).

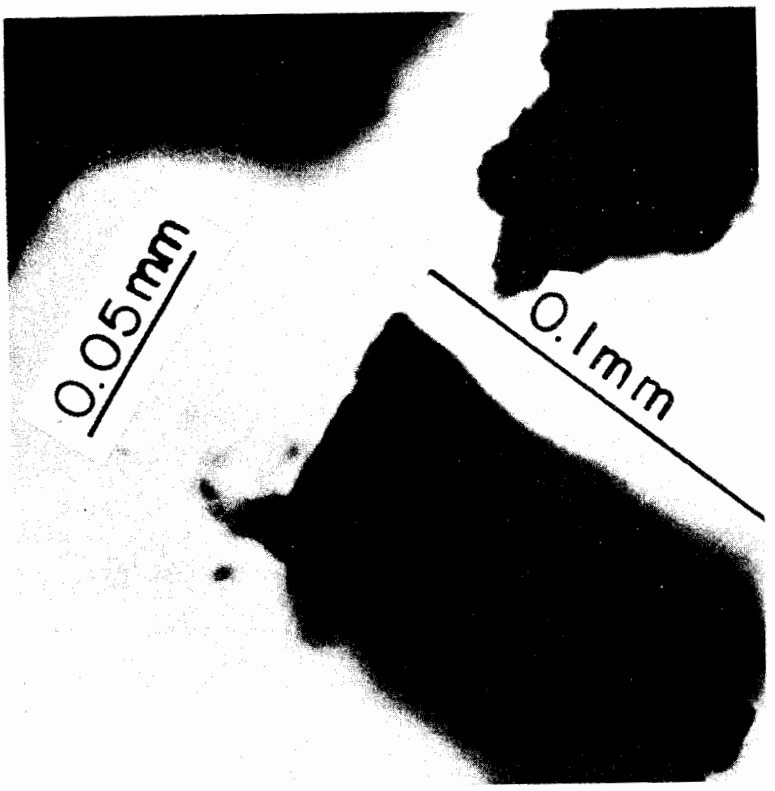
2. A. F. H. Anderson and M. Calvin, manuscript in preparation.

3. P. B. Sogo, M. Jost, and M. Calvin, Radiation Res., Suppl. 1, 511 (1959).



ZN-3900

Fig. 22-1. Electron micrograph of chlorophyll a crystals. (Photograph taken by Terry Trosper and R. B. Park of this Laboratory).



ZN-3866

Fig. 22-2. Optical micrograph of chlorophyll a crystals.

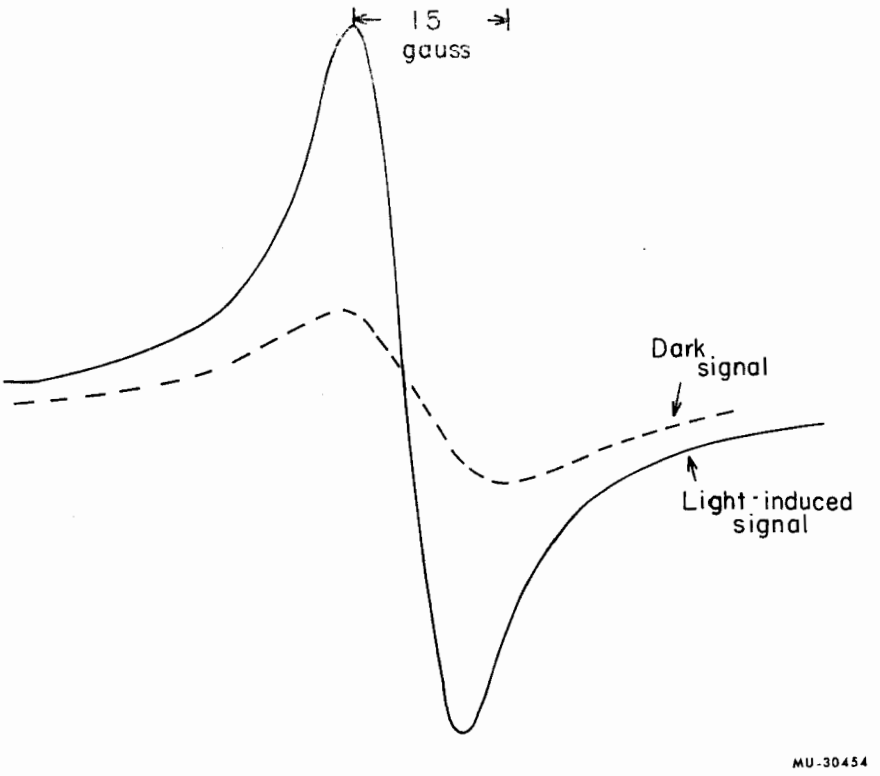


Fig. 22-3. ESR signals in chlorophyll a crystals.

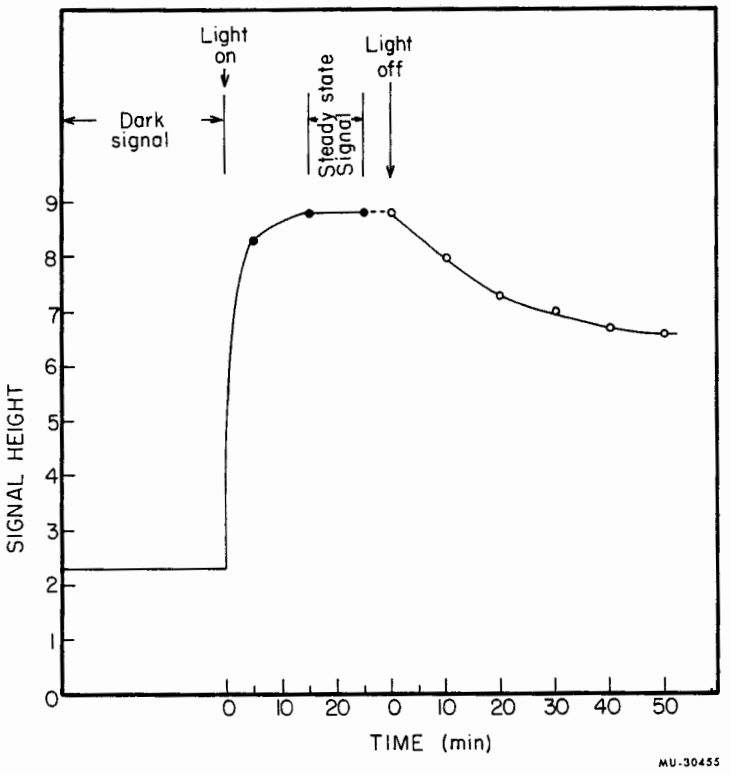


Fig. 22-4. Time dependence of ESR signals in chlorophyll a crystals.

the following experiment. Chlorella algae were washed twice with distilled water and then extracted with 100% methanol at room temperature. The extract was made into samples suitable for testing in the ESR spectrometer in three ways: (a) the solvent was removed from the extract, and the resultant sticky mass of pigments (chlorophyll a associated with chlorophyll b, carotenoids, and quinones) was applied to a silvered copper rod; (b) the solid mass of pigments obtained in (a) was treated with 2 ml of petroleum ether, which was then evaporated off in a cylindrical quartz tube (8 mm o.d.) to give a 0.5-mm-thick and 1.5-cm-long film of pigments on the bottom of the tube; (c) a phase separation of the methanol extract was carried out with petroleum ether, which took up the chlorophylls and carotenes, leaving the bulk of the xanthophylls in the methanol. The petroleum ether phase was then made into a film as in (b).

Films prepared by method (a) gave only small dark signals when dry, but when treated with water vapor gave white-light-induced signals; these were not reproducible in their intensities and decay times but had a constant line width of about 6 gauss.

Films prepared by method (b), where the preparation was carried out in dim light and the film had been degassed for 6 h at 10 microns, gave small dark signals, and white-light-induced signals could not be obtained. When the same film was exposed to varying vapor pressures of deoxygenated water vapor, then white-light ESR signals were induced. Each addition of water vapor was carried out after the sample had been degassed and shown not to have light-induced signals. Since the line width of the signals remained constant as they decayed from their steady-state value, the concentration of spins was taken to be proportional to the signal height. A typical white-light-induced signal is shown in Fig. 22-5, the steady-state concentration of photoinduced spins as a function of water-vapor pressure in Fig. 22-6, and the rates of decay of the spins at given water vapor pressures in Fig. 22-7. The steady-state population of unpaired spins in the film goes through a maximum when the ambient water-vapor pressure is 4.7 mm. For a given method (b) of producing the sample films, the decay rates, as shown by the slopes of the curves in Fig. 22-7, are relatively independent of the different vapor pressures. However, a sample prepared by method (c), and with ambient water-vapor pressure equal to 4.7 mm Hg, showed the equilibrium amplitude (value at t = 0) and the rate of decay to be markedly different from signals produced by the film (b), as shown by the curve V in Fig. 22-7. A solution of the extracted pigments in petroleum ether did not give light-induced signals.

It would be presumptuous to draw any strong conclusions from the two experiments described above, but they have clearly demonstrated the importance of two variables in the ESR properties of chlorophyll a; first, the necessity of water for the production of signals from crude chlorophyll a; and, second, the effect of purity on the signals from chlorophyll a. Holmogorov et al. have also shown that water is necessary for the production of spins in preparations of chlorophylls.[4] Because we can induce signals

4. V. E. Holmogorov, A. N. Sidorov, and A. N. Terenin, Dokl. Akad. Nauk. U.S.S.R. 147, 954 (1962).

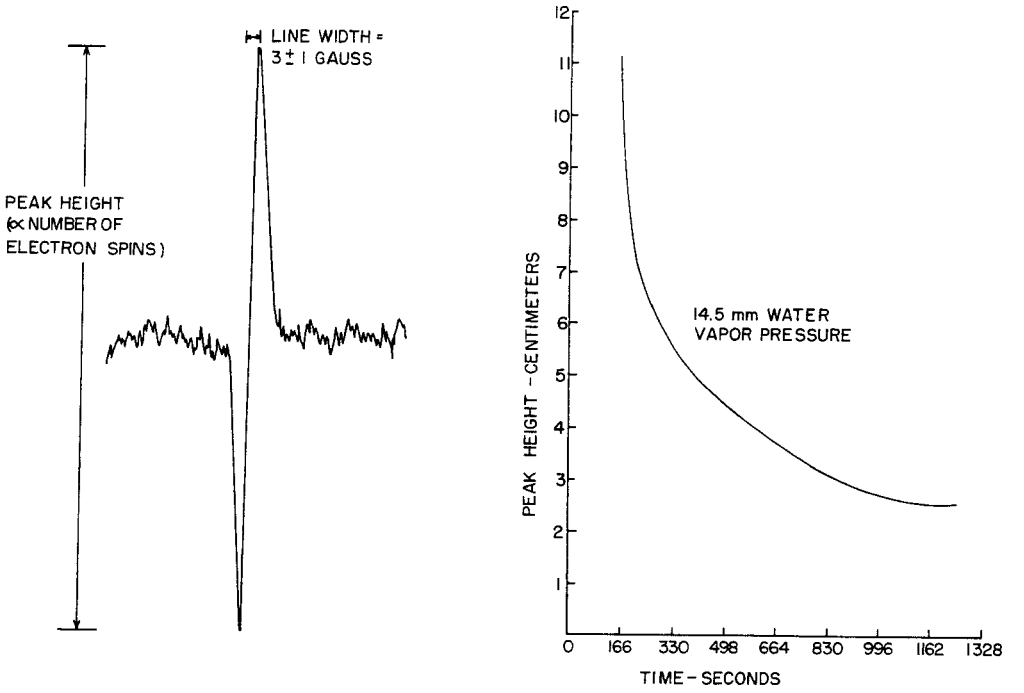


PHOTO INDUCED ESR OF METHANOL EXTRACT OF *CHLORELLA*

MU-19450

Fig. 22-5. Typical ESR signal from plant extract.

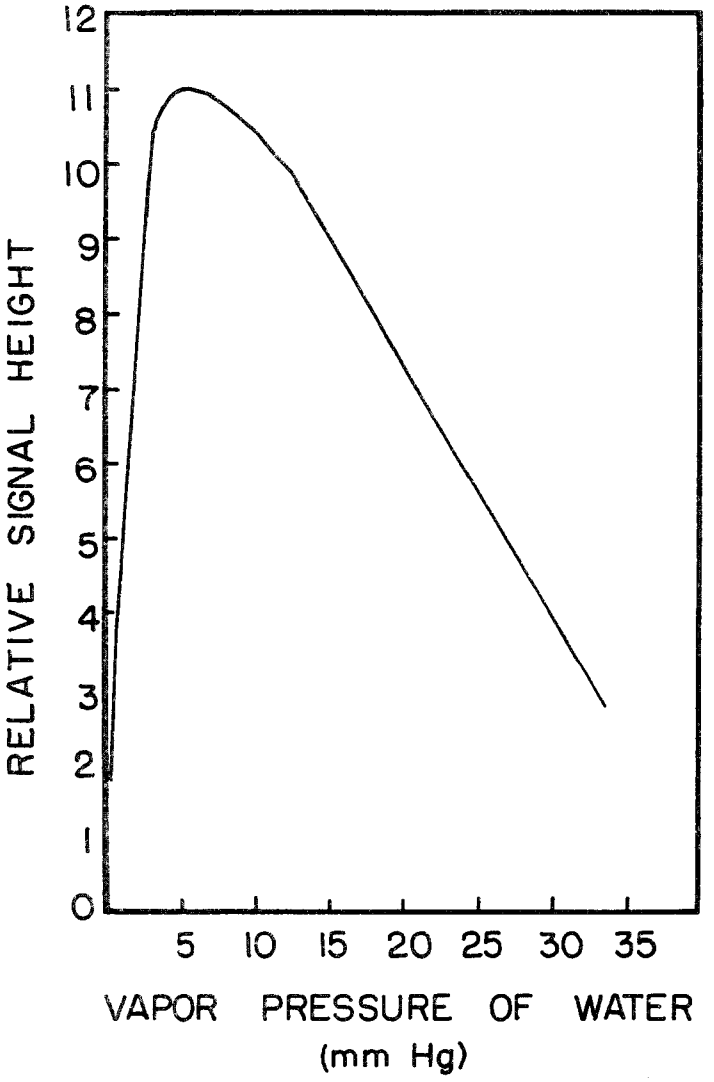


Fig. 22-6. Concentration of spins in an extract as a function of water-vapor pressure.

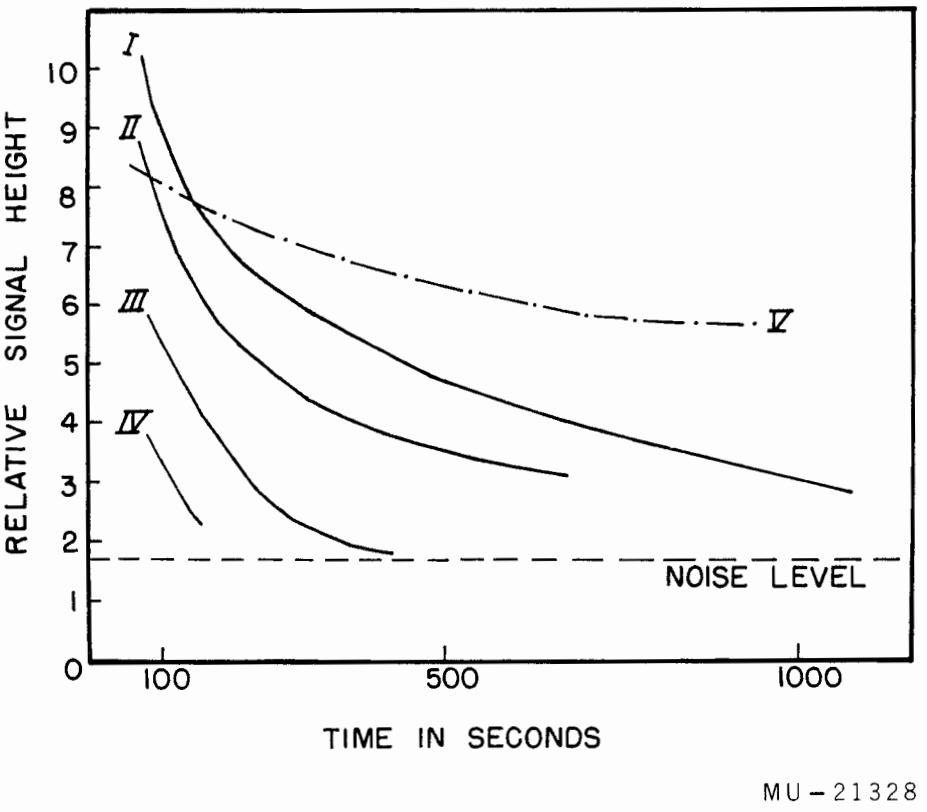


Fig. 22-7. Rates of decay of spins in an extract at a given water-vapor pressure. I, 4.67 mm Hg H_2O; II, 14.5 mm Hg H_2O; III, 19.8 mm Hg H_2O; IV, 31.8 mm Hg H_2O; V, 4.67 mm Hg H_2O.

in crystalline chlorophyll a without the addition of water, we feel that water may be making small crystallites or micelles of chlorophyll a in the films that give rise to the signals. A similar conclusion has recently been reached by Terenin to interpret the effect of water vapor on what he reported to be originally crystalline chlorophyll.[5] The possibility that a group of water molecules may act as an electron acceptor[6] is still with us, but should be investigated on samples of chlorophyll a whose crystallinity and purity are established.

The character of the signals we obtained from our crystalline chlorophyll a differs from those obtained by Brody et al.[7] using crystals prepared by a different procedure. Whereas the shape of the dark- and light-induced signal are the same in our crystalline sample, indicating the presence of one type of electron, those reported by Brody are markedly different, suggesting the presence of an impurity in his preparation (possibly xanthophyll) or different crystallinity. The light-induced signal in our avowedly impure films is much narrower (approx 3 gauss) than that produced in the crystals. The signals in the impure sample more closely resemble those seen by the other workers.

Only studies on highly pure crystalline forms of chlorophyll a can be deemed significant in determining the nature of photoinduced electrons in chlorophyll a itself, and indeed all of its electronic properties.

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5. V. Holmogorov and A. Terenin, Naturwissenschaften 50, 299 (1963).
6. J. Weiss, Nature 186, 751 (1960).
7. S. S. Brody, G. Newall, and T. Castner, J. Phys. Chem. 64, 554 (1960).

23. THE INFLUENCE OF STATIC ELECTRIC AND MAGNETIC FIELDS ON THE OPTICAL PROPERTIES OF POLYMERS*

Ignacio Tinoco, Jr., and C. Allen Bush

[Abstract of a paper in Biopolymers, Symposium Section (in press)]

Synopsis

The optical properties of molecules in static electric and magnetic fields are reviewed. The influence and effect of these fields upon the (a) energy levels, (b) electron wave functions, (c) distribution among energy levels, and (d) orientation of molecules is discussed. The rotation of light in electric and magnetic (Faraday rotation) fields is considered in detail. Faraday rotational strengths are defined and their magnitudes are discussed. The class of molecules that should give large Faraday rotational strengths is pointed out. Equations are presented which can be used to predict the change of Faraday rotation with conformation.

*Work supported in part by Public Health Service Research Grant GM 10840 and by an unrestricted grant from Research Corporation.

24. THE INTERACTION BETWEEN Mn^{IV} HEMATOPORPHYRIN IX AND WATER*

Paul A. Loach and M. Calvin

Mn^{IV} hematoporphyrin IX, prepared from Mn^{III} hematoporphyrin IX by addition of one equivalent of a strong oxidant in alkaline solution, reverts to Mn^{III} hematoporphyrin IX in the absence of added reducing agents and at a rate which is strongly pH-dependent. A study of the kinetics revealed a first-order dependence upon the concentration of Mn^{IV} hematoporphyrin IX at pH 13 and second-order dependence at pH 10. The $\Delta H^{\ddagger}$ = 20 kcal/mole and $\Delta S^{\ddagger}$ = - 25 eu were determined at pH 13.

Evidence for the formation of a percarboxylic acid was provided by infrared spectra. Free molecular oxygen or hydrogen peroxide was not formed under the conditions of the reaction. A scheme is presented to account for the observations.

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*Abstract of paper submitted to J. Am. Chem. Soc., June 1963.

25. VARIATION OF ELECTRON PARAMAGNETIC RESONANCE SIGNALS OF PHOTOSYNTHETIC SYSTEMS WITH THE REDOX LEVEL OF THEIR ENVIRONMENT*

Paul A. Loach, Gaylord M. Androes, Ann Maksim, and M. Calvin

The redox dependence of the light-induced electron paramagnetic resonance signal at g = 2 in R. rubrum, R. spheroides, and Chromatium chromatophore particles and quantasome particles from spinach chloroplasts has been qualitatively determined over the range -0.3 to +0.6 volt and quantitatively determined over the range +0.3 to +0.6 volt. A light-induced electron paramagnetic resonance (EPR) signal has been titrated and demonstrated to have a midpoint potential, E_m, of +0.44 volt at pH 7 and 20° C. No concentration dependence over a 20-fold range or pH dependence from 5 to 10 was found for this transition in R. rubrum chromatophores. A plot of the E_m for the transition vs log ionic strength revealed a linear dependence with higher E_m values occurring at higher ionic strength. In addition to the dark signal which complementarily replaces the light signal, another dark signal, occurring in the same location as the light signal, has been demonstrated to occur at high potential.

Selective chemical oxidation with K_2IrCl_6 of chromatophore particles from each bacteria resulted in the removal of about 95% of the absorbance in the near infrared due to supplementary pigments and left all photoactive pigments.

Two light-induced EPR signals were found in quantasome particles by their dependence upon the redox level; of particular interest is a signal observed at quite high potential--e.g., +0.6 volt. It was demonstrated that oxygen evolution occurred at the same rate at +0.55 volt as at +0.40 volt.

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*Abstract of paper submitted to J. Photochem. and Photobiol., June 1963.

26. ABSORBANCE CHANGES IN BACTERIAL PHOTOSYNTHESIS*

Irwin D. Kuntz, Jr., Paul A. Loach, and Melvin Calvin

The magnitude and the rise and decay kinetics of photoinduced absorption changes in bacterial chromatophores (from R. rubrum, R. spheroides, and Chromatium) were studied at the redox potential of the solution was varied from -0.3 to +0.8 volt. No reversible light-produced changes could be observed above +0.5 V or below -0.1 V at the wavelengths studied (400 to 950 mμ). The loss of photosignals at the higher potential was centered at +0.435 volt, and followed a one-electron transition. Photo-oxidizable material lost at any given potential in this range (+0.3 to +0.55 V) was quantitatively replaced by material that was chemically oxidized. A similar replacement was not apparent as the potential was lowered below 0 volt.

Chromatophore solutions maintained above +0.55 volt showed irreversible bleaching of the bulk pigments. The material obtained after loss of 90% of the near ir and visible absorption still retained the full complement of photo-induced absorption changes except those associated with the carotenoids. These bleached chromatophores showed similar redox properties to the unaltered chromatophores. In addition, a redox bleaching centered at about +0.60 V and associated with an absorption band at 850 mμ could be detected in R. rubrum.

Kinetic studies on deaerated chromatophore solutions of R. rubrum revealed no differences in the rise times at 433, 792, and 865 mμ, decay being some 6 to 8 times as fast as that at 433 mμ. These differences must reflect different pigment systems. The simplest kinetic analysis of the rise and decay reactions is a first-order (or psuedo-first-order) forward reaction and two parallel first-order (or pseudo-first-order) decay reactions. Rise times proved insensitive to the environmental redox changes. However, the decay constants increased five- to tenfold as the potential was lowered below 0 volt. Concentrations of ferrocyanide above 0.1 M had a similar effect. The increased rates are not sufficient to explain the loss of photosignal below -0.15 V.

The results are interpreted as supporting and extending the concept of a photosynthetic unit.

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*Abstract of paper submitted to Biophys. J., June 1963.

27. PRELIMINARY LOW-TEMPERATURE EPR STUDIES

Donald R. Gentner

Numerous problems have been encountered in the attempt to see photo-induced electron paramagnetic resonance (EPR) signals in biological systems at liquid helium temperature. The bulk of the effort is still being applied to instrumentation and modification of the existing spectrometer, including installation of a new 60-kc AFC unit and a superheterodyne detection system.

A reversible photoinduced EPR signal was previously observed in Rhodospirillum rubrum at 77° K.[1] In a preliminary run at 4° K, an EPR signal was observed in R. rubrum chromatophores. Upon illumination, however, no change in the signal was observed. By comparison with results at 77° K, only a small light-induced increase would have been expected, and it may have been below the sensitivity of the spectrometer. The instrument has now been considerably improved, and the experiment will be repeated in the near future.

A by-product of this work has been the development of a promising new standard for calibration of EPR spectrometers. It consists of Cr^{+3} atoms in a magnesium oxide lattice. In the cubic lattice of magnesium oxide, the EPR spectrum of chromium is a single line with a g value of 1.98.[2] (There are also four satellite peaks of low intensity due to the Cr^{53} isotope with 9.5% natural abundance.) The line width is controlled by dipolar broadening: a ratio of chromium to magnesium of 1 to 10^3 gives a half-width of about 5 gauss. Briefly, the standard is prepared by scavenging a solution of magnesium chloride with magnesium metal for about 3 h. This is then filtered, and magnesium hydroxide is precipitated from the filtrate by ammonium hydroxide. Centrifugation and washing with distilled water three times, followed by heating, yields purified magnesium oxide. The oxide is redissolved in dilute hydrochloric acid, a known amount of chromic nitrate is added, and the magnesium oxide is precipitated, centrifuged, washed, and heated as before. The magnesium oxide powder, which now contains chromium as a lattice impurity, is mixed with a polyethylene powder and hot rolled, producing a convenient sample which can be cut and weighed as needed.

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1. G. M. Androes, M. F. Singleton, and M. Calvin, Proc. Natl. Acad. Sci. U.S., 48, 1022 (1962).

2. J. E. Wertz and P. Auzins, Phys. Rev. 106, 484 (1957).

28. THEORETICAL AND INSTRUMENTAL COMMENTS ON MAGNETIC OPTICAL ROTATORY DISPERSION (MORD)

Edward Dratz and James Thorne

The phenomenon of magnetically induced optical rotation was first observed by Faraday in 1845.[1] In 1854 Verdet[2] found that the rotation θ is equal to VHL[3] at a single wavelength. Since that time Faraday rotation has been observed by a few scattered investigators.[4-7] However, the published data are as yet too scanty to permit useful empirical correlation between molecular structure and Faraday rotation. The theoretical development has gone quite far,[8-10] but this work has been hampered by lack of experimental data to check existing theory.[8] The current theory predicts that a good deal of useful information may be derived from a careful study of the wavelength dependence of the magnetic rotation (MORD).

Recently Shashoua reported a detailed MORD spectrum rising from a broad featureless visible absorption band.[11] Initial studies in this Laboratory demonstrated that Shashoua's results were largely instrumental artifacts.[12] The original instrument constructed in this Laboratory was useful but not sensitive enough and had a very restricted wavelength range. The recently published work has accomplished little more than to point out the many experimental difficulties associated with measurement of MORD.[11, 12] A highly sensitive MORD instrument is being constructed and is described in this report.

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1. M. Faraday, Phil. Trans. London 136, 1 (1846).

2. Verdet, Compt. Rend. 39, 548 (1854).

3. Here H is the field strength directed along the direction of propagation of the light; L is the sample length for a pure material; V is a constant of proportionality known as the Verdet constant; θ is the rotation of the plane of polarization.

4. A. Cotton and M. Schener, Compt. Rend. 195, 1342 (1932).

5. F. H. Garner, C. W. Nutt, and A. Labbuf, J. Inst. Petrol. 329 (1955).

6. J. R. Partington, review in Advanced Treatise on Physical Chemistry, IV, (Longmans, Green and Co., London 1953) pp. 592-632.

7. L. R. Ingersoll and D. H. Liebenberg, J. Opt. Soc. Am. 46, 538 (1956).

8. I. Tinoco, Symposium on Quantum Aspects of Polypetides and Polynucleotides, Stanford, March 1963, to be published in Biopolymers.

9. M. P. Groenewege, Mol. Phys. 5, 541 (1962).

10. R. Serber, Phys. Rev. 41, 489 (1932).

11. V. E. Shashoua, J. Am. Chem. Soc. 82, 5505 (1960).

12. R. Williams and G. Androes, in Bio-Organic Chemistry Quarterly Report, UCRL-10032, January 1962, pp. 32-44.

Many experimental and theoretical similarities exist in the study of Optical Rotatory Dispersion (ORD)[13] and MORD. Measurements far from molecular absorption bands include large contributions from all the electronic transitions in the molecule in both phenomena. MORD measurements in an absorption band are due mainly to the absorption band of interest, and other nearby bands give relatively small contributions. Therefore, just as in ORD, the greatest amount of specific molecular information may be derived from measurement of rotation in absorption bands.

Non-optically active materials as well as optically active ones can be studied by MORD. The assignment of electronic transitions in complicated molecules may be aided by determining the sign and magnitude of rotation due to the various bands in the spectra. Conformation changes and geometrical structures of optically inactive or weakly active systems may show specific effects in MORD. A short résumé of the basic theory for molecules of low symmetry (less than the threefold axis) and paired electrons will clarify further discussion of the above proposals. The theory for ORD developed by several workers can be used,[14, 15] and the effect of the magnetic field introduced as a perturbation on the wave function of the molecule. In this regard, the formalism developed by Tinoco for MORD is especially useful.[8]

For electronic transitions o (ground state) → a (excited state), the rotation is proportional to

$$\theta \alpha \sum_{a \neq o} \frac{I_{oa}}{\nu_a^2 - \nu^2} .$$

For natural rotation, $I_{oa} = R_{oa} = \mathrm{Im}\ [\underset{\sim}{\mu}_{oa} \cdot \underset{\sim}{m}_{oa}]$ = rotational strength.

For Faraday rotation,

$$I_{oa} = F_{oa} = \mathrm{Im}\left[\underset{\sim}{\mu}_{oa} \cdot \left\{ \sum_{b \neq a} \frac{\underset{\sim}{\mu}_{ob} \times \underset{\sim}{m}_{ab}}{h(\nu_b - \nu_a)} - \sum_{b \neq o} \frac{\underset{\sim}{\mu}_{ab} \times \underset{\sim}{m}_{ob}}{h\nu_b} \right\} \right] =$$

Faraday rotational strength.

For naturally optically active substances in a field, $I_{oa} = R_{oa} + F_{oa}$, where $\underset{\sim}{\mu}_{ij} = -\underset{\sim}{\mu}_{ji} = \int \psi_i^* \underset{\sim}{\mu} \psi_j \, d\tau$ = electric dipole transition moment,

$\underset{\sim}{m}_{ji} = \underset{\sim}{m}_{ij} = \int \psi_i^* \underset{\sim}{m} \psi_j \, d\tau$ = magnetic dipole transition moment,

and $\sum_{b \neq i}$ is a sum over all excited states not equal to i.

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13. C. Djerassi, Optical Rotatory Dispersion, (McGraw-Hill Book Co., New York, 1960).

14. A. Moscowitz, Advan. Chem. Phys. 4, 1 (1952).

15. M. J. Stephen, Proc. Cambridge Phil. Soc. 54, 81 (1958).

The expressions governing the optical properties of interest can be cast into the following simple forms:

$$\text{Absorption:}\quad D_{oa} = (\mu_{oa})^2 = \text{dipole strength},$$

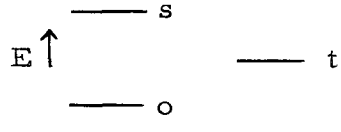
$$\text{Rotation:}\quad R_{oa} = \mathrm{Im}[\underset{\sim}{\mu}_{oa} \cdot \underset{\sim}{m}_{oa}],$$

$$\text{Faraday rotation:}\quad F_{oa} = \mathrm{Im}\left[\underset{\sim}{\mu}_{oa} \cdot \left\{ \sum_{b \neq a} \frac{\underset{\sim}{\mu}_{ob} \times \underset{\sim}{m}_{ab}}{\Delta E_{ab}} - \sum_{b \neq o} \frac{\underset{\sim}{\mu}_{ab} \times \underset{\sim}{m}_{ob}}{E_b} \right\}\right].$$

The transition moments may be considered vectors located on a molecule. Faraday rotation depends on three molecular vectors and the angles between them. Natural rotation depends on two molecular vectors and on an angle. It can be expected that both natural rotation and Faraday rotation are more sensitive to molecular structure differences than absorption spectra, because MORD and ORD depend on a more detailed combination of molecular parameters than does absorption. Conformation changes will affect the relative orientation of transition moments and therefore affect F_{oa}. Note that F_{oa}, unlike R_{oa}, is insensitive to screw axis--that is, inversion of the molecule does not affect the magnetic rotation. MORD should be useful in distinguishing mixtures of optically active enantiomorphs from nonoptically active molecules--e. g., a mixture of right- and left-handed polypeptides from a random coil.

A point of considerable interest is the Faraday rotational strength of a singlet-triplet transition relative to the F_{oa} for a singlet-singlet transition. The detailed theory has not been worked out, but an approximate consideration of the problem will be presented.

Consider the energy level scheme, with the ground state a singlet and the $o \rightarrow s$ transition fully allowed:



Typically $D_{ot} \approx 10^{-6}$ to $10^{-8}\ D_{os}$, and therefore $(\mu_{ot}) = (10^{-3}$ to $10^{-4})(\mu_{os})$.

If the transition moments are mutally perpendicular and m_{st} is taken to be one Bohr magneton,

$$F_{ot} = \mu_o \cdot \mu_{os} \times m_{st},$$

$$F_{ot} \approx (10^{-3} \text{ to } 10^{4})(\mu_{os})^2 \cdot m_{st},$$

and F_{ot} will be 10^{-3} to 10^{-4} times as strong as a fully allowed Faraday rotational strength F_{os}.

It may be concluded that $F_{ot}/F_{os} = (10^3 \text{ to } 10^4)D_{ot}/D_{os}$ in the most favorable case. The Faraday rotation then may be about 1,000 to 10,000 times as sensitive to singlet-triplet transition as are absorption spectra.

The foregoing discussion points out that transitions that are weak in absorption may be relatively strong in rotation, or vice versa. Therefore the ratio of F_{oa}/D_{oa} and the sign of F_{oa} yield much more distinctive information on a transition than does D_{oa} alone. It may be determined, for example, whether closely spaced peaks in an absorption spectra are of vibrational structure or are separate electronic transitions.

The theoretical predictions discussed above have yet to be proved experimentally. The main block to investigation has been the difficult instrumentation involved. The instrument that we have constructed to study the phenomena is described below.

The goal of instrumental sensitivity is $\pm 10^{-4}°$ with a range of 12,000 to 1,850 Å. A schematic drawing of the instrument appears in Fig. 28-1.

Because of the extreme sensitivity of the instrument, it must be very rigidly mounted. The spectrometer is secured to an optical bench made of 2-in. steel bars clamped in a very stiff frame. The light sources are 900-watt xenon or 1,000-watt Xe-Hg dc high-pressure arcs (Hanovia). The light source used is focused on the slit of a Cary 14 prism-grating monochromator (Applied Physics) by a first-surfaced elliptical mirror (Bausch & Lomb). This combination gives a high-intensity light beam in the wavelength range of interest, and has less than 35 Å/mm bandwidth and about 10^{-4}% stray light. The light from the monochromator is collimated by a first-surfaced spherical mirror to maintain achromaticity. All the reflecting optics are magnesium-fluoride-overcoated to maintain uv reflection efficiency. The polarizer is a calcite Glan-Thompson or crystal quartz Rochon prism (Crystal Optics, Chicago, Illinois). The polarized light passes through a 5-cm cell filled with water mounted inside a commercial solenoid. An alternating magnetic field in the solenoid modulates the plane of polarization by the Faraday effect. The solenoid is capacity-coupled in a series-resonant circuit to a 250-watt power amplifier, a 60-watt preamplifier, and a signal generator. The azimuth of polarization is modulated at 740 cps about $\pm 5°$ at 3,500 Å. The light then passes through two large dc magnets with their 1,750-gauss fields opposed.

The sample is placed in the first field, the solvent in the second. The second field, containing the solvent cell, subtracts directly the rotation of the cell and solvent holding the sample. This direct subtraction is important because the rotation of the solvent is typically 100 to 1,000 times that of the solute. Differences in field strength and shape and the difference in lengths of the cells are compensated by placing both cells filled with solvent in the magnets and setting the net rotation to zero by trimming the field in the sample magnet with a variable shunt resistor.

A compensator magnet containing a water cell will be used to rotate the plane of polarization an amount equal and opposite to the sample rotation. The light passes through an analyzer, the azimuth of which is set at $90°$ to the polarizer, and then through a lens to the photomultiplier detector

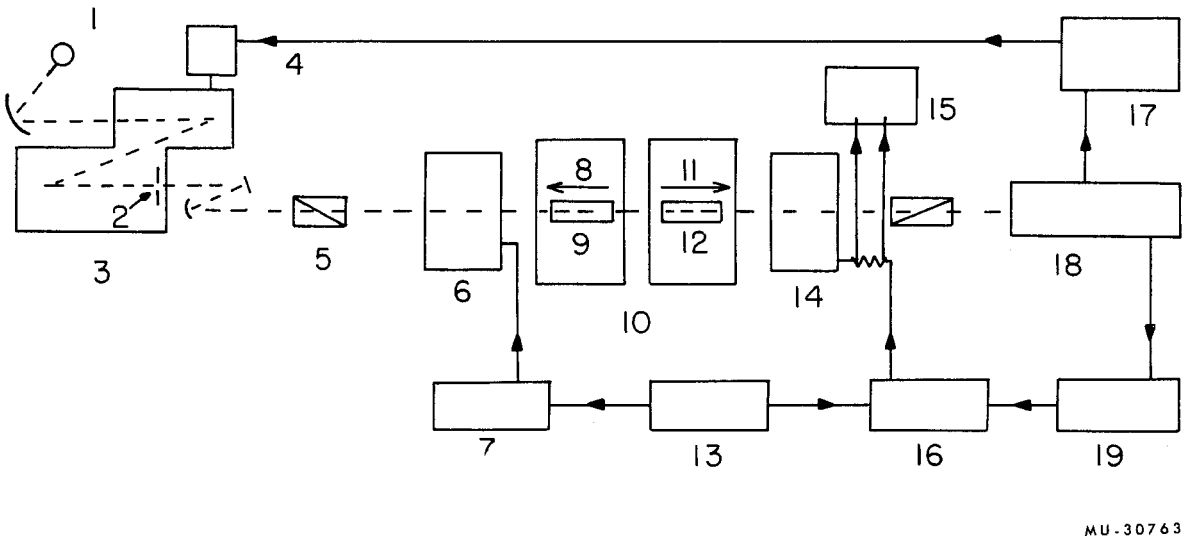


Fig. 28-1. 1. dc Arc lamp. 2. Monochromator exit slit. 3. Cary Model 14 monochromator. 4. Slit width control. 5. Polarizer. 6. Modulator. 7. Power amplifier. 8. Field direction in sample magnet. 9. Sample cell. 10. 1,750-gauss magnets. 11. Field direction in solvent magnet. 12. Solvent cell. 13. Signal generator. 14. Compensator. 15. Recorder. 16. Lock-in amplifier. 17. Slit servo amplifier. 18. Photomultiplier tube. 19. Preamplifier.

(EMI 6256B, 9558B). The photomultiplier signal is ac-coupled to a battery-powered low-noise preamplifier (Tektronix Type 122) with the bandpass set at 80 to 1,000 cps, and is fed into a lock-in amplifier along with a phase-corrected signal from the modulation oscillator. The bandwidth of the lock-in is adjustable from 10 to 0.05 cps. The lock-in acts as a phase-sensitive rectifier, and its output, which is proportional to the sign and magnitude of the rotation, is read on a VTVM (vacuum tube voltmeter, Kiethly 610A). The VTVM acts as a dc amplifier with a gain of about 1,000; the output will be fed into the programmable power supply driving the compensator magnet. The current in the compensator magnet will be determined from the voltage drop across a precision series resistor. The compensating current will in turn be placed on a strip chart recorder as the wavelength is scanned linear with time. The sign of the rotation will be recorded, although the peak magnitudes of rotation will have to be corrected for the approximate inverse-square wavelength dependence of the Verdet constant of the compensating material. A chopper-stabilized dc amplifier and a servo motor control the slit width of the monochromator to maintain constant energy on the photomultiplier detector, thereby avoiding phase shifts in the electronics ahead of the lock-in.

The signal picked up by the photomultiplier will be dependent on the angle between the polarizer and analyzer, the sum of the rotations of sample and compensator, and the modulation angle and frequency.

F_o is the light flux emerging from the polarizer; θ is the sum of the angle of rotation of the sample, the angle of rotation of the compensator, and the angle between the polarizer and analyzer. At null, $\theta = 90^\circ$; ϵ is the instantaneous angle of modulation.

The basic equation is the $\cos^2$ law of Malus for light flux transmitted by a pair of polarizers:

$$F = F_o \cos^2 (\theta + \epsilon). \tag{1}$$

Expansion by trigonometric identities gives

$$F = 1/2\ F_o(\cos\ 2\theta \cos\ 2\epsilon - \sin\ 2\theta \sin\ 2\epsilon + 1). \tag{2}$$

The angle ϵ has the time dependence

$$\epsilon = K \sin wt. \tag{3}$$

The terms in Eq. (2) containing functions of ϵ can be expanded to

$$\sin \epsilon \approx \epsilon,$$

$$\cos \epsilon \approx 1 - \epsilon^2/2. \tag{4}$$

This manipulation leads to a useful expression,

$$F = 1/2\ F_o(1 + \cos\ 2\theta - K^2 \cos 2\theta + 2K^2 \cos\ 2\ wt \cos 2\theta - 4K \sin\ 2\theta \sin wt). \tag{5}$$

The dc terms are not needed and the ac terms are. Thus,

$$F = F_o K^2 \cos 2wt \cos 2\theta - 2 F_o K \sin 2\theta \sin wt.$$

The phase-sensitive rectifier is locked to the fundamental frequency component sin wt. Setting the sin wt term to zero sets $\theta = 90°$, nulling the instrument. Note that the sign of rotation as well as the magnitude is sensed by the sin wt sin 2θ term.

The termination of the expansions of sin ϵ and cos ϵ are not exact and inclusion of higher terms will give rise to higher harmonics. For example, if sin ϵ is taken to be $\sin\epsilon = \epsilon - \epsilon^3/6$, an extra term of the form arises:

$4/3 \sin 2\theta \, K^3 \sin^3 wt$, and gives $\frac{F_o K^3}{2}$ (sin 2θ sin wt - 1/3 sin 2θ sin 3 wt).

The terms for the higher harmonics will be greatly reduced in amplitude because of the K^n dependence, since K is small. It can be seen that all the odd harmonics approach zero as θ approaches $90°$. The even harmonics behave like the second harmonics with a cos 2θ amplitude dependence; moreover, the phase detector eliminates the even harmonics.

If the modulation signal is larger than the sum of preamplifier noise and analyzer light leak, the sensitivity can be shown to be independent of modulation amplitude.

At null, the ac signal is

$$F = F_o K^2 \cos 2wt.$$

Any sample rotation, θ, will introduce a small term,

$$F_o K \sin 2\theta \sin wt.$$

Assuming that the noise is predominantly shot noise in the photomultiplier detector, the noise is proportional to the square root of the intensity. Essentially all the noise will be introduced by the large cos 2 wt term. If the magnitude of K is doubled, the amplitude of the sin wt term produced by sample rotation will be doubled; however, the magnitude of the cos 2 wt term will be increased four times, and the associated shot noise will double. The net result is that this signal and noise increase by the same amount in response to increased modulation amplitude.

The measurements of rotation in an absorption band are difficult because of the low light levels. It is important to know what the optical density of the sample should be for optimum sensitivity. The dependence of light flux on sample extinction is given by $F_o = F'_o e^{-Elc}$, where E = extinction coefficient, l is sample length, and c is the sample concentration. For solutions, the Faraday rotation is given by $\theta = lcHV$.

The signal of interest is

$$S = F'_o e^{-Elc} K \sin 2\theta \sin wt,$$

and integrating over one modulation cycle, one finds that the signal is proportional to

$$S \propto F'_o e^{-Elc} \cdot K \sin 2\theta.$$

The change in signal with change in optical density at constant E, H, and V is

$$\partial S/\partial(lc) \propto K F'_o e^{-Elc} (2HV \cos 2\theta - E \sin 2\theta).$$

The noise is again assumed to be predominantly due to the shot effect and is introduced by the 2wt term. The noise is integrated over the cos 2wt modulation, and since θ is always very close to 90°, the cos 2θ has a magnitude of very nearly unity. The noise, then, is proportional to the following expressions:

$$N \propto (F'_o e^{-Elc} K^2)^{1/2}$$

$$N \propto F'_o e^{-Elc/2}.$$

The change in signal with change in optical density at constant E, F'_o is

$$\partial N/\partial(lc) \propto F'_o \frac{KE}{2} e^{-Elc/2}.$$

The maximum signal-to-noise ratio can be found from

$$\partial S/\partial N = \frac{\partial S/\partial(lc)}{\partial N/\partial(lc)} \propto \frac{F'_o}{2} e^{-Elc/2} (\sin 2\theta - \frac{2HV}{E} \cos 2\theta) = 0,$$

$$\tan 2\theta = \frac{2HV}{E} \text{ and } \tan 2\theta \approx 2\theta = 2HV\,lc.$$

It may be concluded that Elc = 1 (that is, an optical density of 1) is the optimum condition for maximum signal-to-noise ratio. It is possible to show with a more detailed calculation that the condition $0.5 \leq OD \leq 1.5$ maintains 90% of the signal-to-noise ratio, and that the signal-to-noise ratio drops off rather rapidly beyond these limits.

The operation of the instrument at present is manual, i.e., the null measurement is made by mechanically rotating the analyzer and visually reading the angle from a divided scale with the aid of a vernier. This method allows a sensitivity of about 0.001° or ± 0.0005°. The instrument is electronically capable of doing five times as well as this under optimum light intensity conditions if the nulling is done with the magnetic compensator.

Measurements made to date have been aimed at determining the relative stability of the field in time, the suitability of commercial and home-made cells as to birefringence, and matched Faraday rotation properties. The effect of temperature on the magnetic rotation of some common solvents has been investigated to determine the precision of temperature control needed. The temperature effect includes solvent density changes and the temperature coefficient of the Verdet constant. For most solvents, if they

are not used in wavelength regions close to their absorption, temperature control to 0.5° is sufficient. The balance of the magnets and the mechanical stability of the polarimeter have given some trouble; these problems have been solved by straightforward means.

The dispersion measurements made to date have been with the fields off, to check published data on polypeptide dispersion.[16] The ORD of poly-γ-benzyl-L-glutamate (Fig. 28-2) is given. The helical form is stable in ethylene dichloride and the random coil in dichloroacetic acid.

The instrument is now in the debugging stage and should be in full automatic recording operation very soon.

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16. J. T. Yang and P. Doty, J. Am. Chem. Soc. 79, 761 (1957).

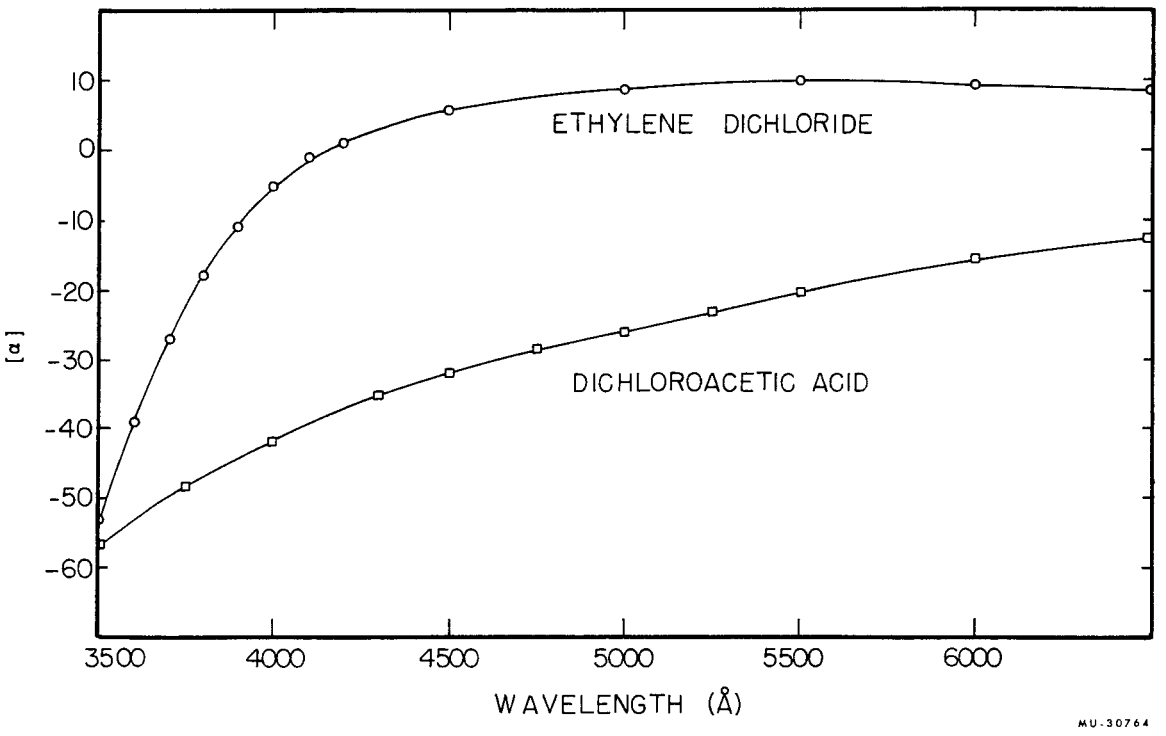

Fig. 28-2. Rotatory dispersion of poly-γ-benzyl-L-glutamate. Molecular weight 100,000 (by intrinsic viscosity measurement).

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