and the solution was acidified with HCl followed by 1 hour at room temperature. Water was added, and the ether phases were washed with water until neutral reaction. If the material present in corresponding areas of unsprayed papers, it was shown that the physiological activity could be fully accounted for in these zones. These eluates were also used to provide material of higher purity for determination of ultraviolet absorption after alkali and acid treatment. Treatment of Fraction A and B with alkali gave an absorption band at 278 nm (4).

Identification of Prostaglandin E₂—It was shown in a preceding paper (14) that the keto group in PGE can be reduced by sodium borohydride with formation of two epimeric alcohols, PGF₁ and PGF₂, of which the former is identical with PGF, isolated previously from sheep vesicular glands. Thus, if Fraction B were identical with PGE, it should be possible to reduce it with tritium-labeled sodium borohydride to labeled PGF₁ and PGF₂, suitable for identification by cocrystallization with authentic material.

To a solution of 3 mg of Fraction B in 1.5 ml of methanol were added 5 mg of tritium-labeled sodium borohydride (12). The reaction mixture was kept for 15 minutes at ice-bath temperature followed by 1 hour at room temperature. Water was added, and the solution was acidified with N hydrochloric acid and extracted with equal volumes of ether three times. The combined ether phases were washed with water until neutral reaction, dried over sodium sulfate, and evaporated under reduced pressure. The residue was separated by reversed-phase partition chromatography with 129 ml of methanol and 171 ml of water as moving phase and 15 ml of isooctanol and 15 ml of chloroform as stationary phase, supported on 4.5 g of hydrophobic Supercel. The radioactive compounds coinciding with the titration peaks were eluted at the positions where PGF₁ and PGF₂ are expected to appear. The material appearing in the PGF₂ region was diluted with unlabeled PGF₂ and crystallized five times from several solvents with measurement of the specific radioactivity after each crystallization. The same procedure was carried out for the other radioactive component after the addition of inactive PGF₁. The specific radioactivity remained constant throughout five recrystallizations after the first crystallization for the other radioactive component after the addition of inactive PGF₁. The specific radioactivity remained constant throughout five recrystallizations after the first crystallization.

The behavior of the isolated compound in column chromatography and paper chromatography and on treatment with alkali is thus indistinguishable from that of prostaglandin E₂. The identity with prostaglandin E₂ was finally established by mass spectrometry and by its conversion to labeled PGF₁ and PGF₂, followed by cocrystallization with the corresponding authentic material.

Prostaglandin E₂ thus occurs in the seminal plasma of man and sheep (13) and, as a closely related compound, occurs in normal sheep and pig lungs (9); this class of physiologically highly active compounds appears to be widely distributed in animal tissues.

REFERENCES

The Photolysis of Diazoaoylchymotrypsin*

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Recently, several groups of investigators (1-3) have reported the use of multifunctional reagents to help map the regions around the active sites of enzymes. This communication reports a new type of bifunctional reagent and a new method of binding this reagent near the active center. Briefly, the method involves introducing an aliphatic diazo group into a reagent, which can then react with the enzyme at its active center. The diazo group is thus held by chemical bonding near the active site; subsequently, this group can be converted by photolysis, under mild conditions of temperature and pH, to a reactive carbene. The carbene will then react irreversibly with the surrounding molecules, even being capable of insertion into a C=H bond (4).

This method has been illustrated by synthesizing p-nitrophenyl diazoeacetate, and allowing it to acylate chymotrypsin. The resulting diazoacetylchymotrypsin was then photolyzed in aqueous solution at low temperatures.

The ester was prepared as follows. Carbobenzoxyglycine was condensed with p-nitrophenol in ethyl acetate solution by means of dicyclohexylcarbodiimide at 0°. The resulting p-nitrophenyl carbobenzoxyglycinate, after recrystallization from dilute ethanol, melted at 128-129° (129° in the literature (5)). The ester was then converted to p-nitrophenyl glycinate hydrobromide (6): 2.8 g of the latter in 6 ml of water and 16 ml of methylene chloride were diazotized under nitrogen with 1.5 g of sodium nitrite in 5 ml of water by the addition of 3 ml of 5% sulfuric acid.
The photolysis both with tungsten lamps and with sun lamps was followed as a function of time; in both cases, the enzymatic activity was progressively regenerated, approaching 80% as a limit. In parallel fashion, the enzyme progressively lost radioactivity. With the sun lamps, the limit was reached after 100 minutes, and the radioactivity and enzymatic activity were the same then as after 240 minutes. The sun lamps thus accomplished in about 2 hours a photolysis which required 48 hours with tungsten. However, the end results were essentially the same with both methods.

The photolysis of diazoacetylchymotrypsin surprisingly leads to a regeneration of approximately 75 to 80% of the enzymatic activity. Presumably, the photolysis proceeds in part according to the equations:

\[
\text{Enzyme-CH}_2-O-CO-CH_N_2 \rightarrow \text{Enzyme-CH}_2-O-CO-CH_2 + N_2
\]

\[
\text{Enzyme-CH}_2-O-CO-CH_2 \rightarrow \text{Enzyme-CH}_2OH + \text{HOCH}_2CO_2H
\]

The isolation of 55% of the total radioactivity as glycolic acid from labeled diazoacetylchymotrypsin provides evidence for this pathway. For the isolation, purified glycolic acid was added.
as carrier to a 0.001 M solution of the labeled enzyme which had previously been photolyzed for 4 hours with sun lamps. After the enzyme had been denatured at 40° and removed, the glycolic acid was isolated as its p-bromophenacyl ester (12), m.p. 138.5-139° (138° in the literature (12)).

Approximately 20 to 25% of the enzymatic activity, however, is not regenerated on photolysis, nor can this activity be regenerated by the action of hydroxylamine on the modified chymotrypsin. The control experiments in the table show that the loss of activity is not caused by the action of light on chymotrypsin itself, nor by allowing the diazoacetylchymotrypsin to stand in solution without photolysis. The permanent loss of 20 to 25% of enzymatic activity is then a direct result of the chemical reaction that occurs on photolysis. Furthermore, this loss of enzymatic activity corresponds quantitatively to the introduction into the photolyzed enzyme of 20 to 25% of the radioactivity originally present. The data thus show that the highly reactive carbene (shown as C in the equations) reacted in part as planned, by an internal attack on the enzyme itself. The photolysis was carried out with solutions from 2×10⁻⁴ M to 8×10⁻⁴ M in diazoacetylchymotrypsin with the same results; the reaction is therefore intra- and not intermolecular. An investigation of the position of this attack is now actively underway.

The essential new feature of this method of modifying an enzyme, and marking it, lies in the use of photolysis to initiate the chemical reaction. Where such a method can be applied, as in the photolysis of a diazoacetyl ester, it allows the formation of an extremely reactive chemical intermediate at any chosen temperature or pH or solvent, and at a rate controlled only by the intensity of the illumination used. Where the wave length of absorption of the photosensitive group is greater than those of the absorption bands of the protein, the photochemical decomposition takes place without damage to the latter. The photochemical method, here illustrated, offers a new method of potential utility in mapping the active sites of enzymes.

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