[CONTRIBUTION FROM THE BASIC SCIENCE RESEARCH LABORATORY, UNIVERSITY OF CINCINNATI]

CHANGES IN THE ULTRAVIOLET ABSORPTION SPECTRUM OF URACIL AND RELATED COMPOUNDS UNDER THE INFLUENCE OF RADIATIONS[1,2]

BY FRANCIS F. HEYROTH AND JOHN R. LOOFBOUROW

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As part of a program involving the correlation of both the growth-promoting and the lethal effects of ultraviolet radiations of various wave lengths with their effects upon the constituents of living cells, we have irradiated nucleic acid and its nitrogenous constituents. The paramount importance of the cell nucleus for certain vital processes makes such a study desirable.

As a criterion for following any constitutional changes induced in these materials, we have utilized their ultraviolet absorption spectra. (In work now in progress, chemical tests, including a color test with the arsenophosphotungstate reagent for uric acid are also being employed.)

A search of the literature reveals little precise information as to the absorption spectra of the pyrimidines. Dhéré in 1906 stated[3] that 4-methyluracil exhibits an absorption band between 2346 and 2801 Å. Since this is very similar to the absorption spectrum he found for yeast nucleic acid, he attributed the absorption of the nucleic acids to their pyrimidine and purine components. Damianovich[4] in 1922 presented spectrographic evidence that the ultraviolet absorption of vitamin B-containing extracts is also similar to that of pyrimidines or purines and, with Williams, has since measured more accurately the absorption of nucleic acid.[5] Frederick Gates,[6] in discussing the lethal action of ultraviolet rays upon bacteria, stated that the curve of the reciprocal amounts of energy required for killing at various wave lengths is more closely related to the absorption spectra of the pyrimidines than to those of the proteins. Macbeth, Nunan and Traill[7] have studied the somewhat similar absorption of barbituric acid, and Holiday[8] has recently reported quantitative data on the ultraviolet absorption of several purines.

Preliminary measurements upon uracil in a concentration of 2 g. per

[1] Paper presented before the Division of Medicinal Chemistry at the Indianapolis Meeting of the American Chemical Society, April 2, 1931.

[2] This research was supported in part by a grant from the National Research Council.

[3] Dhéré, *Séances et Mémoires de la Société de Biologie*, **60**, 34 (1906).

[4] Damianovich, *Anales de la asocn. quím. Argentina*, **10**, 209 (1922).

[5] Damianovich and Williams, *Anal. soc. cient. Argentina*, **98**, 241 (1929).

[6] Gates, *Science*, **68**, 479–480 (1928).

[7] Macbeth, Nunan and Traill, *J. Chem. Soc.*, 1248 (1926).

[8] Holiday, *Biochem. J.*, **24**, 619 (1930).

liter in water showed it to be light sensitive. The sample used was prepared by the method of Wheeler and Liddle[9] from thiourea and sodium formylacetic ester. After exposure for an hour and a half to a quartz mercury vapor lamp, the absorption at wave lengths longer than 3100 Å. was greatly increased over that of the non-irradiated substance. This, together with the discrepancies encountered in comparing samples of uracil prepared under different conditions of illumination, led us to compare the absorption of a sample prepared in the dark from malic acid by the method of Baudisch and Davidson[10] with that of a sample prepared under a skylight. Figure 1 shows the absorption of these preparations in a concentration of 1 g. per liter. The influence of daylight and of soft x-rays in increasing the absorption between 2900 and 3400 Å. of the preparation made in the dark is also indicated. Figure 2 shows the effect of irradiation with full ultraviolet upon a sample in a concentration of 1 g. per liter. The remainder of this paper deals with a study by improved technique and in more suitable concentrations of the course of the change induced by full ultraviolet irradiation. The curves in Figs. 1 and 2 are not quantitatively comparable with the more exact measurements reported in the remaining figures, in the making of which more highly purified materials were used.

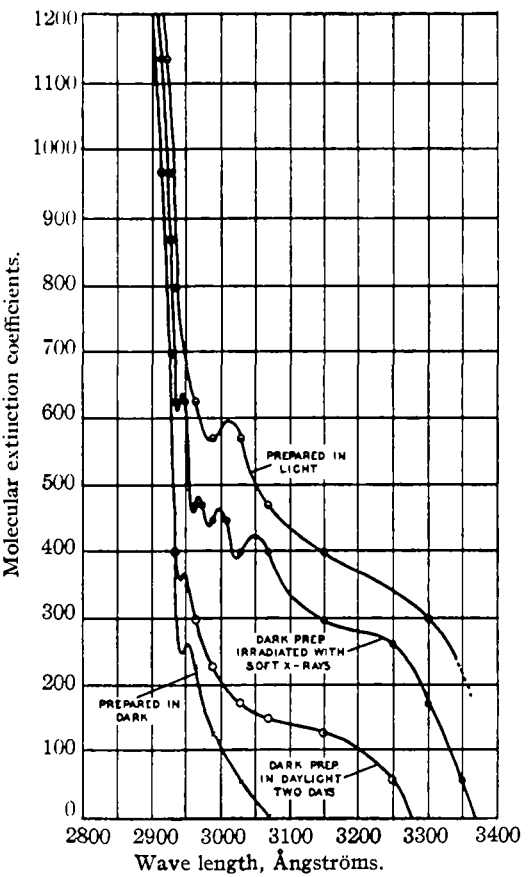


Fig. 1.

Experimental

Materials.—Uracil was prepared in a nearly dark room by the Baudisch–Davidson method and recrystallized five (and in some cases eleven) times from hot water. After

[9] Wheeler and Liddle, *Am. Chem. J.*, **40**, 547 (1908).

[10] Baudisch and Davidson, THIS JOURNAL, **48**, 2379 (1926).

the third recrystallization the mother liquors were colorless. The melting point was 337.5–338°.

2,5-Dichloro-4-methylpyrimidine was prepared by chlorinating 4-methyluracil as described by Gabriel and Colman.[11] It was purified by the aid of norite and was ten times recrystallized from petroleum ether. The crystals had no yellow color and melted at 45.0–45.2°.

Adenine sulfate was prepared by the method of Jones[12] by hydrolysis of yeast nucleic acid. It was recrystallized six times.

Thymus nucleic acid was prepared from calf thymus by the method of Jones. It gave no biuret reaction and was a very fine white dusty powder.

Methods

A. Determination of Absorption Spectra.—A modified photographic match-point method was employed. The absorption spectra were obtained by photographing solutions, of the concentrations indicated, in a 2-cm. brass cell, with quartz end-plates, by the aid of a Gaertner quartz spectrograph. The source was a high-frequency tungsten spark. On each plate (Cramer contrast) four photographs through the solution were taken with exposures of ten, twenty, fifty and one hundred seconds, together with a series of photographs through the solvent (water) in a similar cell with exposures of one to twenty seconds at intervals of one second, and twenty to thirty-five seconds at intervals of five seconds. The plates were developed by a time–temperature method with Rodinal. For greater accuracy in matching, each plate was enlarged on glossy, contrast bromide paper. To eliminate errors due to lack of parallelism of the slit, each of the four enlarged photographs of the solution was cut through the middle and matched directly upon the middle of the comparison spectra. Each spectrum of the solution was then matched at as many as possible of a series of 42 wave lengths corresponding to known lines of the tungsten spark. At each wave length the fraction of the light transmitted by the solution is taken to be equal to the ratio of the time of exposure of the comparison spectrum to the time of exposure of the spectrum of the solution. (This introduces a systematic error due to failure of the reciprocity law, which is small, however, as discussed below.) Since four photographs of the

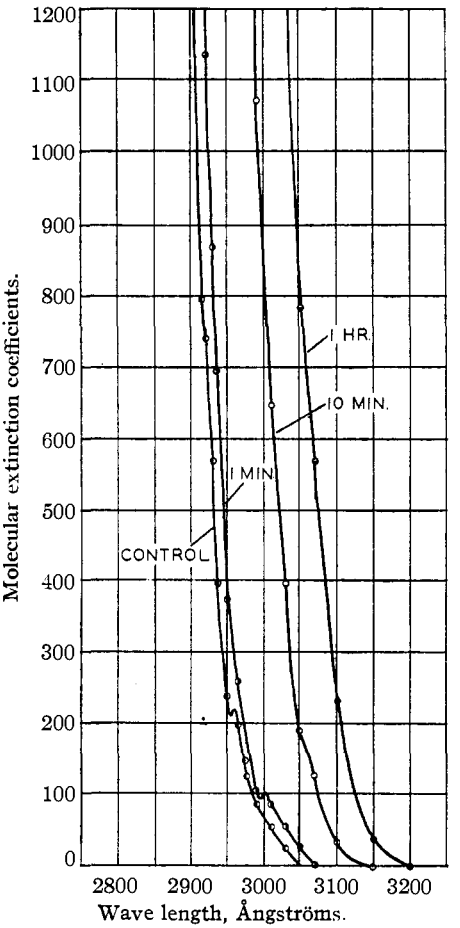


Fig. 2.

[11] Gabriel and Colman, *Ber.*, **32**, 1525 (1899).

[12] Jones, "The Nucleic Acids," 1907.

solution were taken, there were, for most wave lengths, four values for each wave length chosen. This afforded a test of the consistency of the values, and helped to eliminate errors due to over-exposure or under-exposure. The mean (with any inconsistent result eliminated) value for $-I_x/I_o$ was then plotted against wave length on semi-logarithm paper, the results immediately giving the absorption curves without calculations. These curves were traced on ordinary cross-section paper, and a scale of ordinates for the molecular extinction coefficients of each appended by calculating the value of E_m for some ordinate from the relation $E_m = -[\log (I_x/I_o)]/d \cdot e$, where e is the molar concentration, and d (=2 cm.) the thickness of the cell. A detailed discussion of the sources of error and the accuracy attainable by this modified method will be published elsewhere. It is believed that the forms of the curves, and the finer subsidiary maxima indicated, are correct to 3 or 4%. A progressive error in the absolute values is introduced by failure of the plates to follow the reciprocity law (on which is based the assumption of a proportionality between time of exposure and intensity of energy). This probably does not influence the form of the curves, and even the absolute values are not greatly affected with the particular emulsion used. The maximum error, which occurs at 2500 Å. is only about 5%.[13] By means of a curve showing the deviation from the reciprocity law at various wave lengths, the true absolute values may be calculated. No attempt has been made to make this correction in the case of the curves here shown, as we are interested in the changes of the spectra with irradiation rather than in absolute values.

B. **Methods of Irradiation.**—The cells were completely filled with solutions of the materials in boiled redistilled water. The spectra were photographed and then the solutions (without removal from the cell) were exposed at a distance of 2 cm. to the rays of a water-cooled Victor quartz mercury arc. The spectra of the solutions were rephotographed at intervals throughout the period of irradiation. The exposures of the solution to the tungsten spark used in photographing the spectra are not included in the stated times of irradiation. The total energy received during the 180 seconds of total exposure for each set of spectra is roughly equivalent to that furnished by about eighteen seconds of exposure to the Victor arc. The cumulative errors in irradiation time thus introduced are negligible in comparison with the times of irradiation. In the preliminary experiments the irradiation was done in quartz test-tubes and not in the brass absorption cell. As the results appeared to be the same in character, it is very unlikely that the effects described could be attributed to any slow action of the metal of the cell upon the solution during the course of irradiation.

The concentrations selected as best for the cell thickness used were: thymus nucleic acid, 0.035 g. per liter; uracil, 0.01 g. per liter; adenine, 0.02 g. per liter; dichloromethylpyrimidine, 0.03 g. per liter.

Results

Figure 3 shows the successive spectra of uracil during irradiation. (The denomination molecular in all these figures refers only to the original non-irradiated spectra, since nothing is known as to the concentrations or even molecular weights of the material during or after irradiation.) A broad band of absorption extends from 2300 to 2900 Å. and gradually tapers off from that point toward longer wave lengths. From the minimum at 2300 the absorption rapidly rises toward shorter wave lengths. There are three distinct maxima at 2570, 2600 and 2640, with several minor breaks in the

[13] Harrison, *J. Opt. Soc. Am.*, 11, 341 (1925).

curve on either side of these points. The validity of these maxima may be questioned because such variations in extinction approach the limits of accuracy of the method. We prefer including them to smoothing the curve because we find them throughout a given run, in different runs of the same compound, and because they occur, at slightly different wave lengths, in different compounds. The observations of Macbeth, Nunan and Traill also clearly indicate the existence of two peaks in this region of the spectrum of barbituric acid.

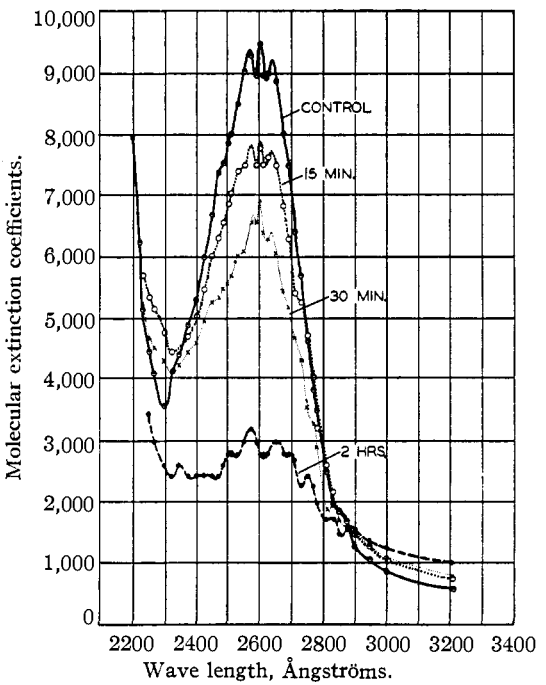


Fig. 3.

During the first few minutes of irradiation there is a definite rise in absorption in the region 2200 to 2300 Å., and in the region of wave lengths longer than 2900 Å. This type of change (effect A) is even more clearly seen in the case of thymus nucleic acid (Fig. 4), where during an irradiation of one hour there was but negligible change from 2500 to 2900 Å., while at about 2300 Å. there has been an increase of nearly 45% in extinction coefficients. The position of the minimum shifts slightly toward longer wave lengths during this period. This effect A is also evident, but less distinct, during the irradiation of dichloromethylpyrimidine (Fig. 5).

During the later periods of irradiation a different effect (B) is encountered. The absorption in the central region of the band decreases (Figs. 3, 4, 5). In the case of uracil this is noted even in the fifteen-minute expo-

sure. For a time the rise in the regions shorter than 2300 Å. and longer than 2900 Å. continues. In the case of uracil, after fifteen minutes, the absorption from 2200–2300 Å. begins to decrease, but absorption in the region longer than 2900 Å. continues to rise for three hours, after which (not shown in the figure) it declines. The other compounds, in general, show a similar behavior. The three central absorption peaks of uracil remain distinct during the first hour of irradiation. Later three new peaks appear on the leg of the curve from 2300 to the 2570 Å. maximum. Breaks at 2760 and 2830 Å., first seen in the thirty-minute sample, occur as pronounced maxima after two hours of irradiation. Very similar changes occur in the case of the other compounds.

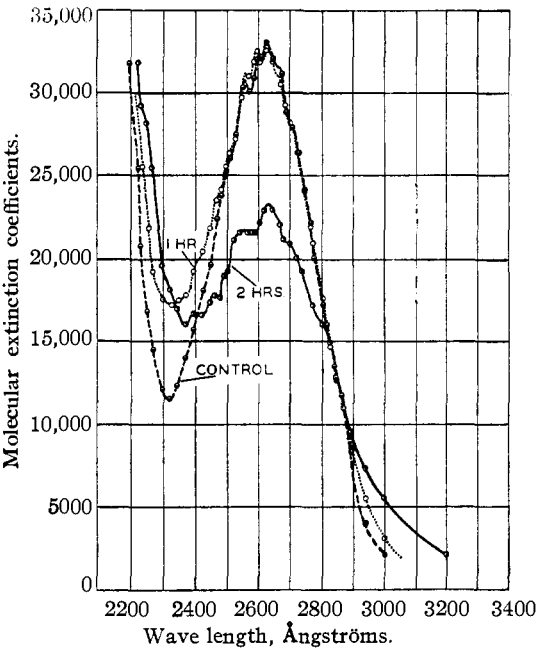


Fig. 4.

The influence of the hydrogen-ion concentration upon the absorption spectrum of non-irradiated uracil is seen in Fig. 6. The reactions were attained by adding very dilute sodium hydroxide or hydrochloric acid; these reagents were used also in the solvents for the comparison photographs. Changes in P_H on the acid side of the neutral point affect the absorption only very slightly. At P_H 2.6 the absorption minimum is possibly shifted slightly toward longer wave lengths. At P_H 7.7, the absorption is considerably lowered throughout. The 2650 peak is lowered by over 20%, the 2270 minimum drops markedly and there is a distinct tendency for the long waved leg of the curve to become more vertical.

At 2840 Å. the curve reaches zero absorption: the negative values at longer wave lengths indicate fluorescence. At P_H 9.2, there is a rise of the 2270 Å. minimum, and slight shifting toward the longer wave lengths (effect A). The absorption in the regions 2300 to 2800 Å. is decreased throughout (effect B). The central peaks are shifted toward longer wave lengths (2600 and 2620). The decrease in extinction of the third peak, originally noted at 2650 but now at 2720 Å. has continued. Fluorescence persists. At P_H 11.0 the most pronounced effect is a broadening and shifting of the whole band toward the longer waves. Effects A and B are both evident.

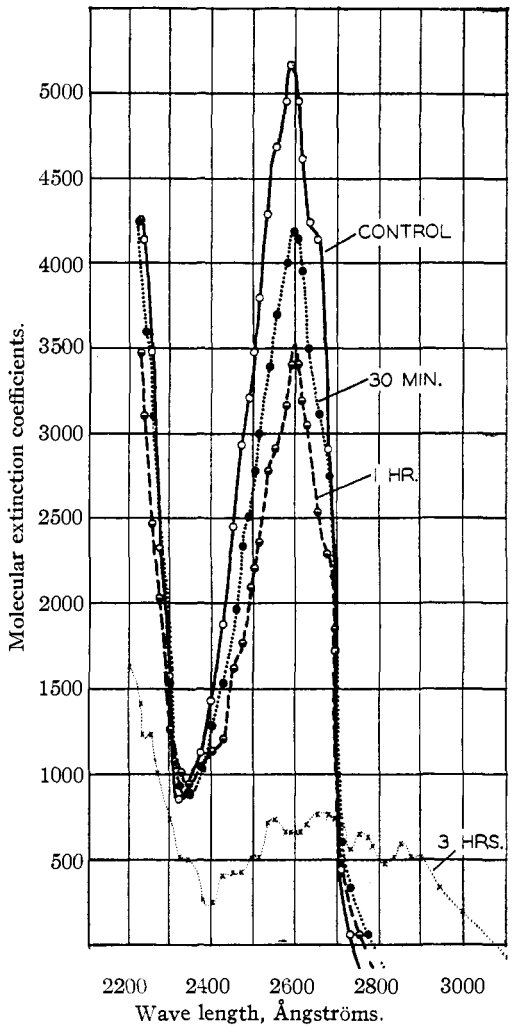


Fig. 5.

Figure 7 shows the spectrographic results of the irradiation of a purine, adenine sulfate. Unfortunately, the concentration selected did not permit the inclusion of the entire band. In this case, the first effect, noted after five minutes of irradiation, was a considerable decrease in the minimum at 2240 Å. and a decrease of the entire leg of the curve longer than 2780 Å. From five minutes to one hour, effect A predominated. The observations after three hours indicate that effect B had then become the more pronounced one, although A still continued as the absorption was increasing at both minima. Adenine sulfate also exhibits fluorescence in the long-wave ultraviolet.

Discussion

Two independent effects are evident in the spectrographs here presented. What we have called effect B, the progressive disappearance of

the selective absorption which characterizes the solutions of compounds containing the pyrimidine ring, must mean removal of pyrimidine compounds from solutions. The mechanism of this removal is at present unknown. There may be either (a) a gradual disruption of the ring with the production of diactinic products of the nature of urea and open-chain

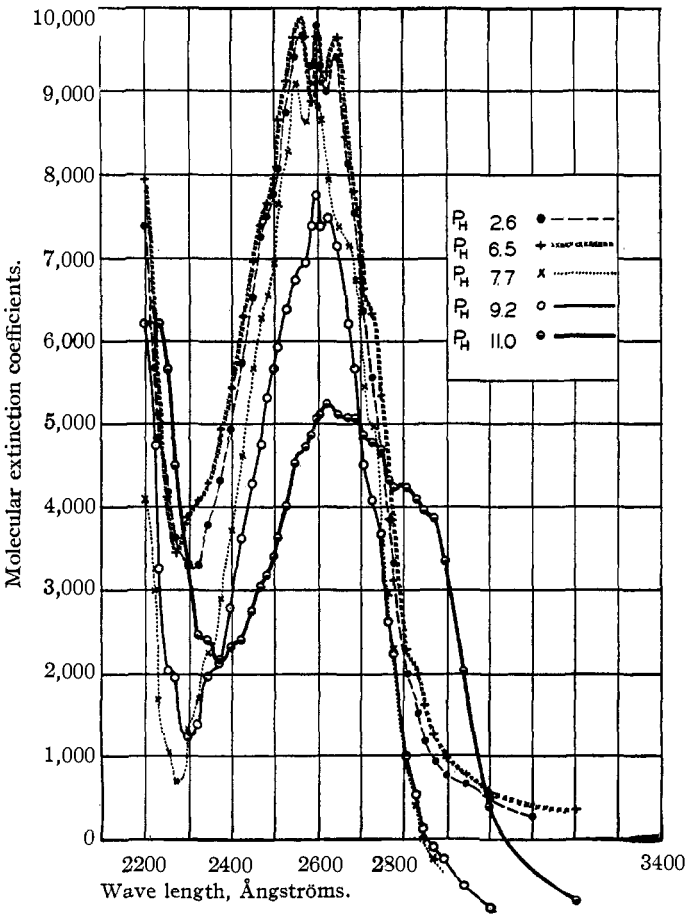


Fig. 6.

three-carbon compounds, or (b) a gradual polymerization. Stobbe[14] has found that in several compounds the effect of polymerization is to lessen the absorption in the ultraviolet. Bass[15] has shown that in the presence of ferrous salts, urea and pyruvic acid are formed in oxygen-containing solutions of thymine by irradiating for twelve hours with the total radiations from a quartz mercury lamp. As yet there is no basis for a choice between

[14] Stobbe, *Ber.*, **44**, 960 (1911).

[15] Bass, THIS JOURNAL, **46**, 190 (1924).

these hypotheses. The biological significance of the destruction of purine and pyrimidine compounds in relation to the lethal action of the ultraviolet will be discussed elsewhere.

Effect A, best seen in Fig. 4, consists in increased absorption in the regions of the minimum about 2300 Å., and in the longer wave region of low ab-

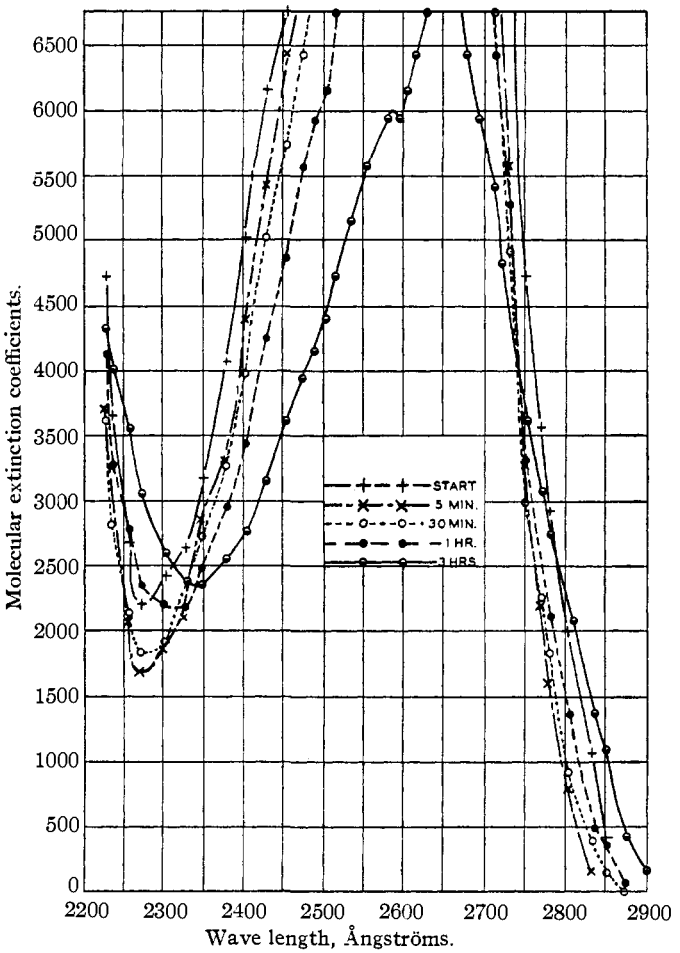


Fig. 7.

sorption. It was the only effect noted in the preliminary observations in the very restricted field shown in Figs. 1 and 2. If the effect of irradiation consisted merely in a lowering of the concentration of the material, there should be no building up in these regions. Holiday has shown Beer's law to hold over a comparable concentration range for several purines. Furthermore, the spectrographs of Damianovich and Williams for various

concentrations of yeast nucleic acid show no signs of such an effect A (increase in absorption at the minima) as a result of dilution. Quantitative considerations of the possible absorption of any decomposition products in these regions preclude such materials as the source of the effect. It would, therefore, seem that effect A is an indication of the production during the first stages of irradiation of some constitutionally altered form of the ring compounds in question. The following possibilities may be considered: (1) the production or destruction of pseudo betaine modifications; (2) the production of closely related products of rearrangement in the course of an oxidation by hydrogen dioxide produced by the irradiation of the solvent; (3) a shift in a lactam–lactim equilibrium of the pyrimidines; (4) some type of intramolecular rearrangement which produces a more unstable ring preceding its disruption or polymerization; (5) disruption of the 4–5 double bond by the addition of water with the formation of dihydropyrimidines as observed in the oxidation of thymine, and other pyrimidines.

(1) There has never been a tendency among workers in the field of the pyrimidines to assign betaine or pseudobetaine structures to these substances, but this possibility cannot be eliminated without further investigation.

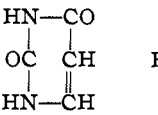
(2) In the case of a spectrographic study of the destruction of quinol by x-rays, M. Reinhardt[16] eliminated the possibility of oxidation as a cause of the change in absorption spectrum by showing that the characteristic absorption band of quinone did not appear. In our preliminary experiments it was found that diffuse daylight produced this effect A in the case of uracil. Although there is as yet no agreement as to the conditions under which hydrogen dioxide is produced from water by light, it seems improbable that daylight produces sufficient quantities to cause the observed change.

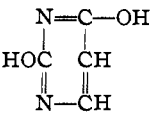
(3) That uracil may exist in lactam and lactim forms is generally recognized. The influence of the hydrogen-ion concentration shown in Fig. 5 was studied to determine whether a shift in lactam–lactim equilibrium could be detected spectrographically, and, if so, whether it could be the cause of effect A. Levene, Bass and Simms[17] have shown the pK of uracil to be 9.45 (dissociation of one lactim OH). It is thus to be expected that the transformations will be completed at reactions acid to P_H 9.5. This is borne out by the results of Fig. 5. Increase in P_H from 6.5 to 7.7 produces two marked changes: (a) the decrease in absorption at 2280 Å. and at wave length 2820 Å. and (b) the decrease in the peak at 2650 Å. A third somewhat less evident change (c) is the relative building up of the absorption of a slight inflection at 2520. These three changes are more marked at P_H

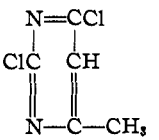
[16] Reinhardt, *J. Cancer Research*, **12**, 289 (1928).

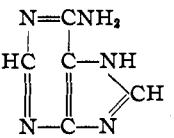
[17] Levene, Bass and Simms, *J. Biol. Chem.*, **70**, 229 (1926).

9.3. It is interesting, although possibly premature, to attempt to assign tentatively the three chief and the various subsidiary peaks, each to a site of unsaturation and therefore, source of absorption, in the mixtures of the two tautomeric molecules. There are at least five peaks which might be expected. The decrease in the 2650 peak might be attributed to the disappearance of a CO group in going from the lactam to the lactim form. The dissimilarities of the changes (a), (b), or (c), to the effect A, eliminates the lactam–lactim transformation as the cause of the change. Additional support for this contention is seen in the fact that dichloromethylpyrimidine, which cannot exist in a lactam form, also shows effect A, although to a lesser extent. The spectrum of this compound, Fig. 3, shows a striking similarity to the alkaline spectra of uracil in that fluorescence is present. This may be characteristic of the presence of three double bonds within the ring. Adenine is also fluorescent.


Lactam


Lactim


Dichloromethylpyrimidine


Adenine

Both effects A and B appear to occur at the higher alkalinities of uracil. That alkali should partially destroy pyrimidines is very likely in view of its destructive action upon uric acid. Feulgen[18] has demonstrated that long treatment of thymus nucleic acid with alkali changes it to a non-gelling β-form.

(4) Blanksma[19] has shown that daylight converts acetylchloroaminobenzene in aqueous solution into chloroacetanilide, and Porter[20] has shown that the change occurs even in the case of the dry crystals. Blanksma has stated that it "appears to be a general rule that radicals Br, Cl, NO_2, NO and O attached to nitrogen change places under the influence of sunlight with a hydrogen atom in the nucleus." A somewhat different intramolecular transformation which might be expected to lead to the production of a less stable form of uracil is a migration of the hydroxyl group from position 6 of the lactim form to position 5. This would increase the weighting of the C atom which is joined to 4 by a double linking, and might therefore be expected to increase the extinction and at the same time lead to a less stable form. The resulting compound, analogous to isobarbituric acid, would be the labile "α-isouracil" which Tafel and Houseman[21] isolated from the products of the electrolytic reduction of uric acid.

Although T. B. Johnson and Caldwell[22] stated in 1929, "various chemi-

[18] Feulgen, *Z. physiol. Chem.*, **90**, 261 (1914); **91**, 165 (1914); **104**, 189 (1918).

[19] Blanksma, *Rec. trav. chim.*, **21**, 366 (1902).

[20] Porter, THIS JOURNAL, **49**, 2145 (1927).

[21] Tafel and Houseman, *Ber.*, **40**, 3743 (1907).

[22] Johnson and Caldwell, THIS JOURNAL, **51**, 873 (1929).

cal aspects of the vitamine problem have stimulated an interest in the chemistry of iso-uracil and its derivatives," no one except Tafel and Houseman has described this substance. In the case of the dichloromethylpyrimidine a similar migration of chlorine might conceivably occur, but less readily. During the first five minutes of irradiation of adenine there is a marked lessening of the absorption about the minima, after which effect A begins immediately. This may indicate a preliminary breaking of the auxiliary ring from position 5, after which an intramolecular migration of the amino group from position 6 to 5 might occur.

That the loss of a double bond should produce a symmetrical increase in extinction seems unlikely. Of the five suggested ways of accounting for the effect A, the fourth seems at present the most likely. A number of ways of testing this hypothesis of an intramolecular rearrangement in the cases of other pyrimidines and purines are readily available, and will be applied in the near future. Attempts are being made to isolate Tafel's α-isouracil from solutions of uracil irradiated at definitely limited wave lengths, and also to test such solutions for growth-promoting potency

Comparison of the Absorption Spectra of the Non-Irradiated Compounds.—It is readily apparent that, as Dhéré stated, the absorption spectrum of nucleic acid is dependent on those of its purine and pyrimidine components, and that the absorption of the purine components is primarily due to their content of pyrimidine rings. There is great similarity between the absorption of uracil, adenine and thymus nucleic acid. The latter, due to its content of two purines and two pyrimidines, has a more complex group of subsidiary peaks in the region of the maximum. In calculating the molecular extinction coefficient of this compound the molecular weight has been taken as 1459.

Only 523/1459 of the molecular weight of thymus nucleic acid (the purine and pyrimidine fraction) represents the fraction contributing to the absorption. Therefore, the molecular extinction at the maximum would be about 523/112 times that of uracil, or 45,360. It was found to be about 33,000, indicating that the weighting of the nitrogenous complexes by the non-absorbing sugar and phosphoric acid diminishes the absorption somewhat. However, adequate criteria for the purity of thymus nucleic acid are not available. In the case of dichloromethylpyrimidine, the introduction of the two chlorine atoms similarly lowers the absorption to 5000 at the maximum. In neither case does the introduction of non-absorbing substituents into the purines or pyrimidines cause more than a very slight shifting along the wave length scale.

We wish to acknowledge the assistance of Mr. Parke Goode, Miss Agnes Helmar, Miss Naomi Frech and Mr. Joseph Maas in the experimental work.

Summary

1. The ultraviolet absorption spectra of uracil, dichloromethylpyrimidine, adenine, and thymus nucleic acid are presented. In each case, the effect of ultraviolet irradiation upon these absorption spectra has been followed.

2. Two effects of irradiation are noted. One, occurring early, consists chiefly in increased absorption in the regions of low absorption adjacent to the absorption band. A second, and later, effect consists in the gradual loss of all noteworthy selective absorption of these materials.

3. Possible explanations of the mechanism of these effects are critically discussed.

4. Possible biological implications of the changes described are alluded to briefly.

CINCINNATI, OHIO

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF UNIVERSITY OF ILLINOIS]

STEREOCHEMISTRY OF PHENYLQUINONES. THE PREPARATION AND RESOLUTION OF 2-(3-BROMO-2,4,6-TRIMETHYLPHENYL)-5-METHYLBENZOQUINONE-3,6-DI-(ACETIC ACID). XVIII[1]

BY D. W. HILL[2] AND ROGER ADAMS

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The success in obtaining stereoisomeric diphenylbenzenes and diphenylquinones when properly substituted has led to the extension of the work to certain phenylquinones.

In this investigation 2-(3-bromo-2,4,6-trimethylphenyl)-5-methylbenzoquinone-3,6 di-(acetic acid) (XV) has been prepared and resolved through the morphine salt. This is the first phenylquinone that has been resolved into optical antipodes and thus the same conclusions may be drawn as from the study of the diphenylquinones[3] that a quinone and benzene ring when attached to each other and when each is properly substituted may have restricted rotation between them. Using the x-ray values previously discussed, this result might be anticipated. Assuming the CH_2COOH group to be essentially the same in hindering effect as the methyl group, the following conditions exist: CH_3, 1.73 Å. + C=O, 1.12 Å. $\longrightarrow$ 2.85 Å.; CH_3, 1.73 Å. + CH_2COOH, 1.73 Å. $\longrightarrow$ 3.46 Å. Thus on one side of the molecule after subtracting the vertical distance between the 2,2′ carbon

[1] For the two previous papers, see Stanley and Adams, THIS JOURNAL, 53, 2364 (1931); Chang and Adams, *ibid.*, 53, 2353 (1931). See also Stanley, *ibid.*, 53, 3104 (1931).

[2] Commonwealth Fund Fellow.

[3] Shildneck and Adams, THIS JOURNAL, 53, 343 (1931).