

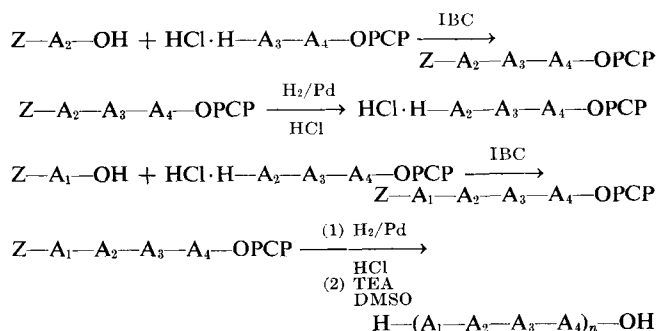
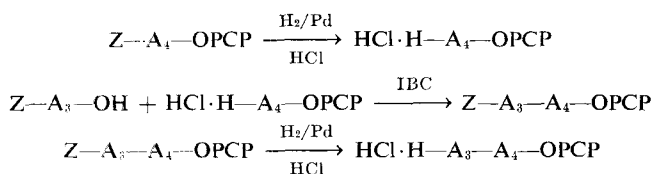
Sequential Polypeptides: Synthesis of *N*-Protected, *C*-Activated Peptides from *C*-Terminal Residues of Amino Acids

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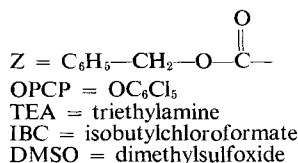
Abstract □ A modified mixed anhydride method is reported from which satisfactory yields are obtained by the coupling of *N*-carbobenzoxy amino acids with di- and tripeptide pentachlorophenyl active ester hydrochlorides. This method provides a convenient approach which limits the degree of racemization during the synthesis of *N*-protected, *C*-activated peptide units used for the preparation of sequential polypeptides.

Keyphrases □ Polypeptides, sequential—synthesis □ *N*-Carbobenzoxy amino acids, coupling—di- and tripeptide pentachlorophenyl active ester hydrochlorides □ IR spectrophotometry—structure

Pentachlorophenyl esters, which are among the most active esters (1), have been successfully used in the synthesis of peptides and polypeptides with a known sequence of amino acids (2, 3). Polymerization of amino acid sequences, simulating the active site of enzymes, leads to model compounds which are useful for comparative biological studies with the parent enzymes. In addition, high molecular weight polypeptides with known repeating sequences of amino acids are of great interest for conformational studies of proteins. Retention of the optically active centers during the synthesis of biologically active peptides and polypeptides is a major concern. It was recently reported that in order to eliminate the problems such as racemization and transesterification associated with the use of alkali during peptide synthesis, pentachlorophenyl active esters of *N*-carbobenzoxy amino acids could be coupled to amino acids and peptides *C*-protected by dicyclohexylamine (4, 5). The peptide chains were lengthened from *C*-terminal residues of amino acids (5). This approach, in addition to affording better yields, would further limit the degree of racemization through oxazolone formation, since the *N*-protected, *C*-activated component used would always be a single amino acid (5-8). In previous work on polypeptides with known sequence of amino acids, the *N*-protected peptide units were converted into their pentachlorophenyl esters (OPCP) with dicyclohexylcarbodiimide and pentachlorophenol (3). This approach leads to racemization, particularly of the *C*-terminal residue of the amino acid in the peptide chain (6-8). However, if the *C*-terminal residue is incorporated as active ester hydrochloride and the peptide chain extended stepwise by coupling through suitable activation, such as the mixed anhydride method, this problem can be overcome as shown in Scheme I.



A₁, A₂, A₃, and A₄, represent amino acid residues in a peptide sequence.



Scheme I

Sequential polypeptides synthesized according to Scheme I, would have the advantage of (a) excluding the use of alkali; (b) providing the activation for polymerization from the onset of the synthesis; and (c) activation for coupling would be for single amino acids only. When the synthesis of *N*-protected, *C*-activated peptides was attempted, following the classical experimental conditions for mixed anhydride coupling (9, 10), it was observed that the yields for coupling of *N*-protected amino acids with single amino acid pentachlorophenyl active ester hydrochlorides were quite satisfactory (5). However, when di- and tripeptide pentachlorophenyl active ester hydrochlorides were coupled to *N*-protected amino acids, an appreciable loss in yields was noted. This was attributed to the possible formation of diketopiperazine derivatives or cyclic and linear polypeptides (5).

For successful completion of a synthesis according to Scheme I, the main problem was to limit the side reactions, mainly intramolecular and polycondensations of the active ester hydrochlorides. The purpose of this paper is to report a detailed study of mixed anhydride coupling of *N*-protected glycine with model pentachlorophenyl ester hydrochlorides, which led to satisfactory reaction conditions for extending the chains of *N*-protected, *C*-activated peptides from *C*-terminal residues of amino acids.

RESULTS AND DISCUSSIONS

Normally, acylation with active esters such as *p*-nitrophenyl (11) and pentachlorophenyl esters (3) leads to optically pure peptides. However, considerable loss of optical activity can occur during the preparation of these active intermediates (12, 13). The problem of

Table I—Preparation of *N*-Carbobenzoxy and *N*-Carbobenzoxy OPCP Esters of Homologs of Glycine

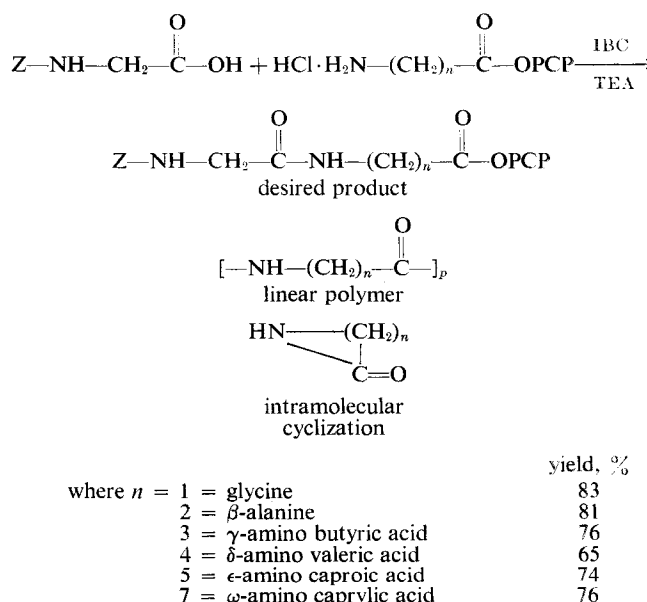
Compd. ^a		M.p., °C.	Yield, %	Anal., %	
				Calcd.	Found
1-Gly-OH	I	119–120 ^b	85		
Z-Gly-OPCP	II	133–134 ^c	75		
Z-β-Ala-OH	III	105–106 ^d	80		
Z-β-Ala-OPCP	IV	117–119	76	C, 43.30 H, 2.57 N, 2.97	C, 43.35 H, 2.51 N, 3.11
Z-γ-Abu-OH	V	66 ^e			
Z-γ-Abu-OPCP	VI	134 ^f	69		
Z-δ-Ava-OH	VII	101	80	C, 62.17 H, 6.77 N, 5.57	C, 62.25 H, 6.69 N, 5.61
Z-δ-Ava-OPCP	VIII	96	68	C, 45.69 H, 3.23 N, 2.80	C, 45.73 H, 3.18 N, 2.87
Z-ε-Aca-OH	IX	55	78	C, 63.38 H, 7.22 N, 5.28	C, 63.41 H, 7.29 N, 5.20
Z-ε-Aca-OPCP	X	82	65	C, 46.77 H, 3.53 N, 2.73	C, 46.69 H, 3.61 N, 2.81
Z-ω-Acap-OH	XI	81–82	65	C, 65.51 H, 7.90 N, 4.77	C, 65.58 H, 7.97 N, 4.69
Z-ω-Acap-OPCP	XII	79–80	65	C, 48.78 H, 4.09 N, 2.58	C, 48.81 H, 4.16 N, 2.63

^a Abu = aminobutyric; Ava = aminovaleric; Aca = aminocaproic; Acap = aminocaprylic. ^b Reference 17. ^c Reference 3. ^d Reference 18. ^e This compound was purchased from Nutritional Biochemicals Corp., Cleveland 28, Ohio. ^f Reference 19.

racemization in the case of *N*-protected dipeptide *p*-nitrophenyl esters was circumvented by Goodman and Stueben (11) by the coupling of *N*-protected amino acids to amino acid *p*-nitrophenyl esters using the dicyclohexylcarbodiimide (DDC) method. This coupling can also be carried out by other faster procedures of acylation such as mixed anhydride (14). The mixed anhydride method was selected because of the easy removal of side products. Since the mixed anhydride coupling of *N*-carbobenzoxy amino acids with OPCP ester hydrochlorides of amino acids proceeded in satisfactory yields and because low yields were obtained only when di- or tripeptide OPCP ester hydrochlorides were used for coupling, it was reasonable to assume that the low yields were mainly due to intramolecular cyclization rather than intermolecular polymerization. The chances for cyclization to relatively unstable "three-membered" lactams from amino acid OPCP ester hydrochlorides would be much less, as compared to fairly stable diketopiperazines in the case of dipeptides and "nine-membered" cyclic structures from tripeptide OPCP ester hydrochlorides. In all the above cases, the chances for linear polymerization would be less favored due to the fairly dilute concentrations used for mixed anhydride coupling.

To gain further information as to the possible reason of low yields involving intramolecular cyclization, it was considered worthwhile to investigate the coupling by mixed anhydride of *N*-carbobenzoxy glycine with homologs of glycine OPCP ester hydrochlorides. Using the standard conditions for mixed anhydride reaction, Z-Gly-OH(1)¹ was coupled with OPCP ester hydrochlorides of glycine(XIII), β-alanine(XIV), γ-amino butyric acid(XV), δ-amino valeric acid(XVI), ε-amino caproic acid(XVII), and ω-amino caprylic acid(XVIII). The yield in the case of δ-amino valeric acid OPCP ester hydrochloride should be the lowest of the series due to the formation of the relatively more stable "six-membered" δ-valero lactam. This observation was confirmed as demonstrated in Scheme II. As the number (*n*) increases, the chances of intramolecular cyclization of the hydrochloride active ester would also increase, reaching a maximum when *n* = 4.

¹ All the abbreviations employed are in accordance with the Recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [*Biochemistry*, 5, 2485(1966)]. All amino acids used in this work were of L-configuration.

Scheme II—Mixed anhydride coupling of *N*-carbobenzoxy glycine with homologs of glycine OPCP ester hydrochlorides.

From the above results, it appeared logical to carry out a systematic study of the reaction conditions for mixed anhydride coupling in order to increase the yields of *N*-carbobenzoxy glycine coupling with δ-amino valeric acid OPCP ester hydrochloride. It was felt that the chances of intramolecular cyclization of the active ester hydrochloride would be considerably limited if it were added slowly to the *N*-protected mixed anhydride activated portion. This would always provide a relative excess of the *N*-protected mixed anhydride-activated portion which would compete for coupling with the amino group liberated by triethylamine neutralization of the active ester hydrochloride. Normally, mixed anhydride coupling is carried out at −5°. However, further lowering of this

Table II—Preparation of OPCP Ester Hydrochlorides of Glycine Homologs

Compd.	M.p., °C.	Yield, %	Anal., %	
			Calcd.	Found
HCl·H-Gly-OPCP XIII	225	94	C, 26.70 H, 1.40 N, 3.89	C, 26.68 H, 1.43 N, 3.91
HCl·H-β-Ala-OPCP XIV	214	93	C, 28.87 H, 1.89 N, 3.75	C, 28.69 H, 1.81 N, 3.81
HCl·H-γ-Abu-OPCP XV	181	94	C, 30.96 H, 2.34 N, 3.61	C, 30.82 H, 2.28 N, 3.69
HCl·H-δ-Ava-OPCP XVI	172-173	87	C, 32.87 H, 2.76 N, 3.48	C, 32.68 H, 2.71 N, 3.53
HCl·H-ε-Aca-OPCP XVII	185-187	83	C, 34.65 H, 3.15 N, 3.37	C, 34.74 H, 3.11 N, 3.42
HCl·H-ω-Acap-OPCP XVIII	144-146	81	C, 37.87 H, 3.86 N, 3.15	C, 37.79 H, 3.91 N, 3.21

reaction temperature should decrease the possibilities of undesirable reactions.

Anderson *et al.* recently reported an extensive reinvestigation of the mixed carbonic anhydride method in the synthesis of peptides (15). In line with their findings, ethyl acetate was selected as the solvent, isobutylchloroformate as the activating reagent, and a reaction temperature of -15° . In the authors' initial work (5), triethylamine was used for the mixed anhydride formation, as well as for liberating the amino groups from the active ester hydrochlorides. The use of amines with reduced "basicity," such as *N*-methylmorpholine, has been suggested to limit the degree of racemization during mixed anhydride coupling (15). Results from this laboratory indicate that *N*-methylmorpholine is a strong enough base not only to neutralize ethyl ester hydrochlorides as reported by Anderson (15) but is also strong enough to neutralize active ester hydrochlorides. Therefore, it was decided to use *N*-methylmorpholine as a base both for the mixed anhydride formation and for liberation of the amino groups of the active ester hydrochlorides.

After many attempts, suitable reaction conditions were worked out and yields, previously obtained as 65%, were raised to 85% in the case of mixed anhydride coupling of *N*-carbobenzoxy glycine with δ-amino valeric acid OPCP ester hydrochloride. The best results were obtained when *N*-carbobenzoxy glycine(1) was activated with two equivalents of *N*-methylmorpholine and one equivalent of isobutylchloroformate in ethyl acetate at -15° . After

5 min. of activation an additional equivalent of *N*-methylmorpholine was added to the reaction mixture. This was followed by an equivalent amount of *N*-methylmorpholine and a suspension of the calculated amount of δ-amino valeric acid OPCP ester hydrochloride (XVI) in ethyl acetate, added alternately in 10 equal portions over a period of 1 hr. One hour after the final addition, the reaction was brought to 10° and worked up as described in the *Experimental section*, to afford 85% of *N*-carbobenzoxy glycyl-δ-amino valeric acid OPCP ester (XXII).

Following the same reaction conditions, *N*-carbobenzoxy glycine was coupled with other homologs of glycine OPCP ester hydrochlorides in yields ranging from 86 to 92%. Tables I-III summarize the experimental data.

In order to establish the applicability of modified mixed anhydride coupling conditions, a number of *N*-protected, C-activated tri- and tetrapeptides were synthesized in satisfactory yields, according to Scheme I. This was demonstrated by the synthesis of Z-Gly-Gly-Ala-OPCP (XXVIII) and Z-Ala-Gly-Ala-Ala-OPCP (XXXIII).

Synthesis of Z-Gly-Gly-Ala-OPCP (XXVIII)—Z-Gly-OH(1) was coupled with HCl·H-Ala-OPCP (XXV) through the modified mixed anhydride method to afford Z-Gly-Ala-OPCP (XXVI) in 95% yield. XXVI was hydrogenated in the presence of one equivalent of hydrogen chloride in anhydrous methanol to afford HCl·H-Gly-Ala-OPCP (XXVII) in 94% yield. XXVII was coupled with

Table III—Coupling of *N*-Carbobenzoxy Glycine with OPCP Ester Hydrochlorides of Glycine Homologs Through Modified Mixed Anhydride Method

Hydrochloride Component	Reaction Product	M.p., °C.	Yield, %	Anal., %	
				Calcd.	Found
XIII	Z-Gly-Gly-OPCP XIX	160°	92	C, 41.98 H, 2.53 N, 5.44	C, 41.92 H, 2.48 N, 5.51
XIV	Z-Gly-β-Ala-OPCP XX	145°	90	C, 43.14 H, 2.84 N, 5.30	C, 43.21 H, 2.90 N, 5.41
XV	Z-Gly-γ-Abu-OPCP XXI	92°	87	C, 44.24 H, 3.13 N, 5.16	C, 44.33 H, 3.21 N, 5.23
XVI	Z-Gly-δ-Ava-OPCP XXII	128°	85	C, 45.28 H, 3.41 N, 5.03	C, 45.31 H, 3.52 N, 5.13
XVII	Z-Gly-ε-Aca-OPCP XXIII	119°	86	C, 46.28 H, 3.68 N, 4.91	C, 46.21 H, 3.72 N, 5.03
XVIII	Z-Gly-ω-Acap-OPCP XXIV	112-114°	89	C, 48.12 H, 4.68 N, 4.18	C, 48.32 H, 4.31 N, 4.71

Z-Gly-OH(1), using modified mixed anhydride conditions and the desired tripeptide, Z-Gly-Gly-Ala-OPCP(XXVIII) was isolated in 85% yield.

Synthesis of Z-Ala-Gly-Ala-Ala-OPCP(XXXIII)—The dipeptide Z-Ala-Ala-OPCP(XXIX) was prepared by modified mixed anhydride reaction of Z-Ala-OH and HCl·H-Ala-OPCP(5) in 91% yield. Hydrogenation of XXIX afforded 94% of HCl·H-Ala-Ala-OPCP(XXX) which was coupled by the modified mixed anhydride method with Z-Gly-OH and the tripeptide Z-Gly-Ala-Ala-OPCP(XXXI) was isolated in 86% yield. Upon hydrogenation of XXXI, HCl·H-Gly-Ala-Ala-OPCP(XXXII) was obtained in 94% yield. Coupling of Z-Ala-OH via modified mixed anhydride method with XXXII produced Z-Ala-Gly-Ala-Ala-OPCP(XXXIII) in 87% yield.

This work presents the incorporation of simple amino acids in *N*-protected, *C*-activated peptides, synthesized from *C*-terminal residues of amino acids, as units for sequential polypeptides. However, the combination of modified mixed anhydride and pentachlorophenyl active ester methods as described above has been successfully extended in the synthesis of sequential polypeptides incorporating polyfunctional amino acids and these results will be presented in forthcoming papers.

EXPERIMENTAL²

N-Carbobenzoxy derivatives reported in this work were prepared by the sodium bicarbonate method (16). *N*-Protected pentachlorophenyl esters were prepared by DCC, and pentachlorophenyl ester hydrochlorides were prepared by hydrogenation in the presence of anhydrous methanol containing hydrogen chloride, according to the procedures previously reported (3).

Modified Mixed Anhydride Method for the Coupling of *N*-Carbobenzoxy Amino Acids with Pentachlorophenyl Ester Hydrochlorides of Amino Acids and Peptides—*Preparation of N-Carbobenzoxy Glycyl-δ-Amino Valeric Acid Pentachlorophenyl Ester (XXII)*—A solution of 2.09 g. (10 mmoles) of *N*-carbobenzoxy glycine in 100 ml. of ethyl acetate was chilled to -15° with stirring. To this was added 2.2 ml. (20 mmoles) of *N*-methylmorpholine followed by 1.35 ml. (10.5 mmoles) of isobutylchloroformate. Five minutes later 1.1 ml. (10 mmoles) of *N*-methylmorpholine was added to the reaction mixture. This was followed by 1.1 ml. (10 mmoles) of *N*-methylmorpholine and a suspension of 4.02 g. (10 mmoles) of δ -amino valeric acid OCP ester hydrochloride(XVI) in 20 ml. of ethyl acetate, added alternately in 10 equal portions over a period of 1 hr. The reaction temperature during the above additions was maintained at -15° . After additional stirring for 1 hr. at -15° , the reaction mixture was allowed to warm to 10° , then extracted three times each with 30-ml. portions of 1 *N* hydrochloric acid, water, 5% aqueous sodium bicarbonate, and water. The ethyl acetate was dried over anhydrous sodium sulfate. The solvent was removed by evaporation *in vacuo*, leaving a residue which was crystallized from methanol-ether-*n*-pentane to yield 4.67 g. (85%) of *N*-protected active ester XXII, m.p. 128° . The IR spectrum showed a peak at 5.61μ , characteristic of pentachlorophenyl esters.

N-Protected active ester derivatives reported in Table III, were prepared using the above procedure.

Preparation of *N*-Carbobenzoxy Glycyl-Glycyl-Alanine Pentachlorophenyl Ester(XXVIII)—Glycyl-alanine pentachlorophenyl ester hydrochloride(XXVII) was prepared in 94% yield by hydrogenation of *N*-carbobenzoxy-glycyl-alanine OCP ester (5) in the presence of anhydrous methanol containing hydrogen chloride, according to the procedure previously reported (3) m.p. $198-199^{\circ}$.

Anal.—Calcd. for $C_{13}H_{16}Cl_5N_2O_3$: C, 30.63; H, 2.32; N, 6.50. Found: C, 30.51; H, 2.28; N, 6.41.

Modified mixed anhydride coupling of 2.09 g. (10 mmoles) of *N*-carbobenzoxy glycine with 4.31 g. (10 mmoles) of dipeptide active ester hydrochloride XXVII, afforded upon crystallization with ethyl acetate-ether, 4.9 g. (84%) of *N*-protected tripeptide active ester, XXVIII, m.p. $174-175^{\circ}$. The IR spectrum showed peaks at 6.05μ (amide I), 6.51μ (amide II) and 5.6μ (OCP ester).

Anal.—Calcd. for $C_{21}H_{18}Cl_5N_3O_6$: C, 43.04; H, 3.07; N, 7.17. Found: C, 43.18; H, 3.11; N, 7.02.

Synthesis of *N*-Carbobenzoxy Alanyl-Glycyl-Alanyl-Alanine Pentachlorophenyl Ester(XXXIII)—Modified mixed anhydride coupling of 4.46 g. (20 mmoles) of *N*-carbobenzoxy alanine with 6.77 g. (20 mmoles) of alanine OCP ester hydrochloride (5) was carried out in the manner described above. The *N*-protected dipeptide active ester XXIX was crystallized from methanol-ether-*n*-pentane. Yield 9.85 g. (91%), m.p. $202-203^{\circ}$.

Anal.—Calcd. for $C_{20}H_{17}Cl_5N_2O_5$: C, 44.24; H, 3.13; N, 5.16. Found: C, 44.08; H, 3.19; N, 5.22.

Carbobenzoxy protection of dipeptide XXIX, was removed by hydrogenation in the presence of anhydrous methanol containing hydrogen chloride and alanyl-alanine pentachlorophenyl ester hydrochloride(XXX) was obtained in 94% yield, m.p. $180-181^{\circ}$.

Anal.—Calcd. for $C_{12}H_{12}Cl_5N_2O_3$: C, 32.36; H, 2.70; N, 6.29. Found: C, 32.12; H, 2.68; N, 6.32.

N-Carbobenzoxy glycyl-alanyl-alanine pentachlorophenyl ester(XXXI) was prepared by modified mixed anhydride coupling of 3.14 g. (15 mmoles) of *N*-carbobenzoxy glycine and 6.68 g. (15 mmoles) of dipeptide active ester hydrochloride(XXX). Recrystallization from methanol-ether afforded 7.75 g. (86%) of the tripeptide(XXXI), m.p. $146-147^{\circ}$. IR spectrum showed peaks at 6.04μ (amide I), 6.5μ (amide II), and 5.59μ (OCP ester).

Anal.—Calcd. for $C_{22}H_{20}Cl_5N_3O_6$: C, 44.04; H, 3.34; N, 7.01. Found: C, 44.15; H, 3.28; N, 6.93.

Hydrogenation in the presence of anhydrous methanol containing hydrogen chloride of *N*-protected tripeptide active ester(XXXI), afforded glycyl-alanyl-alanine pentachlorophenyl ester hydrochloride(XXXII), in 94% yield, m.p. $151-153^{\circ}$.

Anal.—Calcd. for $C_{14}H_{15}Cl_5N_3O_4$: C, 33.47; H, 2.99; N, 8.37. Found: C, 33.42; H, 2.91; N, 8.27.

From modified mixed anhydride coupling of 2.23 g. (10 mmoles) of *N*-carbobenzoxy alanine and 5.02 g. (10 mmoles) of tripeptide active ester hydrochloride XXXII, 5.83 g. (87%) of *N*-carbobenzoxy-tetrapeptide-OPCP ester XXXIII, was isolated after crystallization from ethyl acetate-ether, m.p. $192-193^{\circ}$. IR spectrum: 6.05μ (amide I), 6.49μ (amide II), and 5.6μ (OCP ester).

Anal.—Calcd. for $C_{25}H_{23}Cl_5N_4O_7$: C, 44.74; H, 3.73; N, 8.35. Found: C, 44.69; H, 3.76; N, 8.29.

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² All melting points are uncorrected and were taken on a Hoover Unit-Melt apparatus. The microanalyses were carried out by Drs. G. Weiler and F. B. Strauss, Oxford, England. IR spectra were determined in KBr pellets with a Beckman IR 8 spectrophotometer.

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Release Rates of Salicylates from Cocoa Butter I

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Abstract □ The release of *N*-methyl salicylamide, methyl salicylate, and salicylic acid across a hydrophobic membrane from cocoa butter to an aqueous layer was studied at a temperature of 37°. The release of the drugs at the interface was diffusion-controlled. The Higuchi model was modified to account for the fact that the release rates were not constant, but varied inversely with the respective partition coefficients. Since the transference across the interface was a function of the partition coefficient, it was found that as the partition coefficient decreased, the release rate appeared to approach a limiting value.

Keyphrases □ Salicylates—release rates, cocoa butter □ Partitioning salicylates—cocoa butter—water □ Release rates, salicylates—continuous flow cell □ UV spectrophotometry—analysis

Cocoa butter has long been used as a base for suppositories, but the manner by which drugs are released from this base has not been shown. In the absence of a suitable model, several empirical relationships (1, 2) have been developed for the determination of the safe and efficacious amount of drug to be incorporated into the base; yet none has proven to be totally satisfactory.

The continuous release of salicylic acid, *N*-methyl salicylamide, and methyl salicylate from cocoa butter was tested for the applicability of the Higuchi model (3) to these systems. These compounds were chosen because (a) the difference between their respective diffusion coefficients should be insignificant, and (b) they have a range of solubilities in the cocoa butter. The partition coefficients of these drugs were studied to determine any effect on the release pattern of this system.

EXPERIMENTAL

Measurement of Partition Coefficients—A direct method was used to determine the partition coefficient of the drugs between cocoa butter and water. The aqueous phase was adjusted to a pH at least two units below the pKa value of the drug to limit the form of the partitioning drug to a single species. Deionized water was used for methyl salicylate, while *N*-methyl salicylamide and salicylic acid required HCl-adjusted solvents to pH 4 and 1, respectively.

Equilibrium was attained in from 24–48 hr.; however, with time, some of the cocoa butter became dispersed in the water phase resulting in a cloudy appearance. Therefore, contact time of the two phases should be minimized. The portion of drug which was expected to be in each phase upon reaching equilibrium was dis-

solved in the cocoa butter and water separately. The two immiscible solutions were combined and shaken well. The two phases quickly separated and were then maintained for 12–15 hr. in a constant-temperature gyrotory water bath¹ at $37 \pm 0.1^\circ$. After equilibration, the phases were placed in separate containers and assayed for drug content.

As much cocoa butter as possible was removed from the aqueous layer which was placed in a conical flask, covered with a polyethylene film to limit evaporation, and allowed to remain at 20° for 5 hr. A pipet (T.C.), with its tip covered with fine glass wool, was used to withdraw a sample of solution. The sample was discharged into a 100-ml. volumetric flask and the pipet washed repeatedly with the appropriate solvent directly into the flask which was then filled to volume. The solutions were then analyzed at 302 and 360 mμ spectrophotometrically.² Two wavelengths were required to correct the drug analysis for the cocoa butter. This was accomplished by taking the absorbance at 360 mμ, where only cocoa butter absorbed light, and from a predetermined calibration curve for cocoa butter alone obtaining its absorbance contribution at 302 mμ where the drugs also absorbed light. By difference, the absorbance due to the drug was obtained at 302 mμ. The absorptivities using the Beer-Lambert equation for a 1-cm. cell were 0.2349 for methyl salicylate, 0.2039 for *N*-methyl salicylamide, and 0.5170 for salicylic acid. The concentration was expressed in mg./100 ml.

Each drug in the cocoa butter was assayed by a nonaqueous titration procedure similar to the one described by Fritz (4) and previously shown to be accurate to within 1%. To remove the water which remained in the liquefied cocoa butter, anhydrous sodium chloride was added and the mixture was shaken and centrifuged. The oleaginous layer was decanted and powdered anhydrous sodium sulfate added. This was shaken for a period of 2 min., centrifuged, and placed in a 40° oven for 1 hr. The cocoa butter was finally decanted into a tared flask and weighed. Ninety milliliters of pyridine was added for the titrimetric determination of methyl salicylate and *N*-methyl salicylamide, using azo-violet as the indicator. In the case of salicylic acid, 100 ml. of chloroform was added with thymol blue as the indicator. In all cases standardized sodium methoxide (0.1 *N*) was used as the titrant. The concentration of drug in the base was calculated in terms of mg. of drug/g. of drug-cocoa butter solution.

Release Rate Determination—A continuous-flow method similar to that of Sjogren and Ervik (5) was utilized to study the release rate of each drug from cocoa butter. A continuous-flow cell³ was used in the monitoring spectrophotometer. The partitioning cell⁴ (Fig. 1) and a pump (model T-8 Sigma) were connected to the continuous-flow cell by means of tubing [Tygon, 0.635-cm. (1/4-in.) i.d.].

¹ Model G-76, New Brunswick Scientific Co., New Brunswick, N. J.

² Beckman DK-2 recording spectrophotometer.

³ Beckman model No. 92522 continuous-flow cell.

⁴ Loaned from the Sandoz Pharmaceutical Corp., Hanover, N. J.