THE CHEMISTRY OF THE POLYMYXINS AND RELATED ANTIBIOTICS

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INTRODUCTION

The polymyxins are the most powerful and most specific antibiotics against gram-negative bacteria. The polymyxins are very effective in the treatment of several diseases (dysentery, gastroenteritis, meningitis, etc.) due to gram-negative microorganisms. A very valuable property of the polymyxins is their ability to suppress the growth of *Pseudomonas aeruginosa*, which is almost unaffected by other antibiotics and frequently produces suppurating and intestinal infections. The polymyxins comprise a group of antibiotics, very closely related both chemically and biologically, produced by various strains of *Bacillus polymyxa* and related species[1].

The polymyxins were discovered at the same time in 1947 by three independent groups of research workers in America and Great Britain[2-6]. The polymyxins A and D, which were first isolated, although highly active therapeutically, were also nephrotoxic. The polymyxins B, E, and C, prepared later, had a similar therapeutic activity but the first two were not nephrotoxic[7-10]. The related antibiotics, denoted A, B, C, D, and E, were combined in one polymyxin group[6,10,11].

In 1946 a strain of *Bacillus polymyxa*, which produced a new type of polymyxin termed polymyxin M, [12,13] was isolated from Moscow soil. Further studies showed that polymyxin B consists of two closely related antibiotics, polymyxin B_1 and polymyxin B_2. [14,15] In addition to the polymyxins, the related antibiotics circulins A and B, [16,17] the colistins (colimycins) A, B, and C, [18-20] and the polypeptins[21] may be assigned to this group.

As all types of polymyxins show great similarities in structure and properties, and they have always been studied in parallel, it is useful to examine them together, dealing with other representatives of this antibiotic group in separate paragraphs.

1. THE POLYMYXINS

General characteristics. The polymyxins are readily formed during the growth of *Bacillus polymyxa* on a medium containing various nutrients. The antibiotic may be isolated from the fermentation broth by methods based on precipitation, extraction, or sorption[22-28].

The similarity in physicochemical properties is due to the closely related chemical structures of this group of antibiotics. All the polymyxins are basic polypeptides that form a series of salts with acids[3,13,14,22,26-34], some of which have been obtained crystalline. They all give characteristic biuret and ninhydrin reactions, and negative Mollisch, Sakaguchi, Pauli, and Hopkins–Cole reactions[3,32-35]. All the polymyxins are optically active: $[\alpha]_D^{25}$ for polymyxin B_1 = -85.1° (C = 2.33 in 75% ethanol)[15], and for polymyxin M $[\alpha]_D^{25}$ = -48.1° (C = 2.505 in water)[13]. The most extensively studied amorphous salts, the sulphates and the hydrochlorides, are readily soluble in water and in methanol. Polymyxin M sulphate is insoluble in methanol†[26]; the hydrochlorides and sulphate are sparingly soluble in ethanol and other organic solvents[3,14,26,34,36,37]. The picrates are insoluble in water, readily soluble in methanol, ethanol, and acetone[34]. A compound of polymyxin B with formalin and $NaHSO_3$ is considerably less toxic than the sulphate or hydrochloride; similar compounds have also been obtained with other aldehydes[3,38-40].

Dry polymyxin salts retain their activity for several years. Aqueous polymyxin solutions are also fairly stable and retain their activity at room temperature for long periods at pH 3–5. Alkaline solutions are much less stable than acid solutions[3,33,36]. Silaev[41] found that a solution of polymyxin M in 0.1 N ammonia at 37°–38° lost nearly all its biological activity in three days; his report includes a preliminary discussion of the mechanism of inactivation of polymyxin M.[41] A report[42] has also appeared on the thermal decomposition of polymyxin B.

As has been pointed out above, all the antibiotics of this group are similar, both biologically and chemically. A more detailed study has revealed some differences in their composition and properties. All the antibiotics of the group are basic polypeptides, the basicity being due to the presence of considerable amounts of $\alpha\gamma$-diaminobutyric acid ($\alpha\gamma$-DABA); they all contain threonine and a fatty acid, identified, with rare exceptions, as 6-methyloctanoic acid (MOA). The absence of free carboxyl and α-amino-groups indicates that they have a cyclic structure; this has been demonstrated for most of the polymyxins B (B_1 and B_2)[14,15,43-46], and D[30] and M[13] that have been investigated. Polymyxin A, previously known as aerosporin‡, has been studied less[30,33,49-53], and there are only a few published references to the polymyxins C and E.[10,11,29,54,55]

In this review, work done in recent years on the structure of the polymyxins B and M and on circulin A will be examined in detail and data on the other antibiotics of this group will be referred to briefly.

The polymyxins differ from one another both quantitatively and qualitatively in their amino-acid composition (Table 1), infra-red absorption spectra[60], mobility on paper chromatograms, as well as in several other properties[11,13,61-63]. Because of differences in basicity, the polymyxins B and E are precipitated from aqueous solutions of their salts, when these are made alkaline,

† A commercial sample of polymyxin B sulphate (7757 units mg^{-1}) behaved in the same way: there is therefore some doubt about the data of Buckwalter[36] on the solubility of polymyxin sulphate in methanol.

‡ "Aerosporin" is now a trade mark [of Burroughs Wellcome and Co. (Ed. of Translation)] for polymyxin B[47,48].

TABLE. Comparative amino-acid composition of different types of polymyxin and related antibiotics.

Antibiotic	Amino-acid composition: leucine	L-iso-leucine	L-Threo-nine	D-serine	phenyl-alanine	α, γ-di-amino-butyric acid	Aliphatic acid	References
Polymysin								25, 30, 33
A	+D	—	+	—	—	L+D, L	MOA	49, 52, 56
B_1	1L	—	2	—	1D	6(1D+5L)	MOA	15, 25, 56
B_2	1L	—	2	—	1	6	isooctan-oic	
C	—	—	+	—	+L	+	+	10, 25, 56
D	1D	—	3	1	—	5L	MOA	25, 30, 56
E	+D	—	+	—	—	+L	+	20, 25, 56
M	1D	—	3	—	—	6	1	13, 57
Circulin A	1D	1	2	—	—	6L	MOA	17, 58
Colistin	2(L+D)	—	1	—	—	5	MOA	20, 59
Polypeptin*	2L	1	1	—	1D	3L	hydroxy-heptan-oic acid	21, 25, 56

* In addition to the acids indicated, polypeptin contains one molecule of D-valine.

Note: 1. The figures in the columns denote the number of residues of the amino-acids in the antibiotics.
2. The symbol + indicates the presence of the given amino-acid or an unknown aliphatic acid when the number of residues has not been established.
3. The symbol - indicates the absence of amino-acid residues or of an unknown aliphatic acid.

whereas the polymyxins A, C, D, and M remain in solution under similar conditions§.

The properties and chemical composition of this group of compounds have been studied by countercurrent distribution, spectroscopic analysis, different kinds of chromatography, etc. The special part played by chromatographic methods should be emphasised; these have been widely used in detecting both the separate amino-acid components of the polymyxin molecule and the larger low-molecular peptide fragments formed by partial hydrolysis. Compounds of similar composition behaved completely differently in paper chromatography with certain solvent systems, and this has enabled polymyxins A and D, for example, to be separated[30]. Polymyxins A, B, C, D, and E showed different rates of movement in the 1-butanol–15% acetic acid (1:1) system[64]. Polymyxins B and M differed in their mobilities in the butanol–acetic acid–water (2:1:1) system[13,57], etc.

Molecular weight. The molecular weights of the fairly large polymyxin molecules have been determined in different ways; the mean molecular weight is about 1200. Using osmotic pressure measurements, British workers found the molecular weight of polymyxin A to be 1293, and that of polymyxin D 1115.[30] From a study of monomolecular films of the four polymyxins, A, B, D, and E on the surface of an air–water interface, Few and Schulman[65] concluded that the molecular weights of the members of the polymyxin group differ little from one another, the average being 1250. Hausmann and Craig[15] used the method of partial dinitrophenylation, which had previously been used in the study of polypeptin and other peptides[66,67]. Partial dinitrophenylation with subsequent separation of the reaction products by paper electrophoresis has been used[68] to determine the molecular weight (1185 ± 25) of polymyxin M. Other workers[69] obtained similar results by determining the equivalent weight of polymyxin M by ion-exchange sorption. It has been shown that this antibiotic behaves here as a tetravalent ion and the equivalent weight is 280.

Amino-acid composition. The amino-acid composition of the polymyxins was studied using acid hydrolysates from which the fatty acid had first been thoroughly removed. With polymyxin B (B_1 + B_2),[15] the residue after evaporation under diminished pressure was subjected to countercurrent distribution in the system 1-butanol–2-butanol–5% HCl (1:1:2). The fractions containing phenylalanine and leucine were analysed spectrophotometrically (λ = 259 mμ). The mixture of these amino-acids was separated by further countercurrent extraction; $\alpha\gamma$-DABA and threonine, which moved together in the distribution, were also completely separated on redistribution. All four amino-acids were recrystallised and obtained analytically pure. This established that polymyxin B_1 contains $\alpha\gamma$-DABA, threonine, leucine, phenylalanine, and an aliphatic acid, identified as 6-methyloctanoic. Whether the amino-acids belonged to the L or D series was determined by means of acid D-amino-acid oxidase, which selectively destroys D-amino-acids. After the hydrolysate had been treated with the enzyme, phenylalanine was not found on the chromatogram, and the relative amount of $\alpha\gamma$-DABA decreased considerably (from 6 to 5 residues). This showed that all the component amino-acids in the polymyxin B molecule belong to the L series, with the exception of phenylalanine and one $\alpha\gamma$-DABA residue, which have the D-configuration[46]. The configuration of the leucine residue in polymyxin A was established in the same way[33,51].

A study of the amino-acid composition of polymyxin M by qualitative and quantitative paper chromatography, with subsequent isolation and identification of the amino-acids produced by hydrolysis, showed that the molecule contains one D-leucine, 3 L-threonine, and 6 $\alpha\gamma$-DABA residues[13,57]. This was confirmed by countercurrent distribution of the dinitrophenylation products of the hydrolysates of polymyxin M.[13] Polymyxin M therefore differs in its amino-acid composition from all known types of polymyxin and from related antibiotics, with the exception of polymyxins E and A. Polymyxin M differs markedly from polymyxin A in not being nephrotoxic, and from polymyxin E in that it is not precipitated at alkaline pH values[13].

Nature of the free groups. The nature of the free (end) groups in the molecule of polymyxin and related antibiotics was investigated by the methods generally used in examining peptides. Ordinary and formol titration showed[30] that the polymyxin D molecule contains no free carboxyl groups. An attempt to find carboxyl groups in the polymyxin B molecule by the thiohydantoin method[46], or by the action of diazomethane[43] on polymyxin B_1, led to the same results. The electrophoretic behaviour of polymyxin M and its dinitrophenyl derivative also indicates that the molecule contains no free carboxyl groups. This was confirmed[13,57] by the reaction of polymyxin M with hydrazine. All tests showed that a free carboxyl group was definitely absent. The nature of the free amino-groups in the polymyxins was investigated by converting them

§ In earlier papers it was stated that polymyxins A and D can also be precipitated as bases[22,30].

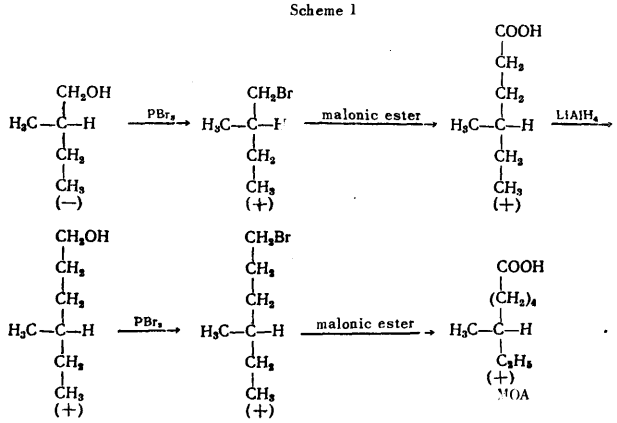
to dinitrophenyl derivatives, which were then hydrolysed [13,30,43,57,70]. γ-(2,4-Dinitrophenyl) (γ-DNP) derivatives of $\alpha\gamma$-DABA were isolated from the hydrolysates and identified by comparison with the dinitrophenyl derivatives of a synthetic specimen of $\alpha\gamma$-DABA. The absence of any other dinitrophenyl amino-acid in the hydrolysates of the dinitrophenyl derivatives of the polymyxins indicates that the free amino-groups in the polymyxin molecule are the γ-amino-groups of $\alpha\gamma$-DABA. However, Bell *et al.* [30] discovered a small amount of unsubstituted L-$\alpha\gamma$-DABA, together with the dinitrophenyl derivatives, in the total hydrolysate of polymyxin D, and since 4 moles of γ-DNP-$\alpha\gamma$-DABA were obtained on quantitative chromatographic analysis of the hydrolysate, it was concluded that the γ-amino-group of this acid was acylated with 6-methyloctanoic acid.

In the same way, it was shown that the hydrolysate of the dinitrophenyl derivative of polymyxin M also contains one molecule of unsubstituted $\alpha\gamma$-DABA, which forms amide links involving its γ- as well as α-amino-group. Polymyxin M [57] may have a branched peptide chain involving one $\alpha\gamma$-DABA amino-group, similar to that in polymyxin B. [43]

Aliphatic acid. As mentioned above, all the polymyxins have an aliphatic acid component; this was extracted from the acid hydrolysate with ether and purified [13,15,30,32] by distillation under reduced pressure, or through its bromothiuronate salt. A comparison of the infra-red spectra and also the analytical results on the amides and silver salts of this acid with those of a synthetic sample, showed that all the known types of polymyxin and related antibiotics contain L(+)-6-methyloctanoic (isopelargonic) acid in the molecule, except for polymyxin B_2 which contains isooctanoic acid [15], identical with the acid previously discovered in the fat isolated from wool [14,30,31,33,52,71-75].

Several methods have been published for the synthesis of racemic and optically active methyloctanoic acid. Syntheses [71,76] from L(-)-2-methylbutan-1-ol, which confirm that it has the L-configuration, deserve attention. A synthesis [75] published in 1960 differs from previous syntheses in being much simpler (Scheme 1).

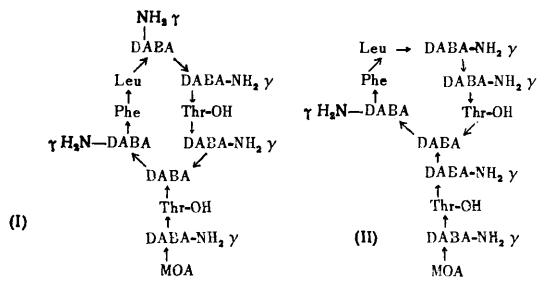
Scheme 1



Amino-acid sequence in the polymyxin B (B_1 and B_2) molecule. Two groups of workers have been independently engaged in studying the sequence of the amino-acid residues in the polymyxin B molecule. Hausmann [43] has given a detailed account of these investigations, which may be summarised as follows. Fully substituted dinitrophenylpolymyxin B_1 was partially hydrolysed with acid, and the resulting mixture of peptides was fractionated by numerous dialyses and countercurrent distributions in different solvent systems; fourteen DNP-substituted peptides were isolated pure. Subsequent detailed examination of these peptides and their dinitrophenyl derivatives, before and after hydrolysis, by means of countercurrent distribution, electrophoresis, and paper chromatography revealed the positions of all the amino-acids. On the basis of these results, the author suggests an empirical formula and two possible structural formulae for polymyxin B_1 (Scheme 2).

Scheme 2

Proposed structure of polymyxin B_1 ($C_{56}H_{98}O_{14}N_{16}$) [43]
(the arrow indicates the direction from CO to the NH group)



Symbols: DABA-$NH_2\gamma$ = $\alpha\gamma$-diaminobutyric acid residue, Leu = leucine residue, Phe = phenylalanine residue, Thr = threonine residue, MOA = 6-methyloctanoic acid residue.

Structure I in Scheme 2 is a large ring consisting of 8 amino-acids with a peptide side-chain terminating in a 6-methyloctanoic acid residue. The only difference in Structure II is that one DABA residue is in the side-chain instead of in the ring, which now contains seven amino-acids. To decide on one of the two proposed structures, it would be necessary to isolate the peptide MOA → DABA → → Thr → DABA → DABA → Phe. However, the peptide bond with the threonine amino-group is unstable to acids, which makes it impossible to separate a peptide without an *N*-terminal threonine.

French workers [46,76-78] used the strongly acidic cation exchanger Dowex-50 to separate the products of partial hydrolysis of polymyxin B. Seven different buffers were used for fractional elution. The peptides separated were determined qualitatively and quantitatively by one- and two-dimensional paper chromatography. The peptides containing phenylalanine were isolated from the acid hydrolysate by chromatography on specially treated activated charcoal. The fractions eluted from the charcoal with distilled water saturated with ethyl acetate and carbon disulphide were examined by paper electrophoresis. Having determined the structure of the peptides isolated, most of which were identical with those isolated and studied by Hausmann, and having established the absence of free α-amino- and carboxyl groups, the authors also arrived at 8- or 7-amino-acid structures with the same sequence of amino-acid residues as had been proposed by the American workers.

The main difficulty in making a choice between the structures is the lack of an enzyme [43,46] that would selectively cleave the peptide at the required link. It was also not clear at which of the two amino-groups (α or γ) of diaminobutyric acid the chain branches.

Several workers attempted to synthesise polymyxin B to confirm the porposed structure. In 1959, a synthesis[75,79,80] of polymyxin B_1 was published. For this purpose several low-molecular peptides were prepared including the pentapeptides I and II:

$$\text{For} \rightarrow \text{L-Leu} \rightarrow \text{L-DABA} \rightarrow \text{L-DABA} \rightarrow \text{L-Thr} \rightarrow \text{L-DABA-OH}; \quad \text{(I)}$$

$$\begin{array}{l} \qquad\qquad\quad \text{H} \rightarrow \text{L-DABA} \rightarrow \text{L-DABA} \rightarrow \text{D-Phe} - \text{OCH}_3. \\ \qquad\qquad\quad\ \uparrow \qquad\qquad\quad\ \uparrow \\ \text{MOA} - \text{D-DABA} \rightarrow \text{L-Thr} \qquad\ \gamma\text{-Tos} \\ \qquad\quad\ \downarrow \\ \qquad\quad \text{CBO} \end{array} \quad \text{(II)}$$

Some of the peptides had been previously known and described, but some were prepared and characterised as new compounds for the first time. Cyclicpeptides giving a negative ninhydrin reaction and containing all the component amino-acids were obtained from the polypeptides I and II by the carbodiimide method, the amino-groups being protected by various residues, such as the formyl (For), the carbobenzyloxy(CBO) and the *p*-toluenesulphonyl(Tos). The free decapeptide was obtained by removing the protecting groups with sodium in liquid ammonia. The structure of the polypeptide fully agreed with the proposed structure I (Scheme 2). Comparative chromatography on paper, and also comparison of the biological activity of synthetic and natural specimens of polymyxin B sulphate showed that their mobilities on paper were close, but the synthetic was less active biologically. According to the authors, further purification of the synthetic preparation and a comparison with polymyxin B_1 are required.

2. CIRCULIN

We shall now describe polypeptide antibiotics closely related to polymyxin, the most extensively studied being circulin, first described in 1948.[16,81-86]

Like the polymyxins, circulin is a basic polypeptide that forms salts with acids. It acts selectively on gram-negative microorganisms, but differs in its amino-acid composition (Table) and also in its behaviour with lipase and with alkali[81,82,85,86], in the presence of which it is completely inactivated; this does not occur with the polymyxins[87,88].

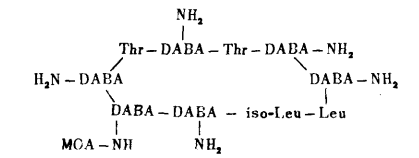
Several papers[82,83,86] have been published on the chemical composition and structure of circulin. However, they are not entirely reliable, as the investigations were made on samples that were not sufficiently homogeneous. It was later shown[17] by paper chromatography that circulin consists of two closely related antibiotics, circulin A and circulin B.

Finally, the amino-acid composition and structure of circulin A were established by Koffler and his coworkers; the results of this work were reported in 1958 at the Fourth Biochemical Congress in Vienna[58]. Five of the six γ-amino-groups of $\alpha\gamma$-DABA were found to be free. It was shown conclusively that the aliphatic acid residue is linked to the cyclicpeptide not through the threonine hydroxy group, as had been previously suggested[86,87], but through the *N*-acyl link with the γ-amino-group of DABA.[89] This agrees well with the following facts: (1) no hydroxamic acid was obtained when circulin A hydrochloride was treated with hydroxylamine; (2) on hydrolysis, in addition to dinitrophenyl-substituted amino-acids and an aliphatic acid, the dinitrophenyl derivative of circulin A gives one molecule of unsubstituted $\alpha\gamma$-DABA; this was not found after hydrolysis of the dinitrophenyl-substituted cyclicpeptide without the aliphatic acid residue; (3) the fragment isolated on partial hydrolysis of circulin A hydrochloride contained DABA and MOA in the ratio 3 : 1; α-amino-γ-(2,4-dinitroanilino)butyric acid and DABA were isolated from the DNP derivative of this peptide in the ratio 2 : 1.

To elucidate the amino-acid sequence in the molecule, circulin was partially hydrolysed in the same way as polymyxin B_1, and the structures of the low-molecular peptides were determined by dinitrophenylation and with the enzyme carboxypeptidase. The following structure was proposed for circulin A (Scheme 3):

Scheme 3

Structural formula of circulin



It was shown by the same workers that circulin is a lipopeptide antibiotic, which destroys the microorganisms that are sensitive to it by acting on the cell envelopes or on the cytoplasmic membranes. To elucidate the relation between its bactericidal action and molecular structure, circulin was subjected to partial changes, and the individual fragments were tested for antibacterial activity in parallel with the intact circulin. It was shown that the cyclicpeptide without the aliphatic acid was several times less active than the intact antibiotic[90]; this also applied to other fragments of the molecule.

3. COLISTIN (COLIMYCIN)

The antibiotic colistin was described by Japanese workers[18,20] in 1950, and it was extensively studied in Japan, Italy, and France under the names colistin and colimycin. Salts of colistin with various acids have been described and also its derivative with formaldehyde and sodium bisulphite, termed sodium colistinmethanesulphonate[20,91-95]. The antibiotic is characterised by thermal stability and very high stability towards acids, pepsin, and pancreatin[96].

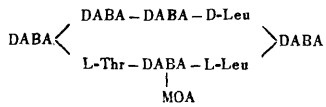
Using paper chromatography, the Japanese workers[19] established the presence of three components, colistins A, B, and C; these were characterised by different R_f values. Examination of the acid hydrolysate by paper chromatography established the qualitative amino-acid composition of colistin and the nature of the aliphatic acid[94,97]. Further studies by a variety of modern methods enabled the quantitative amino-acid composition to be established. Colistin contains L-$\alpha\gamma$-DABA, L-threonine, D-leucine, L-leucine, and methyloctanoic acid(MOA) in the ratios 5 : 1 : 1 : 1 : 1 (Table). Four free amino-groups and one hydroxy-group were found to be present. The molecule does not contain terminal carboxyl or amino-groups, and it has a cyclicpeptide structure, like other members of this antibiotic group.

Seven peptides were isolated[59,91] from the partial-hydrolysis products and characterised by ion-exchange chromatography, paper electrophoresis, and other

methods. The amino-acid sequence was established[63] and the following structural formula was proposed (Scheme 4)[20]:

Scheme 4

Proposed structure of colistin



4. POLYPEPTIN

Polypeptin is an antibiotic of this group that should be considered somewhat separately; in addition to the components common to all members of the group, it contains D-valine[66], a unique component. By countercurrent distribution, the antibiotic was separated into two polypeptides closely related in amino-acid composition[67]. One of these was studied in greater detail, and its molecular weight and amino-acid composition have been established (Table). Polypeptin differs from the antibiotics of the group considered here, not only in its amino-acid composition, but also in the aliphatic acid residue, which, according to Craig[25,26], is probably hydroxyheptanoic acid.

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THE INTERMOLECULAR TRANSFER OF ENERGY DURING THE RADIOLYSIS OF SOLUTIONS OF ORGANIC SUBSTANCES

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1. TRANSFER OF ENERGY DURING THE RADIOLYSIS OF LIQUID MIXTURES

In the chemical action of ionising radiation on a substance the absorption of the energy of the radiation leads to the formation of ions and excited molecules capable of further chemical changes. The decomposition of, or any other change in the ions and excited molecules, taking place with the rupture or formation of a new chemical bond, is called the "primary" radiochemical process, and the resulting products (for example ions, free radicals, or molecules) are called primary radiolysis products. The primary radiolysis products may react with one another or with other molecules to give secondary products.

Unlike light, ionising radiation is not absorbed selectively by substances, since the absorbing power of the latter with respect to radiation is determined by their electron density, *i.e.* by the number of electrons in unit volume. It is therefore usually assumed that when ionising radiation acts on a multicomponent system, the radiation energy absorbed by each component is proportional to its electronic fraction in the mixture. The electronic fraction of a given component (ϵ_i) is defined by the expression

$$\varepsilon_i = \frac{n_i e_i}{\sum_i n_i e_i}, \qquad (1)$$

where n_i is the mole fraction of the i-th component in the mixture and e_i the number of electrons in the molecule of this component.

Accordingly, we can formulate an additivity (averaging) rule, for the radiolysis of multicomponent mixtures, according to which the yield of the primary products of the radiochemical change for each component of the mixture should be proportional to the electronic fraction of the component.

The additivity rule has been confirmed in the study of the radiolysis of binary mixtures of certain organic liquids (benzene–toluene[1], methyl methacrylate–ethyl acetate[2], methyl methacrylate–styrene[3], cyclopentane–cyclohexane[4], etc.).

Muccini and Schuler[4] studied the radiolysis of cyclopentane–cyclohexane mixtures and showed that in the presence of iodine, which reacts with the cyclopentyl and cyclohexyl radicals formed during the radiolysis, the yields of cyclopentyl iodide and cyclohexyl iodide are proportional to the electronic fractions of the corresponding hydrocarbons. At a high dose rate ($\sim 2 \times 10^5$ r sec^{-1}) the yields of cyclopentylcyclopentane, cyclohexylcyclopentane,