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Steroids. LXVI[1]. Microbiological Hydroxylation of Steroids in Position 21

Following the discovery by PETERSON *et al.*[2] of the transformation of progesterone into 11α-hydroxy-progesterone by molds of the Mucorales family, a number of hydroxylations of steroids by microorganisms have been reported. The introduction of the hydroxy group into position 6β[3], 7β[4], 8ξ[5], 11α[6], 11β[7], 14α[8], 15ξ[9], 16α[10], and 17α[11], has been described.

The recent publication by MEYSTRE, VISCHER, and WETTSTEIN[12] describing the hydroxylation of steroids in position 21 by fungi of the Ophiobolus family has prompted us to report on our own independent work on the introduction of the hydroxy group into position 21, by *Aspergillus niger* ATCC 9142. Progesterone was incubated with a 48 h growth of Aspergillus niger ATCC 9142 on a peptone-molasses medium for 96 to 144 h; extraction of the fermentation liquor with chloroform and chromatography on silica gel yielded desoxycorticosterone, identified by its physical constants (m.p. 142–143°, $[\alpha]^{20}D + 185°$ (ethanol) and by its infrared spectrum.

Fermentation of the following steroid substrates with Aspergillus niger ATCC 9142 gave the corresponding 21-hydroxy derivatives:

Substrates	*Conversion Products*
19-nor-progesterone	19-nor-desoxycorticosterone
11-keto-progesterone	11-dehydrocorticosterone
11α-hydroxy-progesterone	11-epicorticosterone
11β-hydroxy-progesterone	corticosterone
6β-hydroxy-progesterone	6β-hydroxy-desoxycorticosterone
14α-hydroxy-progesterone	14α-hydroxy-desoxycorticosterone

In all cases, paper chromatography indicated the presence of more polar compounds in addition to the conversion products. The characterization of the oxidation products was based on comparison of chromatographic behavior[1], sulfuric acid chromogen curve[2], melting point, optical rotation and infrared spectra with those of the known compounds.

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Research Laboratories, Syntex S. A., Mexico D. F., February 8, 1955.

Zusammenfassung

Die Einführung einer Hydroxylgruppe in die 21-Stellung gewisser Pregnanderivate mittels *Aspergillus niger* ATCC 9142 wird beschrieben.

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[2] A. ZAFFARONI, Recent Progr. Hormone Res. *8*, 51 (1953); J. Amer. Chem. Soc. *72*, 3828 (1950).

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[2] D. H. PETERSON and H. C. MURRAY, J. Am. Chem. Soc. *74*, 1871 (1952).

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[7] D. R. COLINGSWORTH *et al.*, J. Amer. Chem. Soc. *74*, 2381 (1952). – F. R. HANSON *et al.*, J. Amer. Chem. Soc. *75*, 5369 (1953). – G. M. SHULL *et al.*, U. S. Pat. 2,658,023 (Nov. 3, 1953).

[8] P. D. MEISTER *et al.*, Abstr. 123rd Meet. Amer. Chem. Soc., Los Angeles, Calif., March 15–19 (1953), p. 5C.

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[10] D. PERLMAN *et al.*, J. Amer. Chem. Soc. *74*, 2126 (1952). – E. VISCHER *et al.*, Helv. chim. Acta *37*, 321 (1954).

[11] P. D. MEISTER *et al.*, J. Amer. Chem. Soc. *76*, 4050 (1954). – C. MEYSTRE *et al.*, Helv. chim. Acta *37*, 1548 (1954).

[12] C. MEYSTRE *et al.*, Helv. chim. Acta *37*, 1548 (1954).

Studies on the Action of X-Rays on Aqueous Solutions of Nucleic Acids and Some Nucleotides

It has been known for some time that the action of ionising radiations on desoxyribonucleic acid (D.N.A.) solutions leads to a loss of viscosity[1]. The significance of the slow post-irradiation viscosity loss ("after effect") noted by several workers[2], has been investigated in detail by BUTLER and CONWAY[3]. These authors concluded that it occurred only if oxygen was present during irradiation and suggested that it may be due to oxidative processes involving hydrogen peroxide and to the slow decomposition of primarily formed hydroperoxides.

We have suggested[4] that chemical reactions of the free radicals produced by the action of the radiation[5] can

[1] A. H. SPARROW and F. M. ROSENFELD, Science *104*, 245 (1946). – B. TAYLOR, J. P. GREENSTEIN, and A. HOLLAENDER, Arch. Biochem. *16*, 19 (1948). – G. C. BUTLER, Can. J. Research. *B 27*, 972 (1949). – D. B. SMITH and G. C. BUTLER, J. Amer. Chem. Soc. *73*, 258 (1951). – G. LIMPEROS and W. A. MOSHER, Amer. J. Roentgenol. Radium Therapy *63*, 681 (1950).

[2] B. TAYLOR, J. P. GREENSTEIN, and A. HOLLANDER, Arch. Biochem *16*, 19 (1948). – G. C. BUTLER, Can. J. Research. *B 27*, 972 (1949). – G. LIMPEROS and W. A. MOSHER, Amer. J. Roentgenol. Radium Therapy *63*, 681 (1950).

[3] J. A. V. BUTLER and B. E. CONWAY, J. Chem. Soc. *1950*, 3418; *1952*, 834.

[4] G. SCHOLES and J. WEISS, Nature *171*, 920 (1953).

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Change of viscosity of 0·1% (w/v) aqueous solutions of D.N.A. (thymus) after irradiation with X-rays (200 kV). Total dose 7,100 rep (dose rate 1185 rep/min; $G(Fe^{3+}) = 15{\cdot}6 \pm 0{\cdot}8$).

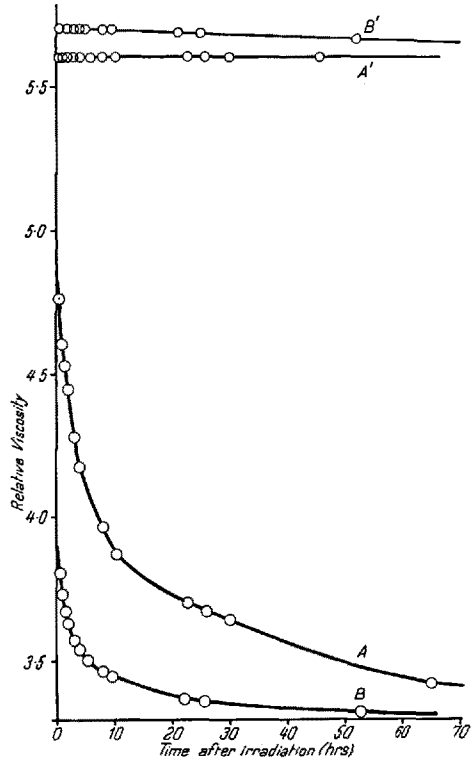

Fig. 1.–Curve *A*. Irradiation in the presence at atmospheric oxygen Curve *B*. Irradiation *in vacuo*. *A'* and *B'* refer to the corresponding unirradiated controls.

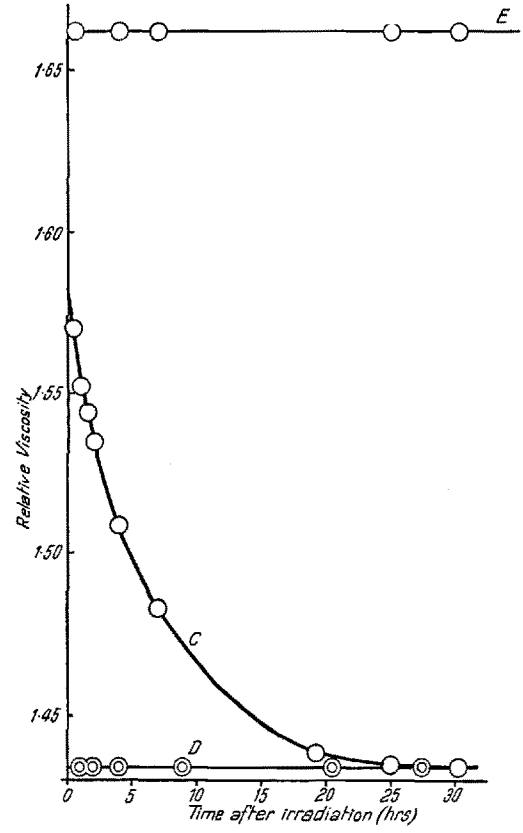

Fig. 2.–In solutions made 0·1 M with respect to sodium chloride after irradiation. Curve *C*. Irradiation in the presence of atmospheric oxygen. Curve *D*. Irradiation *in vacuo*. *E* is the unirradiated control.

lead to the labilisation of carbon-phosphate linkages (i.e. intermediate formation of labile phosphate esters) which subsequently may undergo slow hydrolytic cleavage giving rise to (i) a post-irradiation viscosity loss in D.N.A. solutions and (ii) in the case of nucleotides and other phosphomonoesters, post-irradiation release of inorganic phosphate.

We wish now to report the results of some further investigations on the action of X-rays (200 kV) on aqueous solutions of nucleic acids and some nucleotides, which lend further support to the latter interpretation.

The viscosity changes in D.N.A. solutions on irradiation have been studied under a variety of conditions, with special reference to the effects of oxygen present during irradiation, and also when excess neutral salt was added after irradiation. Figs. 1*B* and 2[1] give some representative results obtained from five samples of D.N.A., equilibrated solutions (0·1% w/v) of which were irradiated with a dose of approximately 7,000 rep. All viscosity measurements were carried out at 25°C.

These graphs show the presence of a considerable after-effect in D.N.A. solutions, when irradiation is carried out in the presence of oxygen.

In oxygen-free solutions no after-effect is observed when the viscosity measurements are made in the presence of sodium chloride (0·1 M).

It must be concluded, therefore, on the basis of current views on the effects of neutral salts on polyelectrolytes[1], that the after-effect *in vacuo* is not due to a change in the structure of the molecules but to a slow re-equilibration of the D.N.A. particles consequent upon irradiation; this may be attributed to an association occurring when insufficient electrolyte is present to complete the counter-ion atmosphere of the D.N.A. particles[2]. These results have since been confirmed by CONWAY[3].

Another feature is that the total percentage loss of viscosity, for any one sample, is the same in the presence or absence of oxygen; this is independent of the presence of sodium chloride, when added after irradiation. However, the initial loss of viscosity on irradiation is less when oxygen is present. The total loss of viscosity for all the samples investigated is a linear function of the initial viscosity i.e. the percentage loss at constant dose is the same for all samples.

Freeze-dry experiments have shown that the extent of the after-effect is independent of any hydrogen peroxide produced in the solution by irradiation. In all cases the viscosity after-effect can be satisfactorily represented by the relationship:

$$\frac{1}{\zeta_t - \zeta_\infty} - \frac{1}{\zeta_0 - \zeta_\infty} = k\,t \qquad (1)$$

[1] In a similar graph which was published previously [M. DANIELS, G. SCHOLES, and J. WEISS, Nature *171*, 1153 (1953)], there is a misprint in the time axis corresponding to a displacement of the time scale by 1 unit (= 2½ h) which is hereby corrected.

[1] Cf. P. DOTY and G. EHRLICH, Ann. Rev. Phys. Chem. *3*, 81 (1952); J. Polymer. Sci. *12*, 159 (1954).

[2] F. T. WALL, J. R. HUIZENGA, and P. F. GRIEGER, J. Am. Chem. Soc. *72*, 2636, 9228 (1950).

[3] B. E. CONWAY, Brit. J. Radiol. *27*, 49 (1954).

[where ζ_0, ζ_t, ζ_∞ are respectively the relative viscosities immediately after irradiation ($t = 0$), at time t and infinity] thus allowing the evaluation of the initial loss and the experimental rate constant governing the decay.

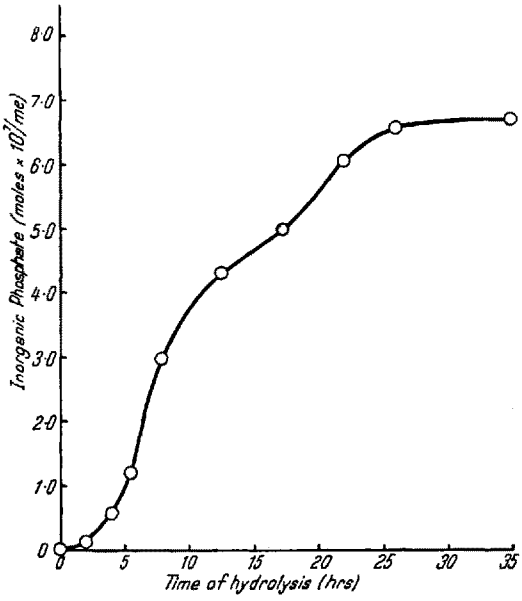

Fig. 3.–Irradiation of aqueous solutions of D.N.A. (0·1% w/v) with X-rays (200 kV) in the presence of oxygen (1 atm). Release of inorganic phosphate on hydrolysis (90°C; pH 6·8). Total dose = $3{\cdot}56 \times 10^4$ rep.

All these observations are not inconsistent with the assumption that irradiation in the presence of oxygen leads in part to a random labilisation of certain bonds in the polynucleotide chain (e.g. carbon-phosphate linkages) which may then undergo slow hydrolytic cleavage resulting in a post-irradiation viscosity loss. In the absence of oxygen, however, it appears that only immediate fracture of the chain takes place, this manifesting itself in the high initial viscosity loss.

In order to obtain some further insight into the chemical nature of the after effect, investigations have been carried out on aqueous solutions of some ribonucleotides. In particular, it has been found that, in addition to formation of inorganic phosphate on irradiation, hydrolysis at 25°C leads to the production of further amounts of phosphate at a measurable rate. This hydrolysis, which can be attributed to the formation of labile phosphate compounds during irradiation, was found to be of first order over the whole range of pH (0·5–13·0) and also exhibited a characteristic variation with pH. The rate and the extent of phosphate release was unaffected after freeze-drying. Hence, here again it is apparent that this process does not involve secondary oxidations by hydrogen peroxide. The pH-variation of the rate constant of the hydrolysis reaction suggests that the lability of the nucleotide is probably due to the formation of an activating carbonyl group in the sugar component[1]. When the irradiations were carried out in the absence of oxygen, some labile phosphates were still formed, though to a smaller extent; the rate constant of the hydrolysis was identical with that found in the oxygenated solutions.

[1] A. DESJOBERT, Thesis (Paris 1951); Bull. Soc. Chim. *111*, 809 (1947). – D. E. KOSHLAND, J. Amer. Chem. Soc. *74*, 2286 (1952).

Similar experiments with D.N.A. and also with ribonucleic acid solutions have also shown some interesting features. Very little post-irradiation phosphate release could be observed at 25°C; this result is not unexpected since chain fission, following oxidative attack on a sugar component which is initially diesterified, would involve the formation of a phospho-monoester, except when attack occurs at an end group. On hydrolysis at 90°C, however, the yield of phosphate release from nucleic acids was increased to many times the initial value; this hydrolysis was acid and base-catalysed and exhibited a complex kinetic behaviour. A typical hydrolysis curve for an irradiated D.N.A. solution is shown in Fig. 3.

These observations demonstrate clearly the presence of labile phosphate bonds in irradiated solutions of nucleic acids.

A full report of this work is in course of preparation.

We are indebted to Professor R. SIGNER (Berne) for samples of D.N.A. Our thanks are due to the Northern Council of the British Empire Cancer Campaign and to the Rockefeller Foundation for financial support.

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Zusammenfassung

Die Wirkung von Röntgenstrahlen (200 kV) auf wässerige Lösungen von Nukleinsäuren und Nukleotiden wurde unter besonderer Berücksichtigung des Einflusses von molekularem Sauerstoff und Wasserstoffsuperoxyd untersucht. Der so gefundene, nach der Bestrahlung einsetzende Abfall der Viskosität von D.N.A.-Lösungen (after-effect) kann als Hydrolyse der durch die Bestrahlung labilisierten Kohlenstoff-Phosphat-Bindungen angesehen werden. Untersuchungen über die Hydrolyse von bestrahlten Nukleotiden, -P.N.A.- und D.N.A.-Lösungen stehen in vollem Einklang mit obiger Annahme.

Living Smears from Endosperm

Studies on the endosperm *in vivo*[1] show clearly that it is excellent material for experimental researches on mitosis in the living cell.

The advantages of endosperm in comparison with other plant material are great: the course of mitosis is much better visible than in other tissues, the lack of cellulose walls allows not only better penetration of chemical substances, but also polarization and micrurgical studies. It seems probable that it may be used as a modification of the *Allium* test *in vivo*. Among genera studied so far: *Iris*, *Leucojum*, *Haemanthus*, *Clivia* and *Colchicum* appeared to be the best, although *Ornithogalum*, *Zephyranthes*, *Helianthus* and *Daphne* genera are also very suitable. A longer list with some methodical notes is given elsewhere[2]. It is evident that the endosperm of some species appeared to be much better than that of others, e.g. *Allium cepa*, *Vicia faba* and *Secale cereale* are not suitable at all. However, hundreds of plants are excellent–so that the material with the exact properties required so far as chromosome dimensions,

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[2] A. BAJER and J. MOLÈ-BAJER, Acta Soc. Bot. Poloniae *23*, 69 (1954).