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Chemical Action of X-Rays on Nucleic Acids and Related Substances in Aqueous Systems

1. DEGRADATION OF NUCLEIC ACIDS, PURINES, PYRIMIDINES, NUCLEOSIDES AND NUCLEOTIDES BY X-RAYS AND BY FREE RADICALS PRODUCED CHEMICALLY

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It is now generally considered that the chemical action of ionizing radiations on aqueous solutions is indirect and involves the primary formation of free radicals which are produced from the water according to the net process (Weiss, 1944, 1947a, b; Stein & Weiss, 1949):

There are also some definite indications that the formation of these radicals *in vivo* may be responsible for some of the biological effects of these radiations (cf. Barron, Dickman, Muntz & Singer, 1949; Barron & Dickman, 1949). As evidence has accumulated to emphasize the important role which the nucleic acids play in growth processes it appeared of interest to study the chemical action of X-radiation on these substances in aqueous systems.

Several investigators have dealt with the action of X-rays on solutions of deoxyribonucleic acids (DNA) and have observed physical effects such as a loss of viscosity and of streaming birefringence (Wegmüller, 1942; Sparrow & Rosenfeld, 1946; Taylor, Greenstein & Hollaender, 1948). The results obtained indicated that the effect of the radiation was to break the long asymmetric molecules into shorter fragments.

We have previously reported our preliminary observations on the chemical effects of X-rays on aqueous solutions of both DNA and RNA (ribonucleic acid) (Scholes, Stein & Weiss, 1949; Scholes & Weiss, 1950) and have interpreted the mechanism of this action as an indirect one. This we confirmed by showing that chemically produced OH radicals

effected similar changes in the nucleate complex. The OH radicals were generated in solution by the hydrogen peroxide-ferrous salt (Fenton's) reagent, where they are formed according to the following reaction (Haber & Weiss, 1934):

During the preparation of this paper several publications bearing on the work have appeared. Butler (1949), by irradiation of DNA with X- and γ -rays and measurements of the viscosity changes in the presence of protective agents, has also concluded that the mechanism of the attack is of an indirect nature. Further evidence is the observed depolymerization of DNA with chemically produced free radicals (Butler & Smith, 1950; Smith & Butler, 1951; Limperos & Moscher, 1950), and the inability of X-rays to depolymerize the nucleate in the dry state, in ethylene glycol and in a frozen aqueous solution (Limperos & Moscher, 1950).

Some investigations have also been made on the influence of oxygen in the irradiation of DNA solutions (Butler & Conway, 1950) and on the molecular sizes, as determined by sedimentation and diffusion-constant techniques, of the degraded nucleate fragments (Conway, Gilbert & Butler, 1950). These studies have shown that after irradiation, the mean molecular weight of DNA is considerably lowered and that the material becomes much more inhomogeneous. Although molecular oxygen does not appear to have any influence on the immediate effects of the irradiation these authors have shown that in the presence of oxygen the DNA suffers a further progressive fall in anomalous

viscosity over a period of several hours. This confirms the previous findings of Taylor *et al.* (1948) who also observed this 'after effect'.

Stern, Brasch & Huber (1949) have found that solutions of DNA are very sensitive to high-intensity electron bombardment; considerable decreases of viscosity were observed. Barron & Flood (1950) and Barron & Bonzell (1950) demonstrated a decrease in the absorption band at 2600 Å. when the nucleic acids were irradiated with X-rays in the absence of oxygen; this they have attributed to the oxidation, by the radicals, of the chromophoric groups in these substances.

More recently, Grinnan & Moscher (1951) have observed an X-ray induced depolymerization of highly polymerized RNA obtained from calf and rat liver and similar degradations of this material were also effected by the H_2O_2 - Fe^{2+} reagent and by treatment with ultrasonic vibrations.

EXPERIMENTAL

Materials. Commercial samples of the sodium salts of (thymus) DNA (BT1 and BT2) and of (yeast) RNA (BY1-BY4) were supplied by British Drug Houses Ltd. A specimen of highly polymerized DNA (mol.wt. $\sim 1 \times 10^6$) was generously given by Prof. R. Signer (Berne).

RNA was purified as follows: the material was deproteinized by shaking the neutral solution with a mixture of 4 vol. CHCl_3 + 1 vol. amyl alcohol (Sevag, Lackman & Smolens, 1938) and the acid then precipitated in an ethanol-HCl mixture (Fletcher, Gulland, Gordon & Dibben, 1944). The product was dialysed through cellophan (Vischer & Chargaff, 1948) and finally dried by successive washings with solvents (Fletcher *et al.* 1944). Two such purified samples, BYC and BYD, contained N, 16.2; P, 9.2 and N, 16.1; P, 9.5%, respectively. The commercial sample, BY3, contained N, 15.8 and P, 9.1%. (Microanalysis by Weiler and Strauss, Oxford.)

Adenine, guanine, xanthine, and uracil, were pure commercial samples (Roche Products Ltd.). Guanine was further purified by three recrystallizations of the hydrochloride from 2N-HCl followed by regeneration of the base. Xanthine and uracil were twice recrystallized from water.

The specimens of adenosine and uridine were kindly supplied by Prof. A. R. Todd. Yeast adenylic acid was obtained from Roche Products Ltd.

The FeSO_4 used for Fenton's reaction was of A.R. quality and the H_2O_2 solutions were prepared by suitable dilution of 90% material which was free of stabilizers. The FeSO_4 solution was slowly added into a continuously stirred mixture of substrate solution and H_2O_2 .

METHODS

Irradiation

A Victor Maximar set operating at 200 kV. and 15 m.a. (without filtration) was used as the source of X-rays, the irradiation arrangements being essentially similar to those described by Farmer, Stein & Weiss (1949). The solutions were prepared in distilled water at room temperature and

100 ml. were irradiated in Pyrex glass vessels. Blank solutions, prepared at the same time, were retained over the period of irradiation (which for doses up to 8×10^6 r. required times up to 40 hr.). No attempt was made to control the pH of the nucleic acid, nucleoside and nucleotide solutions. The solutions of commercial sodium nucleates exhibited, before irradiation, pH values varying between 5 and 6; those containing the RNA samples BYC and BYD were first brought to pH ~ 7 with NaOH. The pure DNA solutions were at pH 6.5. There was always a small decrease in pH on irradiation of the nucleate solutions, this being of the order of 1 unit. Irradiation of the adenosine solutions also resulted in a slight increase in acidity (from pH ~ 6 to pH ~ 5), while in the case of adenylic acid, where the solutions were already slightly acid (pH ~ 3), very little subsequent change could be observed. Because of their limited solubility in water, the purines were irradiated in acid solutions (H_2SO_4).

Dose measurements were carried out by the chemical method of Day & Stein (1949) and were of the order of 3000 r./min. Most of the irradiations were carried out in the presence of air but some were performed in a vacuum (the solution being freed from air by passage through it of purified N_2 followed by evacuation) and some in solutions saturated with O_2 (O_2 being bubbled through the solution for at least 30 min. before irradiation).

Quantitative methods

Determination of ammonia. Samples were steam-distilled in a vacuum at pH 9.2 (Parnas, 1934; Krebs, 1935) and the ammonia determined in the distillate with Nessler's solution and a Spekker photoelectric absorptiometer using an Ilford 602 filter.

Determination of inorganic phosphate. For the estimation of inorganic phosphate in the presence of labile organic phosphate two methods were employed, that of Lowry & Lopez (1946) and the extraction procedure of Berenblum & Chain (1938). The former was used in the irradiation experiments and the latter in solutions treated with Fenton's reagent. Before analysis by the Lowry-Lopez method most of the unchanged nucleic acid was first removed by precipitation with trichloroacetic acid (TCA): to 1 ml. of the cooled irradiated solution 1 ml. of 5% (w/v) TCA was added, the precipitated nucleic acid centrifuged off and the supernatant immediately treated with 5 ml. 0.1N-sodium acetate and 5 ml. of sodium acetate buffer (pH 4.1). The mixture was finally made up to 20 ml. and treated with 0.2 ml. of a freshly prepared solution of 1% (w/v) ascorbic acid and 0.2 ml. of 0.1% (w/v) solution of ammonium molybdate (in 0.05N- H_2SO_4). Although there was some delay in colour development in this case, the maximum was attained after about 45 min. In both methods extinctions were measured in a Spekker colorimeter (Ilford 608 filter), calibration being made with A.R. Na_2HPO_4 .

Titrateable acid groups. These were estimated by titration, with 0.02N-NaOH, to the first permanent pink colour of phenolphthalein.

Van Slyke nitrogen. This was determined in the apparatus described by Peters & Van Slyke (1931) in solutions previously freed from ammonia. Because of high blank values the accuracy of the method was somewhat reduced and was subject to an error of between 10 and 15%.

Quantitative paper chromatography. The quantitative estimation of the purines and pyrimidines has been achieved

by paper-strip chromatography (Hotchkiss, 1948; Vischer & Chargaff, 1948; Markham & Smith, 1949), and the principles elaborated by these authors were employed for the determination of the purines in the irradiated nucleate solutions. A typical experiment was as follows: to 50 ml. of irradiated RNA solution (0.2%, w/v) were added 4 drops 10% (v/v) acetic acid and 0.5 ml. 8% uranyl acetate solution (Kerr & Seraidarian, 1945). The precipitated uranium compound was removed by centrifugation and the supernatant then treated with 0.2 ml. 10N-H₂SO₄ and 1 ml. m-AgNO₃. After a short while the Ag purines were centrifuged off and washed with a little water. The salts were quantitatively decomposed with H₂S and known volumes of the Ag-free solution (0.01–0.02 ml. as measured by an Alga micrometer syringe), were developed on Whatman no. 1 paper with an 86% butanol–14% (v/v) water mixture. After about 20 hr., segments of the dried chromatogram were eluted with 0.1N-HCl and the extinctions read, at the appropriate wavelength, in a Unicam spectrophotometer. The identities of the bases were also confirmed by their absorption spectra.

For qualitative purposes the position of the purines on the chromatogram were located by the fluorescence-quenching technique described by Holiday & Johnson (1949).

Quantitative paper chromatography was also used for the analysis of irradiated uridine solutions (see below).

Adenine and guanine. Adenine was determined as the picrate according to the method of Hitchings (1941*a*). When this purine was separated as the Ag salt, the latter was decomposed with H₂S and after removal of the Ag₂S, the adenine content of the filtrate then determined as above.

Guanine was estimated colorimetrically by means of Folin's reagent (Hitchings, 1941*b*).

Ribose. This was determined colorimetrically with Bial's reagent according to a slight modification (Stone, 1943) of the Mejbaum (1939) procedure: 3 ml. of the test solution and 6 ml. of the reagent (prepared by dissolving 200 mg. orcinol in 56 ml. conc. A.R. HCl and adding 0.2 ml. of 10% (w/v) FeCl₃ solution) were heated for 30 min. in a vigorously boiling-water bath. The solution was cooled, diluted to 20 ml., and the extinction measured in the Spekker (Ilford filter 608); D-ribose was used as a standard. The ribose contents of the adenosine and adenylic acid solutions were determined after preliminary acid hydrolysis (see below).

Analysis of nucleoside and nucleotide solutions

Adenosine irradiations. The Ag salt of free adenine was precipitated from 20 ml. samples of the irradiated solutions according to the procedure of Kerr & Seraidarian (1945) by the addition of two drops of 2N-H₂SO₄ and 0.4 ml. of m-AgNO₃. The adenine content of the separated (centrifuged) Ag salt was then determined as the picrate.

The supernatants from the above treatment contained unchanged adenosine, the amount of which was determined by estimation of the adenine liberated on acid hydrolysis: the solutions were treated with 6 ml. of 20% (w/v) H₂SO₄ and heated in a boiling-water bath for 45 min. After neutralization with 10% (w/v) NH₄OH (methyl orange), 10 ml. of 1% (w/v) ammoniacal AgNO₃ were added, the precipitated Ag salt separated and the adenine content determined.

In addition, the total purine contents of both the irradiated and blank solutions were determined by similar hydrolysis of 10 ml. samples. The amount of purine in the blank solutions gave a measure of the initial nucleoside con-

centrations and this agreed well with the corresponding values obtained from measurements of total ribose.

Ribose was estimated colorimetrically after hydrolysis of the test solutions (45 min. on a boiling-water bath in 0.5N-HCl).

Uridine irradiations. Samples (75 ml.) of the irradiated (and blank) solutions were concentrated to 1 ml. (30°, under reduced pressure) and 0.01 ml. of this run in an 86% butanol–14% (v/v) water solvent mixture on Whatman no. 1 paper (Markham & Smith, 1949). Segments of the dried chromatogram were eluted with 3.5 ml. of water and the optical densities of the eluates measured in a Unicam spectrophotometer.

The identity of the uracil liberated on irradiation was confirmed by an examination of its characteristic absorption spectrum in both neutral and alkaline solution.

Adenylic acid irradiations. The nucleotide contents of the blank and irradiated solutions were determined by precipitation and separation as the uranium complex (Kerr & Seraidarian, 1945), decomposition of this with acid (Buell & Perkins, 1928) and eventual determination of the liberated adenine as picrate. To a 20 ml. sample were added one drop of 10% (v/v) acetic acid and 0.75 ml. 8% (w/v) uranyl acetate; the resultant precipitate was centrifuged off after 30 min. (the supernatants being retained for free purine analysis). Water (2 ml.) and 2 ml. 10% (w/v) H₂SO₄ were added to the uranyl precipitate and the material hydrolysed by immersion in a boiling-water bath for 30 min. After cooling, the calculated amount of 10% (w/v) NH₄OH was added (just alkaline to litmus). The precipitated uranyl salt was removed, redissolved in acid (0.5 ml. of 10% (w/v) H₂SO₄), and, in order to remove any adsorbed purine, reprecipitated with ammonia. The Ag-purine salt was then precipitated from the combined supernatants on treatment with 3 ml. 1% (w/v) ammoniacal AgNO₃, and the adenine content of the separated salt determined.

Free purine present in the supernatants from the nucleotide-uranium precipitate was estimated, as described above, by separation as the Ag salt from slightly acid solution. Examination of the solutions remaining after removal of the acid Ag salt failed to reveal the presence of any nucleoside.

Total adenine and pentose contents of the irradiated and blank solutions were determined, as described in the case of adenosine, after initial acid hydrolysis.

Inorganic phosphate was determined in 10 ml. samples by the method of Lowry & Lopez (1946).

Detection of oxalic acid, guanidine and urea

To detect oxalic acid, the test solution was acidified, continuously extracted with ether for 20 hr. and the extract, after removal of the organic solvent, treated with a sat. solution of CaSO₄. Precipitation of the Ca salt was allowed to proceed to completion (usually several hours). The salt decolorized permanganate, gave the characteristic colour reaction on reduction and treatment with 2:7-dihydroxynaphthalene (Feigl, 1946) and, in addition, was microscopically identical with pure calcium oxalate prepared under similar conditions. The yields of oxalic acid were obtained by titration of the separated Ca salts with 0.01N-KMnO₄.

Guanidine and urea were detected as follows: the solution was neutralized, evaporated to dryness *in vacuo* and the residue extracted several times with warm ethanol. From

this extract, guanidine was precipitated and identified as the picrate and urea as the dioxanthryl derivative (Fosse, Robyn & Francois, 1914).

Electrodialysis

For the separation of small molecular fragments from unchanged nucleic acid a simple electrodialysis apparatus was used. A central compartment containing the irradiated nucleate solution (50 ml.) was separated, by cellophan membranes, from the anode and cathode chambers, the platinum electrodes of which were connected to an ammeter rectifier and transformer so that 15 V. d.c. could be applied across the membranes. Dialysis was allowed to proceed for up to 50 hr., during which time the solution was frequently stirred and the water contents of the electrode compartments frequently changed; the current usually rose to about 15 ma. The dialysates were then compared with those obtained from the corresponding blank solutions.

RESULTS

NUCLEIC ACIDS

The effects of X-rays on nucleic acids

In order to establish chemical effects and to isolate any products of irradiation, it was necessary to employ fairly high doses of X-rays: aqueous solutions of RNA and DNA were therefore irradiated with $1-4 \times 10^6$ r.

Under these conditions, degradation of the polynucleotides was indicated by the complete loss of viscosity of the DNA solutions and also by a lowering of acid-precipitability in both the RNA and DNA solutions as shown by qualitative experiments. This depolymerization was accompanied by the liberation of titratable acid groups and by the formation of inorganic phosphate. Table 1 shows some results obtained.

The yields were reproducible, but were found to vary with the nature and also with the sample of nucleic acid irradiated. This was demonstrated, for example, with particular reference to some RNA

specimens. Experiments with dialysed RNA solutions (BY4), with highly polymerized DNA (Signer A) and with purified RNA (BYC and BYD) have shown that these changes were not due to the presence of impurities or of small molecular-weight fractions.

Evidence for chemical changes in the constituent purine and pyrimidine bases was gained from the formation of ammonia and also from an increase of the Van Slyke amino-nitrogen values. Some quantitative findings are recorded in Table 2.

The yields of ammonia from the polynucleotide solutions were of the order of $0.1 \text{ m-mole}/10^8 \text{ g.r.}$ (gram-roentgen). Similar results were obtained from the irradiations of specially purified RNA and DNA samples (cf. Table 2) all of which were free of protein. It is of some interest to note that the ammonia yield was considerably reduced in the absence of atmospheric oxygen; the significance of this will be discussed in a forthcoming paper.

The recorded Van Slyke nitrogen values, which give the difference between blank and irradiated solutions, indicate ring opening of the constituent bases.

These observations show that the polynucleotides undergo definite chemical changes under the influence of X-radiation. Further experiments were thus concerned with the isolation and identification of any low molecular-weight fragments which may be formed. For the separation of such fragments from unchanged nucleic acid, electrodialysis through suitable 'cellophan' membranes was employed. The irradiated nucleate solutions were electrodialysed for 40-50 hr. Un-irradiated blank solutions were treated in exactly the same manner. In this way an increase in dialysable organic phosphate and also in inorganic phosphate was detected in the positive-pole section. The cathode compartment yielded on treatment with silver nitrate in acid solution (Kerr & Seraidarian, 1945)

Table 1. *Formation of inorganic phosphate and titratable acid groups by irradiation of aqueous solutions of nucleic acids with 200 kV. X-rays*

Solution			Dose (r. $\times 10^{-6}$)	Inorganic phosphate formed (moles $\times 10^5$ / 100 ml.)	Titratable acid formed (equiv. $\times 10^5$ / 100 ml.)	Remarks
Compound	Sample	Concn. (% w/v)				
RNA	BY2	2.0	4	4.0	25.0	In air
RNA	BY3	2.0	4	3.3	19.9	In air
RNA	BY3	2.0	4	2.9	21.0	In air
RNA	BY4	2.0	4	5.0	30.0	In air
RNA	BYC	0.2	1	0.9	—	(after dialysis) In oxygen (1 atm.)
RNA	BYD	0.2	1	0.8	14.0	In oxygen (1 atm.)
DNA	BT2	1.0	4	3.6	18.0	In air
DNA	Signer A	0.2	1	0.45	10.0	In oxygen (1 atm.)

Table 2. *Production of ammonia and increase in Van Slyke nitrogen by irradiation of aqueous solutions of nucleic acids with 200 kV. X-rays*

Solution			Dose (r. $\times 10^{-6}$)	Ammonia formed (moles $\times 10^4$ / 100 ml.)	Increase in Van Slyke N (moles $\times 10^4$ / 100 ml.)	Remarks
Compound	Sample	Concn. (% w/v)				
PNA	BY1	1.0	2	0.56	—	In vacuo
PNA	BY1	1.0	2	1.405	1.10	In air
PNA	BY2	2.0	4	2.34	2.50	In air
PNA	BY3	2.0	4	2.30	—	In air
PNA	BY3	2.0	4	2.35	—	In air
PNA	BY4	2.0	4	2.00	2.10	In air
						(after dialysis)
PNA	BYC	0.2	1	0.45	—	In oxygen
PNA	BYD	0.2	1	0.46	—	In oxygen
						(1 atm.)
DNA	BT1	0.5	2	0.896	0.50	In air
DNA	BT2	1.0	2	1.20	0.98	In air
DNA	Signer A	0.2	1	0.39	—	In oxygen
						(1 atm.)

small amounts of the silver salts of the purines. The individual bases were subsequently identified as adenine and guanine by paper chromatography. No hypoxanthine or xanthine could be detected on these chromatograms. (The yields obtained by such a dialysis method (Scholes *et al.* 1949) do not, however, represent the total inorganic phosphate release. Subsequent experiments have shown that the phosphate is incompletely dialysed out under the conditions described.)

On dialysis of the irradiated RNA solutions the negative-pole section always gave a positive pentose test (Bial's reagent) and, after removal of the purines as above, a second silver salt could be obtained in alkaline solution (Kerr & Seraidarian, 1945). This salt contained ribose, a basic component (which formed a picrate) but no organic phosphate. These findings appear to indicate the presence of small amounts of material of a nucleosidic character.

This alkaline silver salt contained, in addition, a substance which gave a strong Sullivan colour test. This reaction (Sullivan, 1935), which is characteristic of guanidine compounds, was also given by the irradiated nucleate solution (after removal of ammonia). It is probably due to the presence of a guanidine derivative resulting from partial degradation of the guanine ring system. Repeated experiments, however, failed to reveal the presence in these solutions of any detectable amounts of very low molecular-weight basic fragments.

From the irradiated RNA solutions the liberated free purines were separated and estimated without the use of electro dialysis. They were precipitated as the silver salts after removal of unchanged polynucleotide and other higher weight fractions with uranyl acetate. The adenine and guanine contents of the isolated salts were finally determined by quantitative paper chromatography. Table 3 shows

the yields obtained on irradiation of two samples of RNA. It can be seen that the amounts of free bases formed are quite small, being of the order 4×10^{-6} moles of purine/ 10^6 g.r.

Table 3. *Formation of free purine bases by irradiation of RNA solutions with 200 kV. X-rays in the presence of oxygen*

(RNA soln., 0.2%, w/v; 1 atm. O_2 ; dose 1×10^6 r.)

Sample	Yield of purine moles $\times 10^6$ /100 ml.)	
	Adenine	Guanine
BYC	2.0	1.8
BYD	2.2	1.7

Action of OH radicals produced chemically

The nucleic acids are also degraded by Fenton's reagent. Ammonia and phosphoric acid could be readily detected among the products of the reaction; Table 4, records the yields of these substances obtained on oxidation of RNA under the conditions indicated.

Table 4. *Formation of ammonia and inorganic phosphate by the action of Fenton's reagent on RNA*

(RNA (BYC) 0.2%, w/v; 25 ml. treated with 5 ml. $FeSO_4$ soln. and 1 ml. H_2O_2 .)

H_2O_2 added (moles $\times 10^4$)	Fe^{2+} added (moles $\times 10^5$)	Yield of ammonia (moles $\times 10^5$)	Yield of inorganic phosphate (moles $\times 10^6$)
1.8	1.2	0.20	0.23
7.2	10.0	1.10	1.56
3.0	—	0.0*	0.0*

* After 20 hr. at room temperature.

Even with these relatively low concentrations of hydrogen peroxide (approx. 0.01M) the chemical

changes were quite apparent. As 0.01M-hydrogen peroxide alone had no detectable effects on these RNA solutions (cf. Table 4) it was evident that the chemical effects observed with Fenton's reagent were a result of reactions involving the OH radicals produced in this system (eqn. 2). In this respect, Taylor *et al.* (1948) have also shown that hydrogen peroxide up to concentrations of 0.01M has little effect upon the viscosity of DNA solutions. Since, therefore, similar effects are induced both by the ionizing radiation and by free radicals generated chemically, it is confirmed that the mechanism of the action of X-rays on the nucleates in aqueous solution is an indirect one.

With greater amounts of hydrogen peroxide it was possible to degrade the polynucleotides to much lower molecular species. From the RNA solutions, for example, urea and guanidine could be isolated and from DNA solutions, urea, under similar conditions. These were characterized as the dioxanthryl derivative and as the picrate respectively. The formation of such products, however, evidently represents a far-reaching degradation of the basic components.

PURINES, PYRIMIDINES, NUCLEOSIDES AND NUCLEOTIDES

The production of ammonia and increased Van Slyke-nitrogen values found on irradiation of the nucleic acids with X-rays indicated degradation of the constituent purine and pyrimidine bases. A study of the effects of X-rays on aqueous solutions of the bases themselves was therefore made to gain some insight into the mechanism of the attack on these substances. Furthermore, an investigation of the chemical changes occurring on irradiation of the purine and pyrimidine nucleosides and nucleotides was considered to be of some value in any interpretation of the X-ray-induced decomposition of the nucleic acids. However, as the deoxyribose

nucleosides and nucleotides are as yet comparatively rare materials, experiments had to be confined to the ribose derivatives.

To confirm the indirect nature of the action of the radiation on these various substances experiments with OH radicals, produced chemically by the hydrogen peroxide-ferrous salt (Fenton's) reagent, were again carried out.

Action of X-rays

Purines and pyrimidines. The liberation of ammonia from some of the purine and pyrimidine bases is shown in Table 5. The presence of an amino group in the molecule enhanced the formation of ammonia as shown by a comparison of the yields obtained from xanthine and from guanine and also by the very small yields from uracil. Other more qualitative experiments have, in addition, shown only a slight ammonia yield from thymine but a relatively high yield from cytosine. The yield of ammonia, however, depended upon the pH of the irradiated solution, the figures quoted for the purines adenine and guanine, for instance, showing that deamination increased with increasing acidity.

Determination of the ratios ammonia formed/base destroyed gave values greater than unity (Table 5). Under these conditions, therefore, the nitrogen atoms of the purine ring systems also contributed to ammonia formation and the small but definite yields from uracil (which contains no primary-amino group) indicated the occurrence of a similar process in the pyrimidine system.

The results were indicative of a rather severe degradation of these compounds. Indeed, in all cases the formation of carbon dioxide was observed. The purines yielded small amounts of oxalic acid (isolated as the calcium salt), guanine in addition being degraded with the formation of small amounts of guanidine (isolated as the picrate). Table 6 summarizes these results.

Table 5. *Irradiation of aqueous solutions of purine and pyrimidine bases with 200 kV.
X-rays in the presence of air*

Substance	Solution		Dose (r. $\times 10^{-6}$)	Ammonia formed (moles $\times 10^4$ / 100 ml.)	Moles of ammonia formed/ mole of base destroyed
	Concn. (% w/v)	pH			
Adenine	0.2	2.00	4	7.74	—
Adenine	0.2	3.00	4	5.75	—
Adenine	0.2	3.50	4	4.47	—
Adenine	0.1	1.75	4	8.34	1.75
Adenine	0.1	2.75	4	6.50	1.5
Adenine	0.1	4.80	3.2	3.07	—
Guanine	0.2	0.62	4	6.5	—
Guanine	0.2	1.03	8	11.0	—
Guanine	0.1	1.28	4	4.74	1.45
Uracil	0.1	7.0	8	0.19	—
Xanthine	0.1	0.20	4	3.5	—

Table 6. *Irradiation of aqueous solutions of purine and pyrimidine bases with 200 kV. X-rays in the presence of air*(Solutions, 0.1%; dose, 4×10^6 r.)

Solution		Ammonia formed (moles $\times 10^4$ / 100 ml.)	CO ₂ formed	Oxalic acid formed (moles $\times 10^6$ / 100 ml.)	Guanidine formed (moles $\times 10^6$ / 100 ml.)	Titrateable acid formed (equiv. $\times 10^4$ / 100 ml.)
Substance	pH					
Adenine	1.75	8.34	+	5	—	—
Guanine	1.28	4.74	+	1.8	5.0	—
Uracil	7.0	0.13	+	—	—	2.7

Table 7. *Irradiation of aqueous solutions of adenosine with 200 kV. X-rays in the presence of air*

Substance (moles $\times 10^4$ /100 ml.)	Exp. (a) (3.30×10^6 r.)	Exp. (b) (3.18×10^6 r.)	Exp. (c) (3.06×10^6 r.)	Exp. (d) (2.94×10^6 r.)
Adenosine (initial)	3.270	2.905	3.140	2.900
Adenosine (final)	0.338	0.588	0.668	0.594
Total adenine* remaining	0.634	0.884	1.060	1.020
Total ribose remaining	1.130	1.006	1.115	1.130
Free adenine formed	0.296	0.296	0.389	0.424
Ammonia formed	2.75	2.2	—	—
Titrateable acid formed (equiv.)	1.70	1.40	1.12	1.40

* Determined after acid hydrolysis.

Deamination with the formation of the corresponding hydroxy compounds (i.e. hypoxanthine and xanthine) did not apparently take place under these conditions, as these substances could not be detected by paper chromatography, but certain substances containing the primary amino group, e.g. adenine, guanine and cytosine, yielded on irradiation with X-rays small amounts of hydroxylamine (Scholes & Weiss, 1951). For example, from adenine solutions which have received a dose of approx. 1×10^6 r. one obtains approx. 1×10^{-7} moles of hydroxylamine/100 ml. (at pH 7, in air).

The irradiated uracil solutions showed a significant increase in titrateable acid (cf. Table 6) and on treatment with 2:4-dinitrophenylhydrazine yielded a precipitate of insoluble hydrazones one of which (m.p. 315°) was soluble in sodium carbonate, indicating the presence of a functional acid grouping. Although no urea or oxalic acid could be isolated from the irradiated pyrimidine solutions, these substances were readily formed on subsequent mild alkaline hydrolysis with sodium bicarbonate (at boiling-water bath temperatures). The irradiation of uracil therefore leads to the intermediate formation of carbonyl compounds and to substances susceptible to alkaline hydrolysis.

Ribosidic compounds. D-Ribose was degraded with the formation of carbon dioxide, lower aldehydes, small amounts of oxalic acid and presumably also some hydroxy acids. Evidence of acid formation was also obtained from a marked increase in titrateable acid. The production of aldehydic compounds was indicated by the ready formation of 2:4-dinitrophenylhydrazones. Oxalic

acid was isolated as the calcium salt and the formation of hydroxy acids was suggested by the yellow colour produced on treatment of the (concentrated) irradiated solution with neutral ferric chloride.

Determination of the amount of ribose which had disappeared on irradiation showed the sugar to be a relatively good acceptor. For example, on irradiation of 100 ml. of a 6.6×10^{-3} M-ribose solution with a dose of 4×10^6 r. (in air), 0.6 m-mole of the sugar was destroyed and at the same time 0.604 m-equiv. of titrateable acid and 0.005 m-mole of oxalic acid were produced.

The effects of X-rays on aqueous solutions of adenosine and of yeast adenylic acid have also been studied in some detail; 100 ml. samples of 0.1% solutions were irradiated with doses in the region of 3×10^6 r.

Apart from changes resulting in the formation of carbon dioxide, ammonia and titrateable acid, in both cases the liberation of free purine bases was observed. In the nucleoside solutions free ribose was detected chromatographically. The nucleotide was dephosphorylated, but did not yield any uncombined ribose or any of the corresponding nucleoside.

The quantitative results from irradiations of adenosine are recorded in Table 7. In Table 8 the yields are calculated in moles per cent. As it has not yet been possible to determine the actual yields of uncombined ribose in the presence of adenosine, figures for the 'free' ribose formed (Table 8) may also include substances consisting of sugar attached to an 'altered' purine residue.

Table 8. *Irradiation of aqueous solutions of adenosine with 200 kV. X-rays in the presence of air*

Substance (moles/100 moles)	Exp. (a)	Exp. (b)	Exp. (c)	Exp. (d)
Total adenine destroyed	89.90	87.25	84.24	82.25
Total ribose destroyed	73.00	82.25	82.00	77.60
'Free' ribose formed	27.00	17.75	18.00	22.40
Free adenine formed	10.10	12.75	15.75	17.75

Table 9. *Irradiation of aqueous solutions of yeast adenylic acid with 200 kV. X-rays in the presence of air*

Substance (moles $\times 10^4$ /100 ml.)	Exp. (a) (2.93×10^6 r.)	Exp. (b) (3.06×10^6 r.)	Exp. (c) (3.06×10^6 r.)
Adenylic acid (initial)	2.490	2.635	2.550
Adenylic acid (final)	0.289	0.297	0.283
Total adenine* remaining	0.710	0.680	0.753
Total ribose remaining	0.587	0.533	0.393
Free adenine formed	0.400	0.390	0.448
Inorganic phosphate formed	1.765	1.905	1.995
Ammonia formed	2.70	2.94	—
Titrateable acid formed (equiv.)	2.05	2.20	2.15

* Determined after acid hydrolysis.

Similarly, Tables 9 and 10 give the results obtained on irradiation of yeast adenylic acid. Table 10 shows that both 'combined' ribose and 'combined' phosphate were present in the irradiated adenylic acid solutions and this is highly suggestive of the formation of a ribose phosphate as an intermediate in the reaction mechanism. The 'combined' phosphate was always higher than the 'combined' ribose and this may be interpreted as being due to the production of other unidentified organic phosphate compounds. There was also a significant liberation of inorganic phosphate from the nucleotide.

Table 10. *Irradiation of aqueous solutions of yeast adenylic acid with 200 kV. X-rays in the presence of air*

Substance (moles/100 moles)	Exp. (a)	Exp. (b)	Exp. (c)
Total adenine destroyed	81.00	83.80	78.50
Total ribose destroyed	82.75	86.00	95.00
Free adenine formed	18.80	16.20	21.50
'Combined' ribose formed	17.25	14.00	5.00
Inorganic phosphate formed	80.25	81.50	88.20
'Combined' phosphate formed	19.75	18.5	11.8

The analytical figures from the nucleoside and nucleotide experiments show that while under these conditions both the base and sugar components underwent considerable decomposition, these processes did not lead to any marked formation of free purines. This is similar to the case of the nucleic acids.

Some investigations have also been made on the effects of X-rays on aqueous solutions of the pyrimidine ribosidic compounds. Uridine, a typical pyrimidine nucleoside, gave, among other products, uracil and free pentose. Table 11 shows some figures obtained on duplicate irradiations. Comparison with Table 7 shows that uridine was almost twice as reactive as adenosine towards the available radicals produced in the irradiation.

A more quantitative estimate of the reactivity of a particular substance (S) towards OH radicals can be obtained by investigating the reaction in the

presence of ferrous ions. The latter are known to react relatively rapidly with OH radicals according to the reaction

and when both these reactants are present in the solution, the OH radicals will divide themselves between reaction (3) and the reaction

depending on the relative reactivities. Thus, by determining the inhibitory action of a substance towards the oxidation of ferrous ions one obtains a relative measure of the reactivity of this substance

Table 11. *Irradiation of uridine solutions with 200 kV. X-rays in the presence of air*

(Uridine soln., 0.1%; dose, 1.8×10^6 r.)	
Uridine decomposed (moles $\times 10^4$ /100 ml.)	Uracil formed (moles $\times 10^4$ /100 ml.)
0.32	0.0165
0.30	0.0150

towards OH radicals. The reactivities of different substances (e.g. adenine and uracil) may therefore be compared if one studies the inhibitory effect towards the oxidation of ferrous ions of each of these substances separately under otherwise similar conditions. Ferrous sulphate solutions of constant concentration were irradiated, *in vacuo*, in the presence of various amounts of either the purine or the pyrimidine base and the extent of oxidation of

the ferrous ion determined. The results (Fig. 1) show quite definitely that uracil reacts more rapidly than adenine. A comparative study of the ammonia and phosphate liberation from yeast adenylic

Fig. 1. Ferrous ions oxidized by irradiation of ferrous sulphate solutions with X-rays in the presence of different concentrations of adenine (curve A) and uracil (curve B) *in vacuo*. Total dose, 5.52×10^4 r.; pH, 2.4; 1.9×10^{-4} M- Fe^{2+} .

and cytidylic acids has also demonstrated the greater reactivity of the pyrimidine derivatives. On irradiation of nucleic acids, therefore, the pyrimidine components will obviously suffer a greater attack.

Action of OH radicals produced chemically

On treatment with Fenton's reagent both adenine and guanine yielded carbon dioxide and ammonia, the guanine in addition being oxidized with the formation of small amounts of guanidine, urea and oxalic acid. Uracil yielded carbon dioxide, traces of urea, traces of oxalic acid and a mixture of 2:4-dinitrophenylhydrazones (an alkali-soluble hydrazone being apparently identical with the corresponding derivative obtained from the irradiation). As with the irradiation of uracil, the major amounts of urea and oxalic acid were obtained on subsequent mild hydrolysis of the oxidized base. These observations on the pyrimidine confirm those of Pfaltz & Baudisch (1923) who have shown that uracil, on treatment with ferrous sulphate and hydrogen peroxide, yielded intermediate substances, which, when hydrolysed with sodium bicarbonate were split to form urea.

The results of our experiments are shown in Table 12, the recorded yields of urea and guanidine being obtained by direct weighing of the isolated dioxanthidryl-urea and guanidine picrate respectively. As only traces of ammonia were detectable in adenine solutions treated with hydrogen peroxide alone (cf. Table 12) it is evident that these degradations must proceed via the free radicals formed according to reaction (2). The qualitative nature of the products formed is strikingly similar to those obtained on irradiation of the bases; the action of the radiation must therefore be interpreted on a mechanism involving free radicals.

Qualitative examination of the mode of oxidation of some pentose compounds with Fenton's reagent has also shown similarities to the chemical effects induced by X-rays. Ribose itself, for instance, was decomposed with the liberation of carbon dioxide and the formation of lower carbon compounds. Among the latter were aldehydes, hydroxy acids and some oxalic acid. The deamination of the basic component of adenosine and of yeast adenylic acid was readily observed in the treated solutions and, in each case, some free adenine was liberated and could

Table 12. The effects of Fenton's reagent on purine and pyrimidine bases

Solution			H_2O_2 added (moles $\times 10^3$ / 100 ml.)	Fe^{2+} added (moles $\times 10^4$ / 100 ml.)	Ammonia formed (moles $\times 10^4$ / 100 ml.)	Urea formed (moles $\times 10^5$ / 100 ml.)	Guanidine formed (moles $\times 10^5$ / 100 ml.)	Oxalic acid formed
Substance	Concen- tration (% w/v)	pH						
Adenine	0.2	2.0	2.2	4	0.58	.	.	Trace
Adenine	0.2	2.0	6.6	12	1.40	.	.	Trace
Adenine*	0.25	3.0	5.5	.	Trace	.	.	.
Guanine	0.1	1.0	2.2	4	0.25	+	+	+
Guanine	0.2	0.5	20.0	25	1.50	1.0	4.0	+
Uracil	0.1	4.0	1.0	1.25	.	Trace	.	Trace

* 24 hr. at room temperature.

be isolated and identified (via the silver salt). In addition, the nucleotide suffered some dephosphorylation; e.g. treatment of 100 ml. of a 0.1% solution of yeast adenylic acid with 3.6×10^{-3} mole of hydrogen peroxide and 0.45×10^{-3} mole of ferrous salt resulted in the liberation of 1.6×10^{-5} mole of inorganic phosphate.

DISCUSSION

When aqueous solutions of the nucleic acids and related substances are irradiated with X-rays, definite chemical changes take place which result from interactions between the solute molecules and the free radicals formed by the action of the radiation on the solvent (water) (eqn. 1). In general, the results obtained appear to indicate that the following changes take place in the nucleic acids: (a) a deamination and ring opening of the constituent bases; (b) some fission of the glycosidic linkages with the liberation of free purines; (c) some breakage of the ester linkages, leading to the formation of inorganic phosphate and presumably to an increase in titratable acid. It is also certain, at least in the irradiation of RNA, that fragments corresponding in size to nucleosides are produced to some extent.

The effect of the free radicals, therefore, is to induce structural changes in the polynucleotides; the breakage of primary linkages results in a fragmentation which can lead to the elimination of structural units from the polymer chain.

The yield of a radiochemical reaction (the 'ionic yield' or yield per radical pair) can be conveniently expressed as the number of molecules of a specified substance formed or destroyed per ion pair or radical pair produced. On the basis that the energy required to produce one radical pair in water is

in solution, where yields approaching unity are common, these values may appear to be rather small. However, with large molecules of the nucleic acid type, the presence of a number of possible sites of attack will most likely lower the yield of a given single product. Even so, small chemical changes in the polynucleotide molecule may be sufficient to account for gross physical changes such as loss of streaming birefringence and precipitability by acids.

The changes occurring on irradiation of the purine and pyrimidine bases can likewise be interpreted by a mechanism involving free radicals. As OH radicals are known to attack ethylenic centres it is most likely that the action of these radicals on the purines may involve some attack on the central 4:5-ethylenic bond. Similarly, because of the marked ethylenic character of the 4:5-pyrimidine bond, and as the pyrimidines are known to form glycols (cf. Johnson & Dyer, 1933) it is highly probable that intermediates of this nature are formed on irradiation of the pyrimidines; further oxidative fission of such pyrimidine glycol intermediates could lead to the formation of aldehydes and to substances responsible for the production of urea and oxalic acid on subsequent hydrolysis, as indicated by our experiments.

In the case of the nucleosides and nucleotides the experimental observations present a rather complicated picture, but, within the scope of the available data it appears permissible to draw some general conclusions about the mechanism of these reactions. If A, R, P, A* and R* indicate adenine, ribose, the phosphate group, 'changed' adenine and 'changed' ribose respectively, then some probable reactions may be represented schematically as follows:

19.4 eV. (Rigg, Stein & Weiss, 1952), it can be shown from the values given in Tables 1-3 that the yields per radical pair for the formation of ammonia, inorganic phosphate and free purines, are of the order of 0.1, 0.02 and 0.01 respectively. In comparison with other known radiochemical reactions

Primary attack by the radicals is followed by some hydrolytic processes, as indicated above. Secondary radical attacks may also take place on the primary products of the reaction, this latter effect being operative to a greater extent when larger doses of X-rays are employed. It is very likely that with

both the nucleoside and the nucleotide, attack takes place by the two different routes (1) and (2); from a comparison of the yields of the various products, however, it appears that route (1) is the predominant path of degradation.

SUMMARY

1. It has been shown that some well-defined chemical changes take place when aqueous solutions of the nucleic acids (RNA and DNA) are irradiated with X-rays. Fragmentation of the polynucleotides is accompanied by an increase in the number of titratable acid groups and by a liberation of inorganic phosphate and of small amounts of free purine bases. Liberation of ammonia and some ring fission of the constituent purine and pyrimidine bases also occurs. Glycosidic, ester, and internucleotide linkages are thus broken. Corresponding chemical effects (e.g. liberation of ammonia and inorganic phosphate) observed on treatment of the nucleates with the hydrogen peroxide-ferrous salt (Fenton's) reagent have confirmed that the mechanism of the action of X-rays on these dilute aqueous solutions is via the free radicals which are formed by the initial splitting of the water molecules.

2. Aqueous solutions of purines and pyrimidines and of some of their corresponding ribonucleosides and ribonucleotides have been irradiated with X-rays and the nature of the concomitant chemical changes investigated.

3. The presence of an amino group in the base (e.g. in adenine and guanine) enhances the production of ammonia on irradiation. In both the purine and pyrimidine systems, however, the ring nitrogen

atoms may also contribute to the ammonia yield. The purines also yield small amounts of oxalic acid, guanine in addition being degraded with the formation of small amounts of guanidine. Mild alkaline hydrolysis of irradiated uracil solutions results in the formation of urea and oxalic acid. A study of the competition reactions of uracil and of adenine with OH radicals (in the presence of ferrous ions) has confirmed that uracil reacts more rapidly with these radicals than adenine.

4. Chemical changes occurring on irradiation of adenosine and of yeast adenylic acid include ammonia liberation, decarboxylation, an increase in titratable acid groups and the liberation of small amounts of free purine base. From the nucleoside some free ribose is produced. The nucleotide suffers some dephosphorylation, but does not yield any uncombined pentose or any of the corresponding nucleoside. Irradiation of uridine results in the formation of some free uracil and some free ribose.

5. It has been shown also that somewhat similar effects are induced on treatment of these various substances with the hydrogen peroxide-ferrous salt (Fenton's) reagent. Possible mechanisms of the degradation of these compounds by ionizing radiations have been discussed.

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Urinary Steroids

1. FORMATION AND DETERMINATION OF 3:5-DINITROBENZOATES OF ALCOHOLIC STEROIDS

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Very few general methods have been suggested for the estimation of the non-ketonic alcohol fraction from urine. Dobriner, Lieberman & Rhoads (1948) employed an electrometric titration method depending on the formation of half esters of the alcohols with phthalic anhydride and the titration of these with dilute alkali. In model experiments with pure compounds good results were obtained, but the method did not prove satisfactory when applied to urinary extracts. Tompsett & Oastler (1948) weighed the half succinates of the neutral non-ketonic fraction of urinary extracts. The use of the antimony trichloride reaction (Pincus, 1943) has been suggested (Pincus, 1948), but no reports of its application have appeared.

Engel, Patterson, Wilson & Schinkel (1950) described a colorimetric method for steroid alcohols which has been applied to clinical material. By analogy with the Zimmermann reaction a colour would be expected if a steroid complex containing the *m*-dinitrophenyl group was treated with alkali in the presence of excess of ketone. This group of workers tried the 3:5-dinitrobenzoates but found them very sparingly soluble in either aqueous or ethanolic potassium hydroxide. However, the hemi-3:5-dinitrophenylates obtained from primary and secondary alcohols were soluble in alkali, and on treatment with methanolic potassium hydroxide gave a red coloration in the absence of a ketone. No separation of individual alcohols was attempted.

The object of the present work was to prepare derivatives of the steroid alcohols which would lend themselves both to chromatographic separation and quantitative estimation.

Acetates and benzoates have already been employed in isolation studies. The former have been used for chromatographic separations but do not lend themselves to quantitative estimation; they can, however, be characterized in the finger-print region of the infrared spectrum (1200–800 cm.⁻¹) (Jones & Dobriner, 1949). Benzoates have not been widely used for separating steroid alcohols, but recent work by Brooks, Klyne & Miller (1951) has shown that many pairs of related benzoates can be separated by chromatography on alumina. By virtue of the phenyl radical such esters absorb in the ultraviolet region of the spectrum and could be detected and estimated.

The steroid alcohols can be esterified with 3:5-dinitrobenzoyl chloride and the resulting esters, when treated with dilute alkali in the presence of a ketone, give an intense purple colour characteristic of the *m*-dinitrophenyl group. Colour development is complete within 5 min. and acetone will act both as the solvent medium and as the ketone required by the reaction. A method has been developed by studying the conditions for the production of maximum optical density, and has been used for the determination of dinitrobenzoyl esters.