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Reaction of β-Propiolactone with Amino Acids and its Specificity for Methionine

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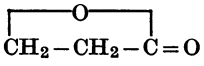
1. The reactions of β-propiolactone with amino acids were investigated under various conditions of pH and temperature to find those under which the reagent acted with specificity. 2. At pH 9·0 and 22°, after 15min. of reaction, at least 85% of each amino acid had reacted, methionine and cystine being the most reactive. 3. At pH 7·0 and 22° most amino acids reacted; methionine, cystine and histidine reacted almost entirely, and proline and lysine to a significantly smaller extent. 4. At pH 3·0 and 22° further specificity was obtained; methionine and cystine were the only reactive amino acids. 5. Reaction at pH 3·0 and 0° was specific for methionine; it was the only amino acid modified even after 145hr. of reaction.

β-Propiolactone is a compound with extremely interesting biological properties. It has been shown to be a carcinogen in rats (Dickens & Jones, 1961) and mice (Searle, 1961*a*). It induces chromosomal aberrations and genetic mutations (Smith & Srb, 1951), and it is bactericidal, fungicidal and a virus inactivator, and has been widely used in this connexion (LoGrippo & Hartman, 1955; LoGrippo, 1960; Sever, Castellano, Pelon, Huebner & Wolman, 1964).

Compounds with four-membered rings such as β-propiolactone (I)



(I)

are highly reactive owing to the strong tendency of the ring structure to open. The highly strained ring will open on reaction with amines (Gresham, Jansen, Shaver, Bankert & Fiedorek, 1951), thiol groups (Gresham, Jansen, Shaver & Gregory, 1948*a*; Gresham, Jansen & Shaver, 1948*b*; Gresham *et al.* 1952), sulphides (Blau & Stuckwisch, 1951; Gresham *et al.* 1948*a*), alcoholic and phenolic hydroxyl groups (Gresham, Jansen, Shaver, Gregory & Beears, 1948*d*; Gresham *et al.* 1949), carboxyl groups (Gresham, Jansen & Shaver, 1948*c*) and indoles and pyrrole (Harley-Mason, 1952).

β-Propiolactone has been allowed to react with guanosine, deoxyguanylic acid and yeast RNA, and in all cases the product of acid hydrolysis was 7-(2-carboxyethyl)guanine (Roberts & Warwick, 1963*a*,*b*). It has also been demonstrated (Baugh & Shaw, 1966) that IMP can be alkylated at the 1- or the 7-position by β-propiolactone under the proper conditions to form 1- or 7-(2-carboxyethyl)inosinic acid. It is quite probable, then, that the reactivity of β-propiolactone with nucleic acids can account for its mutagenic, and possibly its carcinogenic, properties.

The highly reactive nature of β-propiolactone and, in particular, its reactivity with groups that are found in amino acids, seem to indicate that it will combine with amino acids. Indeed, β-propiolactone has been allowed to react with L-cysteine and the product was shown to be *S*-2-carboxyethyl-L-cysteine (Dickens & Jones, 1961), and its reactivity with both thiol and disulphide groups of albumins has also been demonstrated (Searle, 1961*b*). In addition, β-propiolactone vapour has been allowed to react with methionine on a paper chromatogram, and two new spots were found (Majumder, Godavaribai, Muthu & Krishnamurthy, 1965).

The purpose of the present work was to undertake a more systematic study of the reaction of β-propiolactone with various amino acids under different conditions to find those under which the reagent acted with specificity. The present paper also describes conditions for reaction under which the reagent is specific for methionine.

MATERIALS AND METHODS

The amino acids used in these studies were of chromatographically homogeneous grade, obtained from Mann Research Laboratories Inc. (New York, N.Y., U.S.A.). A stock solution was prepared containing 2·5μmoles of each

of 18 amino acids (see Table 1)/ml. in 0·2N-HCl. β-Propiolactone was obtained from Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.) and was used without further treatment.

Reaction of amino acids with β-propiolactone. A solution of the amino acids (4ml.) was adjusted on a pH-stat to the appropriate pH (pH9·0, 7·0 or 3·0) with 3N-NaOH. A portion (250μl.) was removed and mixed with 0·2M-citrate buffer, pH2·25 (5ml.), and the solution was frozen immediately. β-Propiolactone (100μl.) was then added to the magnetically stirred amino acid solution, and the pH was maintained at the desired value by the addition of 3N-NaOH on the pH-stat. Portions (250μl.) were removed at intervals, and each was mixed with 5ml. of the above citrate buffer and then frozen immediately. Samples were assayed on a Spinco model 120C amino acid analyser. Reactions were carried out at 22° and pH9·0, 7·0 or 3·0.

For the reaction at 0° and pH3·0, a smaller amount of β-propiolactone was added (10μl./4ml. of amino acid solution).

RESULTS

Reaction of β-propiolactone with amino acids at pH9·0. The reactivities of amino acids are shown in Table 1. It can be seen, although there was some variation in rates, that all the amino acids had reacted after 1min., methionine and cystine being the most reactive. After only 15min. of reaction at least 85% of each amino acid had reacted. Alanine, glutamic acid, leucine and aspartic acid reacted less rapidly under these conditions, probably owing to steric or charge effects of their side groups. The huge loss in total ninhydrin colour formed during amino acid analysis of the reaction mixture suggested that β-propiolactone had reacted primarily with the amino group(s) of each amino acid, in addition to other possible reactive groups. At least eight new derivatives were detected on the analyser within the first few minutes of reaction. The extent of the reaction under these conditions suggested, as expected, that it would prove too drastic for specific modification of proteins. The reaction was therefore next carried out at neutral pH, to decrease the reactivity of the amino groups and attain specificity.

Reaction of β-propiolactone with amino acids at pH7·0. Under these conditions, the reactivities of all the amino acids tested decreased considerably, as shown in Table 2. Methionine, cystine and histidine reacted to a significantly greater degree than other amino acids after 30min. Methionine had reacted completely in 2hr. After 72hr. of reaction, cystine and histidine were almost entirely modified, and proline and lysine also decreased considerably. However, it is clear that all amino acids tested, except alanine and arginine, reacted to some extent. Thus, although some degree of specificity had been achieved, β-propiolactone seemed to be still too reactive under these conditions.

Reaction of β-propiolactone with amino acids at pH3·0 *and* 22°. The results of this reaction are shown in Table 3. It appears from these results that the reaction had been made specific for methionine; however, on prolonged reaction periods cystine also underwent modification, although considerably less rapidly than methionine (after 4hr. cystine decreased to 0·746μmole). In addition, small amounts of several new reaction products appeared, indicating that other amino acids were reacting.

In an attempt to characterize the reaction product of methionine, β-propiolactone (100μl.) was added to a magnetically stirred solution of methionine (50mg. in 5ml. of water), and the reaction was carried out in the pH-stat at pH3·0 and 22°. After 2hr., 20μl. of the reaction mixture was removed, added to 10ml. of cold citrate buffer, pH2·25, and applied to the amino acid analyser. The results indicated that a considerable proportion of the methionine remained unchanged. Another portion was taken after 4hr. and assayed on the analyser. At this stage it was found that methionine had reacted completely. The reaction was continued for another hour (i.e. a total of 5hr.). The temperature was lowered to 0° and the excess of β-propiolactone was extracted five times with equal volumes of ether. The methionine solution was then freeze-dried to a powder. A portion (0·1mg.) of this product was then dissolved and examined on the analyser; the results indicated that some (11%) of the reaction product had reverted to methionine

Table 1. *Reaction of β-propiolactone with amino acids at* pH9·0 *and* 22°

Amounts of amino acids were determined by analysis of the reaction mixture. Experimental details are given in the text.

Amino acid	Reaction time (min.) ...	Amount of unchanged amino acid (μmole) 0	1	2	15
Trp		1·00	0·639	0·419	0·026
Lys		1·00	0·580	0·353	0·016
His		1·00	0·575	0·358	0·009
Arg		1·00	0·689	0·500	0·036
Asp		1·00	0·854	0·716	0·111
Thr		1·00	0·624	0·367	0·017
Ser		1·00	0·629	0·468	0·024
Glu		1·00	0·829	0·719	0·120
Pro		1·00	0·634	0·478	0·055
Gly		1·00	0·609	0·418	0·028
Ala		1·00	0·815	0·692	0·156
CyS		1·00	0·353	0·158	0·000
Val		1·00	0·723	0·575	0·082
Met		1·00	0·236	0·239	0·008
Ile		1·00	0·737	0·586	0·088
Leu		1·00	0·772	0·638	0·116
Tyr		1·00	0·579	0·241	0·011
Phe		1·00	0·604	0·399	0·004

Table 2. *Reaction of β-propiolactone with amino acids at* pH 7·0 *and* 22°

Amounts of amino acids were determined by analysis of the reaction mixture. Experimental details are given in the text.

Amino acid	Reaction time (hr.)	Amount of unchanged amino acid (μmoles)							
		0	0·25	0·5	1	2	4	24	72
Trp		1·00	1·01	0·904	0·725	0·684	0·722	0·619	0·557
Lys		1·00	0·772	0·681	0·481	0·384	0·390	0·385	0·398
His		1·00	0·552	0·340	0·118	0·046	0·050	0·038	0·025
Arg		1·00	1·00	1·00	1·00	1·00	1·00	1·00	1·00
Asp		1·00	0·932	0·760	0·730	0·682	0·603	0·612	0·608
Thr		1·00	0·922	0·817	0·871	0·818	0·708	0·657	0·634
Ser		1·00	0·887	0·791	0·806	0·794	0·877	0·682	0·632
Glu		1·00	1·02	0·956	1·03	1·08	0·922	0·935	0·882
Pro		1·00	0·780	0·735	0·406	0·575	0·536	0·501	0·289
Gly		1·00	0·865	0·773	0·778	0·767	0·804	0·679	0·648
Ala		1·00	1·05	0·979	1·01	1·07	1·07	1·07	1·07
CyS		1·00	0·447	0·365	0·000	0·000	0·045	0·045	0·058
Val		1·00	0·986	0·871	0·914	0·983	0·877	0·851	0·798
Met		1·00	0·209	0·115	0·044	0·000	0·000	0·000	0·000
Ile		1·00	0·976	1·05	1·04	0·980	1·01	0·977	0·962
Leu		1·00	0·976	0·945	0·955	0·958	0·938	0·961	0·977
Tyr		1·00	1·23*	1·28*	1·14*	0·761	0·635	0·699	0·678
Phe		1·00	0·912	0·791	0·621	0·733	0·676	0·767	0·575

* These values represent tyrosine and a reaction product of methionine that frequently overlapped with tyrosine and could not be calculated separately.

Table 3. *Reaction of β-propiolactone with amino acids at* pH 3·0 *and* 22°

Amounts of amino acids were determined by analysis of the reaction mixture. Experimental details are given in the text.

Amino acid	Reaction time (min.) ...	Amount of unchanged amino acid (μmoles)			
		0	15	30	60
Trp		1·00	1·08	0·909	1·29*
Lys		1·00	1·09	1·02	1·04
His		1·00	1·07	1·01	1·08
Arg		1·00	0·927	0·986	0·964
Asp		1·00	1·00	0·963	0·960
Thr		1·00	1·01	1·01	1·02
Ser		1·00	1·01	0·983	1·02
Glu		1·00	0·984	0·972	1·30
Pro		1·00	1·06	1·06	1·05
Gly		1·00	1·04	1·01	1·05
Ala		1·00	1·05	1·04	1·00
CyS		1·00	1·04	0·919	0·990†
Val		1·00	1·01	1·02	1·04
Met		1·00	0·264	0·148	0·129
Ile		1·00	0·970	1·06	0·974
Leu		1·00	0·990	1·02	0·995
Tyr		1·00	1·46‡	1·90‡	1·911‡
Phe		1·00	0·996	1·00	1·01

* The value for tryptophan includes a reaction product that appeared in the same position and could not be calculated separately.

† The value for half-cystine after 4hr. of reaction was 0·746.

‡ These values represent tyrosine and a reaction product of methionine that frequently overlapped with tyrosine and could not be calculated separately.

during ether extraction and freeze-drying. Portions of the reaction product were then subjected to acid (constant-boiling hydrochloric acid *in vacuo* at 110° for 20hr.) or alkaline (saturated barium hydroxide *in vacuo* at 105° for 16hr.) hydrolysis, and in both cases the reaction product was completely hydrolysed to methionine.

Another portion of the reaction mixture was extracted with chloroform at 0°. Analysis at this stage showed very little (1·2%) reversion to methionine. However, on freeze-drying considerable reversion (12%) was obtained. The crude freeze-dried material was crystallized from cold ethanol. Subsequent analysis of this product showed a 50·4% reversion to methionine. The instability of the product made elemental analysis difficult. Fig. 1 shows the relative positions on the analyser of methionine and its reaction product. The latter appeared as symmetrical double peaks, always in the ratio 1:1, suggesting the existence of the product as a mixture of two diastereoisomers. Comparison of the time-interval between methionine and its reaction product on the amino acid analyser with that for methionine and tyrosine indicates that the reaction product of methionine appeared at approximately the same position as tyrosine (Fig. 1). This explains the high values of tyrosine observed in Tables 2, 3 and 4.

Reaction of β-propiolactone with amino acids at pH 3·0 *and* 0°. Under the conditions of this reaction it can be seen that complete specificity for methionine has been achieved. Table 4 shows that

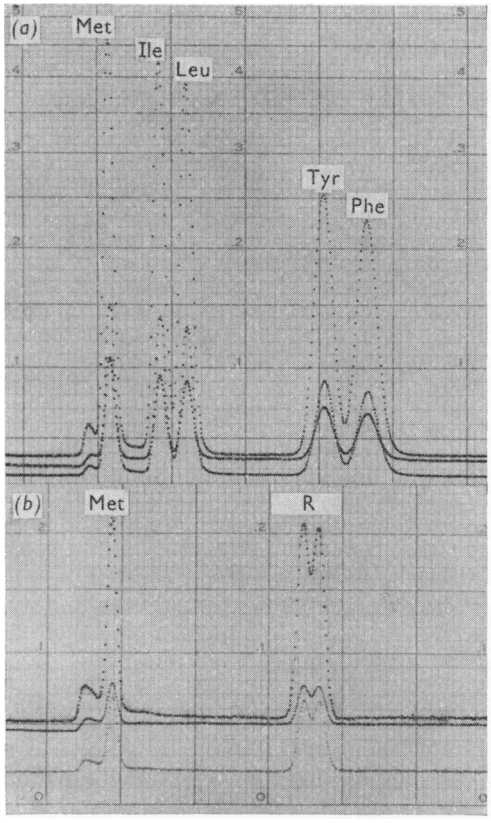
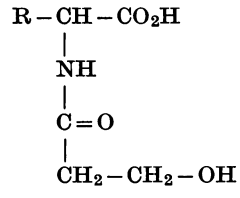


Fig. 1. Relative positions in the amino acid analyser of (*b*) methionine and its reaction product (R) and (*a*) methionine, isoleucine, leucine, tyrosine and phenylalanine. The rate of buffer flow through the column was 68ml./hr. and the temperature was 55°.

methionine was the only amino acid modified at pH3·0 and 0°, even after 145hr. of reaction.

DISCUSSION

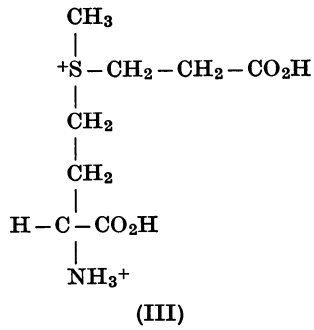
In reaction, the oxetane ring of β-propiolactone can cleave at the acyl–oxygen bond or at the alkyl–oxygen bond. Studies on hyrolysis of β-propiolactone (Long & Purchase, 1950; Bartlett & Small, 1950) have demonstrated that only neutral hydrolysis involves cleavage of the alkyl–oxygen bond. However, acid-catalysed hydrolysis (acyl–oxygen bond cleavage) does not predominate until high H^+ concentrations are attained. Strong and weak nucleophilic reagents can perform displacement reactions of the S_N2 type on the saturated alcoholic carbon atom of β-propiolactone (Bartlett & Small, 1950). However, it should be pointed out that these cleavages involve two competing reactions, which also vary with the amino acid, solvent and order of addition of reactants. It has been demonstrated in reactions with amines (Gresham *et al.* 1951) with water as a solvent that cleavage of the acyl–oxygen bond predominates, with the formation of amides. In contrast, aromatic amines and cyclohexylamines generally give cleavage at the alkyl–oxygen bond in reaction with β-propiolactone (Gresham *et al.* 1951). From this evidence, we postulate that the predominant reaction between β-propiolactone and amino acids at pH9·0 involves acylation of the amino group, giving derivatives of the type shown (II). Acylation at the amino groups would account for the almost complete loss in total ninhydrin colour after about 30min. of reaction. In addition,



(II)

reaction may take place at other positions, since at least eight new derivatives were observed by amino acid analysis in the first few minutes of reaction. Cystine and methionine can react to form the thio ester and acyl sulphonium derivatives respectively, and acylation at hydroxyl groups may also take place.

At neutral pH cleavage of β-propiolactone occurs at the alkyl–oxygen bond giving β-propionic acid residues on the amino groups. Dickens & Jones (1961) have shown that the product of reaction between β-propiolactone and L-cysteine, near neutrality, was *S*-2-carboxyethyl-L-cysteine. The reaction product of methionine at this pH is probably a sulphonium salt (III), since it has been demonstrated (Blau & Stuckwisch, 1951) that dimethyl sulphide and β-propiolactone yield dimethyl-β-propiothetin [$(CH_3)_2\overset{+}{S}\cdot CH_2\cdot CH_2\cdot CO_2H$]. This mechanism presumes a nucleophilic attack of the



(III)

Table 4. *Reaction of β-propiolactone with amino acids at* pH 3·0 *and* 0°

Amounts of amino acids were determined by analysis of the reaction mixture. Experimental details are given in the text.

Amino acid	Reaction time (hr.)	Amount of unchanged amino acid (μmoles) 0	0·25	1	21	48	145
Trp		1·00	0·978	0·965	1·05	1·08	0·935
Lys		1·00	1·01	1·00	1·00	1·00	1·01
His		1·00	0·987	0·980	1·02	1·04	0·970
Arg		1·00	0·989	0·996	0·998	0·996	0·982
Asp		1·00	1·00	0·993	1·00	0·986	0·993
Thr		1·00	1·00	1·03	1·01	1·09	1·01
Ser		1·00	1·01	1·02	1·01	1·00	1·03
Glu		1·00	1·01	1·04	1·03	1·04	1·05
Pro		1·00	0·966	1·01	0·976	0·961	0·961
Gly		1·00	1·01	1·02	1·02	1·02	1·02
Ala		1·00	1·00	0·972	1·01	1·01	0·997
CyS		1·00	0·982	1·11	1·00	0·958	1·00
Val		1·00	1·00	1·06	0·998	1·01	1·01
Met		1·00	0·766	0·718	0·532	0·435	0·351
Ile		1·00	0·999	0·953	1·01	1·00	0·995
Leu		1·00	0·998	0·960	1·01	1·00	1·01
Tyr		1·00	1·20*	1·25*	1·45*	1·55*	1·31*
Phe		1·00	0·997	0·995	0·994	0·996	0·994

* These values represent tyrosine and a reaction product of methionine that frequently overlapped with tyrosine and could not be calculated separately.

sulphur on the β-carbon atom of β-propiolactone. Histidine, which is also quite reactive at pH 7·0, may be alkylated at position 1 or 3 or both of the imidazole group. Proline is most likely to be alkylated at position 1.

At pH 3·0 and 22°, methionine and cystine were the most reactive amino acids. Cystine yielded predominantly the *S*-2-carboxyethyl derivative, whereas methionine presumably gave the sulphonium salt (III). At pH 3·0 and 0°, only the alkylation of methionine takes place (III), and the reaction with β-propiolactone became extremely specific. One would expect the methionine sulphonium salt to be readily hydrolysed under the conditions of hydrolysis used. Instability to milder conditions of hydrolysis has also been reported for the carboxymethyl sulphonium salt (Gundlach, Moore & Stein, 1959). However, the carboxymethyl derivative was stable to isolation and crystallization procedures, whereas the present work suggests that the carboxyethyl sulphonium salt is less stable during these manipulations, possibly owing to the ease of cyclization in the carboxyethyl derivative.

The reactivity of β-propiolactone with amino acids can readily account for some of its actions on cells. However, the specificity of this reaction should provide further means for the specific modification of methionine residues in proteins and for the study of the role of the methionine residues in the activities of proteins.

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